Nanomaterials as Non-viral siRNA Delivery Agents for Cancer Therapy

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A B S T R A C T

Gene therapy has been recently shown as a promising tool for cancer treatment as nanotechnology-based safe and effective delivery methods are developed. Generally, genes are wrapped up in extremely tiny nanoparticles which could be taken up easily by cancer cells, not to their healthy neighboring cells. Several nanoparticle systems have been investigated primarily to address the problems involved in other methods of gene delivery and observed improved anticancer efficacy suggesting that nanomedicine provides novel opportunities to safely deliver genes, thus treat cancer. In this review, various nanoparticle types and related strategies, used in gene delivery for cancer treatment, have been discussed.

Introduction

It is not surprising that genetically modified viruses (viral vectors) are the efficient transporters that are currently being used for transfecting nucleic acids (DNA/plasmids/siRNA) into the mammalian cells.[1,2] Despite their high transfection efficiency, viral vectors face many challenges such as likelihood of biological risks, feasibility and manufacturing cost, so researchers have currently focused on designing synthetic alternatives to viruses as nucleic acid delivery vectors.[3,4] Furthermore, the delivery via viral or synthetic vectors is dependent on simple diffusion, hence prone to be bound to the cell surface constitutes which leads in nucleic acids inactivation by opsonization, immune system recognition and other cellular degradation methods.[5] In cancer treatment, tumor site localization of delivery vehicle is another limitation particularly under *in vivo* conditions, which could further reduce side effects at non-targeted sites.

Currently gene therapy is grasping much attention in research area as an option for therapeutic treatment of genetic or acquired diseases including cancer.[6,7] So far RNA interference technology remains the most promising tool for targeted-gene therapy due to its minimum non-specific effects.[8] However, methods of transfection still remain a big challenge for its successful usage in clinics. Many approaches have been introduced over past few decades which are mainly dominated by viral vectors, posing a high risk of infectivity among mammalian systems. Recently, few non-viral gene delivery systems such as electroporation, chemical methods and gene gun method etc, have been developed; however, efficient delivery, cytotoxicity and safety problems still remain a serious concern.[9-11] Another major obstacle for successful knockdown of concern gene is inappropriate targeting which could insert the therapeutic gene into a patient's reproductive cells and finally produce sperms and eggs and affect the next generation offspring. Generally, siRNA delivery has been used for knocking down the expression of diseased protein; however, plasmids are being used for over-expression of targeted protein missing from the cells responsible for the disease. Nevertheless, nanotechnology-based gene carriers have recently shown promising outcomes in terms of low toxicity and effective delivery to targeted cells/tissues.[12]

Nanotechnology offers several advantages and possible solutions for improved delivery and negligible toxicity that are needed to more effectively translate basic science into clinical practise.[13] Nanoparticles are typically having a diameter between 1-100 nm, at least in one dimension, which makes them to exhibit better (~10% more) EPR (Enhanced Permeability and Retention) effect thus longer circulating in tumor region under *in vivo* conditions.[14,15] Additionally, reports have shown that particle size between 10-100 nm is relatively non-toxic to mammalian cells as nanoparticles smaller than 10 nm and bigger than 100 nm can get entrapped in the reticuloendothelial area of immune system and other interstitial space of body.[16] Furthermore, naked siRNAs

are extremely small in size and if delivered, could be easily escaped from the body, however would be retained in the tumor region if attached to nanoparticles (EPR effect). To explore the full potential of siRNA technology in cancer treatment, few points are of significant consideration such as size of delivery vehicle, easy penetration to the cellular membrane and avoiding degradation by exonucleases in cytoplasm.[17,18] Various types of nanomaterials have already been shown to solve the aforementioned problems. Therefore, this review has focused on the various types of nanomaterials used for successful siRNA delivery into mammalian cells/tissues.

Nanomaterials in gene delivery

Nanocarriers designed for gene delivery can be synthesized from variety of materials including polymers, dendrimers, liposomes, carbon nanotubes, metal and metal-oxide nanoparticles. Nanomaterials exhibit shape, size and composition dependent properties thus can be used in different ways for siRNA delivery. Moreover, the unique optical and physicochemical properties of genes carrying nanomaterials can also be used to destroy cancerous cells which can make the gene therapy more effective.[19]

Liposomes

Liposomes, lipids arranged in lamellar structure, due to their biocompatible and biodegradable nature, have been used for many pharmaceutical and medical applications. Positively charged (cationic) liposomes are best suited for negatively charged nucleotides (DNA and RNA) delivery across the mammalian cell membrane which is generally impermeable to free nucleotides. For short interfering RNAs (siRNAs) delivery, cationic lipid molecules or positively charged liposomes have been used for efficient delivery of short and double stranded RNA molecules which could be incorporated in the RNA induced silencing complex (RISC).[20] Once incorporated in RISC, one strand (sense strand) of the siRNA escapes from complex whereas antisense strand remains attached to the RISC. The antisense strand further serves as a template to bind with complementary messenger RNA (mRNA) which can ultimately be cleaved off resulting in knockdown of the concern protein coded by the mRNA.[21] Several methods have been tried so far to ensure better delivery of siRNA to mammalian cells/tissues. Among them the easiest method is simple mixing of cationic liposomes or lipids with anionic siRNA which results in electrostatic complex formation.[17] Due to stearic hindrance PEGylated lipids molecules in liposome formation result in unilamellar liposome; however, non-PEGylated lipids produce multilamellar structure. Although, multilamellar and non-PEGylated liposomes show advantage over PEGylated liposomes by providing better shielding and thus stability to RNA from serum, reports show that these complexes face difficulty in delivering RNA at targeted site due to compact complexation.[22] Additionally, non-PEGylated liposomes are shown unstable in blood circulation due to protein corona formation.[23] On the other hand, PEGylated liposomes show long circulation in blood as surface exposed PEG molecules protect liposomes from serum proteins and RES recognition.[24] Recently it has been demonstrated that cationic liposomes having high density of PEG result in inadequate complexation of siRNA and face problem with premature release of siRNA when suspended in biological suspensions such as blood stream. Thus, a low density PEG containing liposomes would be ideal siRNA nanocarrier. Non-targeted liposomes face problems with non specific distribution or extended blood stream circulation of nanocarrier which finally gets excreted from body without delivering the siRNA.[25] Therefore, nanocarriers designed to target the specific cells/tissues are required to achieve better results. Thus, liposomes modified with specific antibodies show retention at tumor site thus good anti-tumor efficacy. Antibodies and other biomolecules are frequently used to target specifically the tumor environment. Several methods have been employed to successfully conjugate desired antibody over liposome surface. Among them maleimide based conjugation is easy, so frequently being used. Maleimide-conjugated-DSPE-PEG molecules can be incorporated into liposome bilayer, where maleimide group can be used to covalently bind thiolated antibody to liposome surface.[22]

After successful incorporation of siRNAs in liposomes, cellular uptake and endosomal escape are important steps which would avoid degradation of gene in acidic environment of lysosomes. Therefore, overcoming the endosomal escape strategies are of particular importance. Many devices have been designed to solve this, including membrane-disruptive peptides, some polymers, or fusogenic lipids (Fig. 1).[26-31] Additionally, some methods include the treatment with lysomorphic agents or photochemical internalization.[32-34] Similarly, Kusomoto *et al*. performed a study to confirm that transfection efficiency greatly depends on the type of PEG lipid-anchor used.[35] They found that cholesterol-anchored PEG showed >100 fold DNA transfection activity and enhanced endosomal escape of liposomes. Other strategies involve steps to modify gene carriers to deliver genes directly at the tumor site using target-specific-ligands such as antibodies, growth factors, peptides, transferring, and folate and cell penetrating peptides (including signal sequence-based peptides, and TAT-derived or arginine rich sequences.[36-43] To boost the targeting, Jiang *et al*. demonstrated that a ternary complex containing folic acids, cell-penetrating peptide octaarginine and target gene were composed with β-cyclodextrin and low molecular-weight polyethyleneimine which showed efficient gene delivery to tumor tissues.[44]
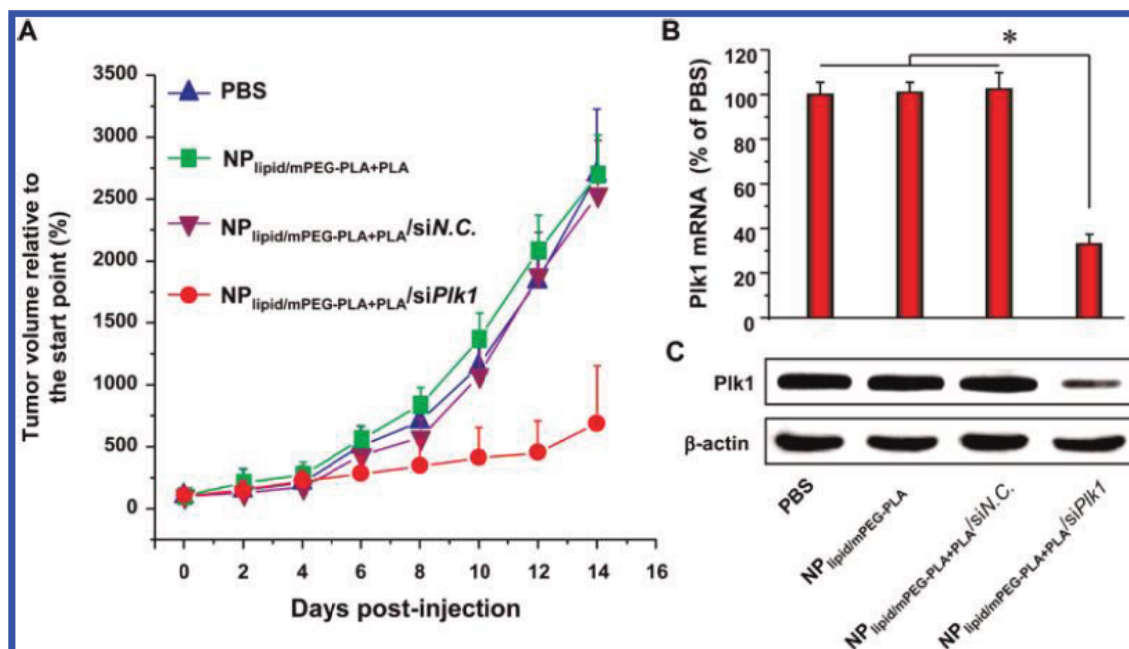
Fig. 1. (A) Inhibition of tumor growth in a murine model with BT474 xenograft after treatment with various formulations (n=6) of cationic lipid-polymer hybrid nanoparticles based systemic siRNA delivery. Analysis of expression of Plk1mRNA (B) and protein (C) in tumors. Reprinted with permission from Ref. 31. Copyright 2012, American Chemical Society.

Organ accumulation is another major obstacle for gene therapy under *in vivo* condition of treatment. Therefore, approaches are required to construct a delivery system which could avoid the deposition in organs but promote the circulation time in blood. In an attempt by Hatakeyama *et al.*, development of a system for nucleic acid delivering nanocarrier by creating a PEG-peptide-DOPE (PPD) that ultimately cleaved in a matrix metalloproteinase (MMP) showed sucess.[45] Here MMP helps in retention of nanocarrier at tumor region thus facilitating the better delivery of targeted nucleic acid. They did not observe any hepatotoxicity or inane immune system which could be correlated with lesser accumulation of nanocarriers in liver and spleen.

Pros and cons of liposomes as gene delivery system

Cationic liposomes despite of being biocompatible, biodegradable and easy to synthesize and store for longer periods of time, exhibit toxicity when used in high amount required to deliver the high pay load of siRNA into mammalian cells. Encorporation of neutral lipids have recently been found to be effective by exhibiting low toxicity. Lack of size control is another limitation with liposomes which largely controls the toxicity, uptake, retention (EPR effect) and organ tissue entrapment of liposomes.

Nanoshells

Nanoshells can be described as thin coatings deposited on the core particle of different materials. These special nanostructures have recently gained considerable attention due to their unusual properties being completely different from their single-component counterparts. Their properties can be tuned by simple variation in core-shell material ratio. Earlier synthesis of monodisperse nanoshells of desired material with expected core-shell ratio was tedious; however, emerging novel techniques and advanced synthesis procedures have made it possible to prepare monodisperse nanoshells of desired shape, size and composition. Due to their extraordinary ability to absorb light in near infra red region (i.e. between 800 nm and 1200 nm), monodisperse nanoshells produce photo-thermal effect in their surrounding environment.[46] Therefore, discharge of carrying nucleic acid can be optically controlled by photothermal effect when nanoshells are illuminated at their resonant wavelength resulting in increase in local temperature due to heat generation by light absorption. When rise in temperature exceeds the critical solution temperature, it leads in nanoshell disintegration followed by the release of encapsulated agents. In an attempt by Huschka *et al.*, genes responsible for green fluorescent protein (GFP) expression in human lung cancer cells (H1299) were knocked down by delivery of GFP gene specific siRNA (siRNA-GFP) carried by poly-L-lysine coated gold nanoshells followed by NIR light (~800 nm) exposure.[47] Braun *et al.* also used gold nanoshells to deliver siRNA by irradiating them with a NIR laser and reported to successfully knock down the gene of interest (GFP) (Fig. 2).[48] They found out the release of siRNA can be

controlled by laser power and time; however, escape of siRNA from endosome was critical and required energy above the critical pulse energy attributed to local heating effect. Thus, functionalized metallic nanoshells have potential to act as powerful nanoplatform in gene delivery applications. However, synthesis and characterization of novel types of nanoshells which could carry a high payload of genes to the targeted cells are still in their early stages. Major hindrance in nanoshells-mediated siRNA delivery is the easy degradation of thin shell which carries the genes of interest. Thus, a premature release of genes happens that ultimately results in inadequate treatment. Therefore, efforts are required to tune the properties of nanoshells as they can identify specific malignant tissues seated either in deep tissues of tumor of superficial and maintain their compact structure within the aqueous environment of cancerous cells to ensure better delivery of genes of concern. As a result, further progress of this area will significantly transform the prospects of cancerous gene knockdown in tumor cells and inhibit the progression of tumors.

Pros and cons of nanoshells as gene delivery system

Hollow nanoshells exhibit high surface area and internal reservoir which have been found to encapsulate the siRNAs or other therapeutics. Additionally, hollow nanoshells of gold show unique optical/photothermal properties that could be controlled to tune the delivery of siRNAs upon irradiation with near infra red light only. Thus, nanoshells offer "leakage free" nanocarrier in solution or in blood, minimizing the systemic exposure for encapsulated and potentially toxic therapeutics. However, the stability of nanoshells is major drawback in this approach, as the thin shell could be broken under physiological suspensions which might leak out the loaded therapeutics, leading to severe side effects.
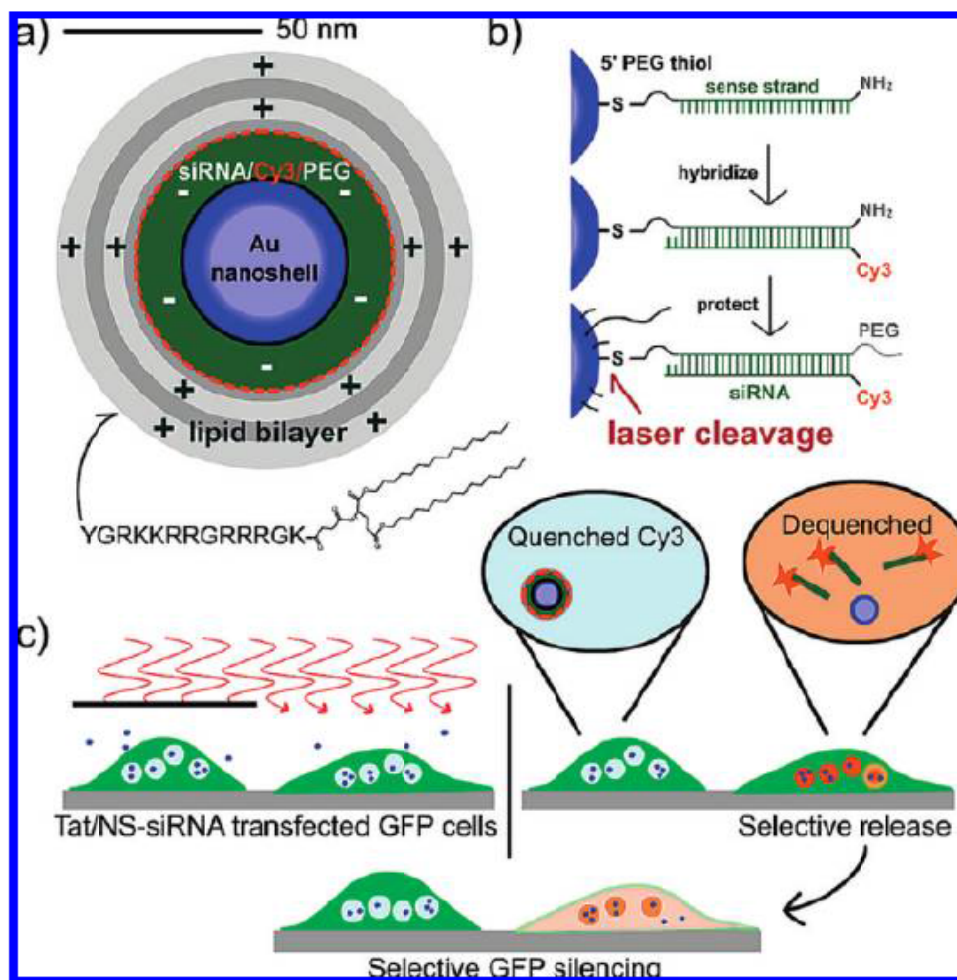
Fig. 2. (a) Diagram of TAT-lipid-coated-NS-siRNA used for transfection and selective release of siRNA. (b) Schematic of the siRNA construct. (c) Scheme of gene knockdown using laser. Reprinted with permission from Ref. 48. Copyright 2009, American Chemical Society.

Fullerenes

Fullerenes are carbon nanoclusters with unique hydrophobic spherical structure. Their exclusive nanostructure is the basis of several unusual properties such as high chemical reactivity, redox property and photosensitivity.[49,50] For biological applications fullerenes can be functionalized with proper hydrophilic residues which can make fullerenes soluble in water.[51] These hydrophilic residues could be amino, hydroxyl and carboxyl residues, which can be attached to the surface of fullerenes by chemical reactions to produce highly water soluble fullerenes.[51] Recently, the synthesis of amphipathic fullerene nanostructures has made them a potential candidate for gene delivery because they can successfully form a complex with genes of interest effectively. In general, fullerenes are made multifunctional by synthesizing derivatives of cationic molecules such as aminofullerenes, poly-N-N-dimethylfulleropyrrolidinium and tetra(piperazino) fullerene epoxide which could effectively deliver the gene of interest under *in vivo* conditions. Some reports show gene complexation and delivery by gene-functionalized fullerenes better than that of commercially available lipid based vectors.[52-54] Fullerene-based gene delivery systems have found to be non toxic than the cationic liposomes or lipofectamine-based common transfection reagents. The reason could be the multifunctional nature of fullerenes which can accommodate more genes to carry than cationic liposomes or lipofectamine reagents. This could greatly affect the toxicity due to the high amount of reagent used for the same extent of transfection. Mechanistically, fullerenes form a protective sheath over bound DNA which protects it from external DNA degrading molecules such as serum, thus increasing the lifetime and chances to incorporate with chromosomes.[53,55] The release of DNA from fullerenes into cytoplasm may occur either due to degradation of fullerenes or loss of binding ability of fullerene functional groups with DNA. Despite great success of fullerenes in gene delivery and non toxicity in the mammalian cells/tissues, further evaluations of long term toxicity must be undertaken to apply the fullerene-based gene delivery systems for clinical testing followed by human use.

Pros and cons of fullerenes as gene delivery system

Despite aforementioned advantages over gene delivery, fullerenes indicate extreme toxicity in mammalian cells/tissues. Due to their hydrophobic nature, they tend to form aggregate into cytoplasm thus accumulate in vital organs. However, thick coating of polymes/lipids or other biomolecules have found to make them biocompatible; non-biodegradable nature poses long term toxicity.

Carbon nanotubes

Carbon nanotubes (CNTs) represent a class of nanomaterials that contain features suitable for different possible biomedical applications including drug and gene delivery capability. CNTs and their bio-functionalized derivatives have shown compatibility with aqueous environment and non toxicity in mammalian cells/tissues. These properties have made functionalized CNTs appropriate for exploring various applications such as drug and gene delivery. Both single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs) have been modified with positively charged biomolecules such as ammonium group and cationic amino acid lysine, which lead to easy complex formation with genes of interest.[56] Reports have indicated that functionalized positively charged carbon nanotubes can condense DNA efficiently, however both nanotube surface area and charge density are critical parameters that determine the interaction and electrostatic complex formation between CNTs and DNA of interest.[56] Positive charge present on nanomaterials have been considered as toxic to the mammalian cells; therefore, CNTs modified with polymers have been found successful for gene delivery applications. In some instances, native molecules have been found more toxic than after functionalization with CNTs. For example, PAA (polyamidoamine) and PEI (polyethyleneimide) are more toxic to the mammalian cells when naked however, they were found less toxic when grafted on CNTs.[57] Further, PAA- or PEI-functionalized CNTs also showed better transfection efficacy than PAA or PEI alone. Due to the non toxic nature and the ease of complexation with DNA/genes of interest, CNTs have shown successful delivery of genes in the treatment of diseases such as brain ischemic insult and cancer.[58,59] Other cationic proteins such as protamines have been incorporated into the CNTs with siRNAs to ensure the nuclear localization for better transfection efficiency.[60] It has been shown that protamines act as a bridge between negatively charged siRNA and positively charged CNTs, so further strengthening the complex and decreasing the toxicity. Another novel concept was introduced by Chen *et al.* where they functionalized CNTs with DSPE-PEG-Amine for easy wrapping of negatively charged siRNA (MDM2) to induce apoptosis in breast cancer cells with a transfection efficiency of 83.5%.[61] In another approach, CNTs were made positively charged by functionalization with polyamidoamine (PAMAM) and showed transfection of GFP genes into mammalian cells.[62] This hybrid nanoconstruct was colloidally stable in aqueous solutions with good transfection efficiency and 38% lower toxicity in HeLa cells when compared with CNTs or PAMAM alone.

Pros and cons of fullerenes as gene delivery systems

Easy functionalization of CNTs makes them cationic which can be used for easy wrapping of siRNAs or other target nucleic acids. However, non biodegradable nature of CNTs raises the cytotoxicity concerns due to deposition in major organ systems.[63] Therefore, comprehensive research is needed before CNTs could be employed for gene delivery in humans. There are a few toxicity data available so far in clear characterization before and after CNTs' sample preparation, limited information about sample preparation, lack of valid positive and negative controls and limited numbers of test parameters examined (Table 1).

Table 1. siRNA delivery by nanomaterials

Nanoparticle type	Gene of interest	Cell line	Result
Liposomes	si-MDR1 (multi-drug resistant gene)	Osteosarcoma (KHOS) and Ovarian cancer (SKOV-3)	Efficient delivery with 5-10 fold higher anti-proliferative activity at 50% inhibitory concentration than free doxorubicin in MDR cells.[64]
Lipid and dextran based polymeric nanoparticles	si-MDR1	Osteosarcoma (KHOS (R2))	Significant suppression of p-gp expression in drug resistant cells.[65]
Liposomes	si-GFP	H411-E and HEPG2	Reduction in GFP protein expression without any cytotoxicity.[66]
Liposomes	si-VEGF	A431 and MDA-MB-231	Effective si-VEGF and GFP plasmid delivery.[67]
Gold nanoshells	si-GFP	H1299 (Lung cancer)	Light triggered si-GFP delivery resulted 47-49% GFP downregulation.[47]
Gold nanoshells	si-GFP	Human cancer	Laser mediated delivery of si-GFP lead to reduced GFP expression.[48]
Fullerene	si-GFP	NIH3T3 and HEK293	Better transfection and significant reduction in GFP expression.[68]
Fullerene	si-EGFP	Female C57/BL6 mice model	Successful delivery of insulin-2.[53]
Carbon nanotubes	si-Caspase-3	Ischemic stroke	si-Caspase-3 delivery reduced neurodegeneration.[58]
Carbon nanotubes	si-hTERT	PC-3 and *in vivo* model	High anti-tumor activity nanotubes cells and in vivo tumor model.[69]
Dendrimers	si-EGFP and luciferase gene	HEK293 and HeLa cells	Low toxicity, low cost and high transfection efficacy.[70]
Dendrimers	si-HIV-1 and si-NEF	SupT1 and PBMC	Efficient delivery and transfection in CD4-T cells as a potential therapy for HIV-1.[71]
Quantum dots	Thymidine Kinase genes	HeLa cells	Apoptosis was induced in HeLa cells by TK gene delivery thus anticancer activity was observed.[72]
Quantum dots	si-HPV18E6	HeLa cells	si-HPV18E6 caused silencing of targeted gene and QDs mediated fluorescence used for intracellular imaging.[73]
Spherical gold nanoparticles	c-myc protoncogene	*In vitro* models, *in vivo* (hydra), *in vivo* (mouse)	This bio-chemical approach was self tracking, non-toxic method for therapeutic RNAi.[74]
Spherical gold nanoparticles	cy5-si-RNA and si-MDR1	HeLa and MCF7	Decreased expression of cy5 and MDR1 genes.[75]
Fe_3O_4	si-VEGF	HUVECs	VEGEF gene delivery was monitored by MR imaging.[76]
Fe_3O_4	si-EGFP	Cancer cells	Cell penetrating peptides were used to enhance gene silencing and MR imaging.[77]

Dendrimers

Dendrimers are large complex molecules with well-defined chemical structures, and nearly perfect monodisperse macromolecules. They are highly symmetric and spherical compounds. Due to having extensive branching systems, dendrimers are dominated by functional groups at their surface which have been exploited to make dendrimers multifunctional for various delivery applications. Unlike polymers, dendrimers can be made hydrophilic by modifying the surface functional groups with charged species or hydrophilic groups. Thus the controllable properties of dendrimers can tune their toxicity as well which make them suitable candidate for biomedical applications. Surface functional groups of dendrimers can be modified to carry positive charge. They have shown to conjugate siRNA followed by successful delivery to mammalian cells/tissues. The repeated functional groups and symmetrical structure make dendrimers better candidate to effectively encapsulate the genes of interest and high payload delivery to targeted sites. Dendrimers are highly flexible, so they can be made amphiphilic using hydrophobic alkyl chain and hydrophilic polyamidoamine, which can produce the molecule carrying lipid and dendrimers properties. Such combined vector molecules have shown the delivery of heat shock protein 27 siRNA which could show efficient gene silencing in prostate cancer models under *in vitro* and *in vivo* experimental model systems.[78] Similarly, PEG modified PAMAM dendrimers were shown to protect the encapsulated siRNA from RNase better than parent dendrimers as well as lipofectamine-2000. These dendrimers showed high transfection efficiency for siRNA and plasmids and are comparable to the lipofectamine-2000.[79] Organ specific gene delivery have also been indicated to be possible by dendrimers through synthesizing lactosylated dendrimers-cyclodextrin conjugate which could selectively deliver siRNA to hepatic tissues for FAP (familial amyloidotic polyneuropathy) treatment in both *in vitro* and *in vivo* model systems.[80] Recently Lee *et al.* have reported the synthesis of dendrimers based delivery vehicle made up of RNA interference polymers self assembled into nanoscale pleated sheets of hairpin RNA forming sponge-like microspheres.[81] Upon entry into cell cytoplasm, these RNAi-microsponges are processed by cellular RNA machinery and converted into stable RNA hairpin to siRNA, thus presenting a novel strategy to provide protection for siRNA during delivery and transport to the cytoplasm. Therefore, the central reason for dendrimers to effectively deliver genes of interest is positive charge present on the surface which makes the protective and effective encapsulation for siRNA. However, established reports have shown that cationic nanoparticles are toxic to the mammalian cells. Therefore, strategies are required to lower the toxicity, which they could be achieved using neutral dendrimers-based nanoparticle systems. Such an attempt was performed by Liu *et al.* by replacing the terminal amines of dendrimers with hydrazine and N-acetylgalactosamine ligands. Thus, it produced neutral glycosylated dendrimers carrying siRNA of interest.[82] The so-obtained dendrimers were complexed with siRNA at pH 5 through electrostatic interaction, however, dendrimers were found neutral at pH 7. Thus, neutral dendritic system showed a new paradigm for designing siRNA delivery systems with better biocompatibility and targeting capability.

Pros and cons of dendrimers as gene delivery system

Dendrimers, no doubt, contain very high surface area required to carry high concentration of siRNA to its target tissues. Dendritic scaffolds have been found suitable carrier for a variety of therapeutics materials with a capacity to improve the solubility and bioavailability of poorly soluble drugs etc. However, the application of dendrites in biological systems is constrained due to the inherent toxicity associated with them. Dendrimer toxicity for biological systems occurs due to their positive charge which interacts with negatively charged surface of biological cells/tissues leading to hematological toxicity.

Quantum dots

Quantum dots are extremely small (1-10 nm) particles with excellent optical characteristics that make them to be applied widely in the area of life sciences. Quantum dots are exceptional candidates for imaging which allow them to overcome the limitations of conventional fluorescent probes such as fluorescent proteins and organic dyes.[83] However, since they are made up of heavy metals, very often they undergo leaching of constituent metals under biological environment, thus causing toxicity in mammalian systems. These toxic properties of quantum dots have recently attracted lots of scientific attention all over the world.[84] Recently introduced strategy of modification of quantum dot surface with different ligands such as mercaptopropionic acid (MPA), N-acetyl-l-cysteine (NAC), and glutathione (GSH) made them comparatively non toxic for bacterial and mammalian cells.[85] Surface modified quantum dots have been so designed to overcome cellular barriers in siRNA delivery such as siRNA protection, cellular penetration, endosomal protection and release and intracellular transport followed by gene silencing.[73] Quantum dots, coated with beta-cyclodextrin and coupled to amino acids, have shown successful siRNA delivery to targeted cells.[86] In another attempt, quantum dots' surface capping molecules were replaced with dihydrolipoic acid (DHLA) derived with an amine terminated PEG spacer, which provided strong coordination to the quantum dot surface and increased stability in aqueous media with conjugation to the siRNA element through amine group. This

nanoconstruct was able to selectively inhibit the expression of epidermal growth factor receptor variant III (EGFRvIII) in targeted human U87 glioblastoma cells.[87] Although surface modifications of quantum dots give them transient biocompatibility, the heavy metal core material is still intact and would be released in biological system during degradation process. This poses the same toxicity concerns about quantum dots as naked ones. Therefore, in order to harness full potential of quantum dots for biomedical uses, there is an urgent need of development of strategies to develop simple and straightforward methodology for the synthesis of non toxic quantum dots devoid of heavy metals. To address these issues, recently, Subramaniam *et al*. have developed a novel sonochemical strategy for high throughput synthesis of a library of biocompatible Zn_xS-$Ag_yIn_{1-y}S_2$ (ZAIS) quantum dots. It could be used as multifunctional nanoparticles for the simultaneous imaging and effective delivery of siRNA to brain tumor cells with negligible cytotoxicity.[88] Furthermore, recently discovered cadmium free quantum dots (CFQD) are another attractive candidates. They are made from rare earth doped oxide colloidal phosphor nanoparticles and show tuneable excitation and emission wavelengths. Although CFQDs can be synthesized under aqueous conditions and offer great biocompatibility, they have not been used in gene delivery applications so far. The oxide surface can be used for chemical functionalization, thus conjugation of siRNAs for effective and biocompatible gene silencing applications. Successful implementation of this technology would make full potential use of QDs in biological applications such as imaging, targeting, diagnostics and gene/drug delivery.

Pros and cons of quantum dots as gene delivery system

Despite having excellent optical properties including broad range excitation, size tunable narrow emission spectra and high photostability and easy surface modification with ligands of interest, QDs face problem in leaching their constituent core material which are mostly heavy metals. Recent research has shown that a thick coating of biomolecules can minimize the leaching thus toxicity. Thick coating can add stability to siRNAs to be delivered against RNA degrading enzymes under *in vivo* condition. However, thick coating will affect the particle size which can alter the properties of siRNA carrying QDs.

Gold nanoparticles
Gold nanoparticles (AuNPs) are highly attended in the biomedical applications especially in bio-diagnostic, bio-imaging and targeted delivery of targeted genes for efficient disease therapy including cancer. The unusual optical properties such as localized surface plasmon resonance (LSPR) under visible range make AuNPs an ideal candidate for bio-diagnostics and other medical applications.[89] Although biofunctionalization is a pre-

requirement for any nanoparticle system prior to their integration in diagnostic applications, AuNPs surface is easy to functionalize and give aggregation-free conjugation of bio-macromolecules. Size and shape controlled easy synthesis of monodisperse AuNPs offers great promise as intracellular delivery of therapeutic delivery vectors. Well described surface properties make AuNPs best suitable particle system for many biomedical applications where selective cell and nuclear targeting are desirable. Non-toxic nature of AuNPs makes it further appropriate for *in vivo* experimental conditions.[90] High reduction potential of Au keeps AuNPs intact during travelling in blood stream, where PEG molecules capping AuNPs show extended retention and make NPs non-opsogenic and stealth. Pre-synthesized AuNPs' surface has been modified to ensure the delivery of variety of siRNAs into mammalian cells in which AuNPs predominantly have undergone surface chemistry modification. In-situ synthesis of AuNPs in presence of targeted capping molecules have recently showed better promises to anchor biomolecules, for e.g. AuNPs reduced and stabilized by chitosan forming a positively charged AuNPs.[75] Further surface modification with PEI (polyethyleneimine) made AuNP system to effectively wrap siRNA electrostatically. Similarly, charge reversal functional AuNPs were shown to carry siRNA and plasmid DNA into cancer cells. The so prepared AuNPs-siRNA complex protected the encapsulated siRNA.[91] Other molecules grafted on AuNPs surface which could show the targeted siRNA delivery are cysteamine, PEI-hyaluronic acid conjugate, hepatoma-derived growth factor, protamines, chitosan and poly-L-lysine.[75,92-96] Lipid-plasmid DNA-AuNPs' hybrids have been recently synthesized and used for siRNA delivery where plasmid DNA is known to provide extra stability to the siRNA-AuNPs complex.[97]

Despite an increase in novel approaches to cancer chemotherapy, there is no cancer treatment method that is 100% effective against cancer. Development of resistance by cancer cells due to excessive usage of anti-cancer drugs and related factors such as individual variations in patients and somatic cell genetic differences in tumors, even those from the same tissue of origin are the main causes. Very frequently, acquisition of resistance to a broad range of anticancer drugs is expression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells. AuNPs have been shown to deliver the siRNAs to block the expression of these transporters thus inhibition of cancer cell growth.[98] Gold nanorods (AuNRds) are another type of AuNPs which are elongated structures and could be synthesized precisely with desired aspect ratios. AuNRds have used for their unusual NIR light absorbing property which can upon laser irradiation increase the temperature

of local tissue environment up to 45-50°C.[99] Furthermore, cancerous cells are prone to slightly increase the normal temperature of their environment and reports have shown that temperature above 40-42°C causes death in cancerous cells while keeping normal cells are unaffected as they can tolerate temperature of ~50°C. Therefore, many cancer therapeutics based on AuNRds mediated siRNA delivery have been shown to shrink tumors of several cancer types.[100-102] Many attempts have made to circumvent P-glycoprotein (p-gp)-based multidrug resistance (MDR) in cancer chemotherapy utilize siRNA delivery through AuNRds to inhibit the expression of p-gp. The AuNRds are co-administered with the anticancer drugs along with si-p-gp.[75]

Pros and cons of AuNPs as gene delivery system: AuNPs are excellent candidates for gene delivery purposes, as they can be synthesized to carry inherent positive charge without any surface molecule coating. Well described AuNPs surface properties further help to modify the surface of AuNPs with cationic molecules which can ultimately conjugate with negatively charged siRNAs by simple mixing. Easy control over AuNPs size makes AuNPs further appropriate nanocarrier for gene delivery. However, other gold nanostructures, such as gold nanorods, require surfactants to keep their rod shape structure intact. These surfactants cause toxicity when used for biological applications. Few strategies have been found to show good results where surfactants were replaced with polyelectrolytes, however not very successful.
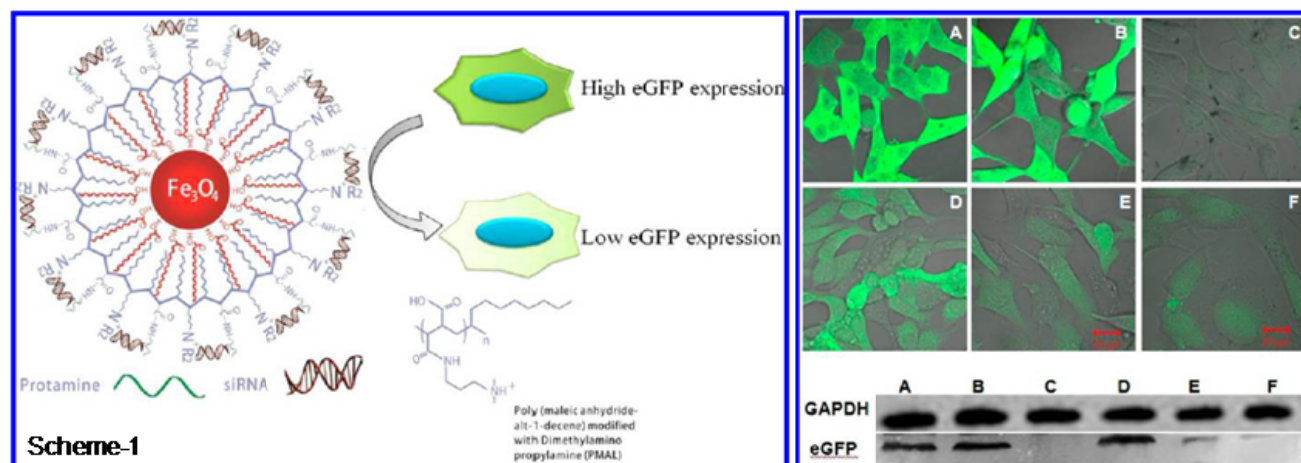
Magnetic nanoparticles

Magnetic NPs have been used primarily as contrast enhancement agents for magnetic resonance imaging (MRI). Moreover, recently novel synthesis methods and easy surface modification techniques have made magnetic nanoparticles as an effective nanocarrier for gene delivery. The biomolecules may be attached to the surface of the particles by employing cleavable linkers or doing electrostatic interactions between particles and genes of interest.[103] Alternatively, the targeted genes can be incorporated into a degradable shell present on the outer layer of nanoparticles which releases the targeted/encapsulated biomolecules upon decomposition.[104] In this persistence, drug targeting and delivery of nucleic acids by magnetic nanoparticles (magnetofection) have shown some successes. Magnetic field mediated directing magnetic nanoparticles follow simple fundamental principle. Magnetic nanocarrier,

containing siRNA/genes, delivered into blood stream can be directed to the targeted tissues by applying magnetic field.[105] Accelerated sedimentation of nucleic acids has been found to be the main cause for enhanced transfection efficiency by magnetic vectors. Thus, about 78% and 66% GFP down-regulation were found when used 32 and 8 nm sized siRNA conjugated magnetic nanoparticles, respectively. Here magnetic nanoparticles were duplexed with PEI and anti-GFP siRNA at Fe-to-DNA ratio of 1:1.[106] In another experiment, magnetic nanoparticles were used for siRNA delivery with LipoMag, consisting of an oleic acid-coated iron oxide core and cationic lipid shells in gastric tumor mice models.[107] Generally, magnetic nanoparticles are encapsulated within a polymer (Fig. 3) or metallic shell or dispersed in matrix of polymers such as silica, PVA or dextran which provides extra stability and easy conjugation to siRNA by attaching carboxyl groups, antibodies, streptavidin, etc. It is generally believed that positively charged magnetic nanoparticles easily and electrostatically attach with siRNA, thus most effective for siRNA delivery. Magnetotransfection has been found effective in delivering multiple siRNA *in vitro* and *in vivo* experimental conditions for cancer therapy under real time monitoring.[108,109] Multifunctional magnetic nanoparticles were synthesized to evaluate gene expression performance under *in vitro* and *in vivo* experimental conditions. Here magnetic nanoparticles were modified with TAT and PEG followed by encapsulation with polymeric liposomes labeled with FITC (fluorescein isothiocyanate).[110] These results showed more enhanced uptake of labeled nanoparticles in MCF-7 cells than unlabelled nanoparticles. Furthermore, investigations showed significant deposition of TAT-PEG-MPLs around the target site which confirmed the targeted delivery of siRNA in the cells/tissues of concern.

Pros and cons of magnetic nanoparticles as gene delivery system

Currently, magnetic nanoparticles have been found to be potentially applied for both diagnostic and therapeutic purposes in biomedical application researches especially in drug and gene delivery. Magnetic nanoparticles offer guided gene delivery with magnetic hyperthermia cancer therapy and magnetic resonance imaging. Along with expanding interest in magnetic nanoparticle researches, their cytotoxic potential has also been discovered. Ionization of iron from magnetic nanoparticles leads to the generation of hydroxyl radicals through fenton reaction with H_2O_2.

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Fig. 3. Scheme 1 shows strategy of the CPMN hybrid nanoparticles for eGFP siRNA delivery. (A) Silencing effect of eGFP siRNA in MCF-7 cells by MNP, CPMN and CPMN under external magnetic field. (B) CPMN with scrambled siRNA. (C) CPMN-eGFP siRNA under magnetic field. (D) lipofectamine with eGFP siRNA. (E) MNP with eGFP siRNA. (F) CPMN with eGFP. Reprinted with permission from Ref. 77. Copyright 2012, American Chemical Society.

Translational potential of nanotechnology into effective nanomedicine

Current nanotechnology research has rapidly been growing, and exploring novel methods to treat deadly diseases such as cancer. Few liposome based drug delivery agents such as Doxil, Doxosome, Estrasorb, Abelcet etc. are already clinically approved and in the market. More translations of such laboratory based nanotechnology research into clinics are required; however, due to some very common limitations with nanotechnology field itself, it is not being translated. Among them, scale-up issue and batch to batch inconsistency are the major drawbacks. Nanoparticle must be available in multi-gram quantity to perform *in vivo* work. Another area which requires great amount of work is minimizing the immune response to circulating nanoparticles. This would ultimately improve the targeting efficiency and treatment efficacy. Similarly, strategies are required to modulate the organ deposition and clearance kinetics of nanoparticles from the body. So mentioned areas require great deal of research and once the concerned questions are answered, there is no doubt about the clinical success of nanomedicine. Nanotechnology has also emerged as potential tool to overcome drug resistance by delivering multiple drugs simultaneously to the targeted tissues which is currently a major limitation ahead. Recent research on interaction of nanomaterials with biological cells/tissues has also been utilized to maximize the clinical potential of nanotechnology for developing nanomedicines.

Conclusions

Nanotechnology has the potential for the production of safe, non-toxic and highly effective medicines for disease treatment at low concentrations. Despite enormous research and development in the area of nanomedicine, efforts are needed to exploit the full potential of this technology in biomedical applications. Smart nanosystems are required which could be easily packed and delivered to the targeted tissues of interest, without any side effects. These strategies could include design of nanosystems capped with tumor homing biomolecules capable of delivering multiple genes of interest simultaneously to target multiple signaling pathways responsible for disease development. Thus, nanomedicine has the potential to develop the system for early detection, prevention and improved diagnosis for better treatment and disease follow-up.

Competing interests

Author declared no competing interests.

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