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Advances in polymeric and inorganic vectors for nonviral nucleic acid delivery

Joel C Sunshine[1], **Corey J Bishop**[1], and **Jordan J Green**[†,1]

[1]Johns Hopkins University School of Medicine, Department of Biomedical Engineering, 400 N. Broadway, Smith Building 5017, Baltimore, MD 21231, USA

Abstract

Nonviral systems for nucleic acid delivery offer a host of potential advantages compared with viruses, including reduced toxicity and immunogenicity, increased ease of production and less stringent vector size limitations, but remain far less efficient than their viral counterparts. In this article we review recent advances in the delivery of nucleic acids using polymeric and inorganic vectors. We discuss the wide range of materials being designed and evaluated for these purposes while considering the physical requirements and barriers to entry that these agents face and reviewing recent novel approaches towards improving delivery with respect to each of these barriers. Furthermore, we provide a brief overview of past and ongoing nonviral gene therapy clinical trials. We conclude with a discussion of multifunctional nucleic acid carriers and future directions.

Nucleic acid therapies have enormous potential in the clinic, from treatment of specific genetic diseases such as cystic fibrosis [1], Leber hereditary optic neuropathy [2], hemoglobinopathies [3,4] and hemophilia [5], to the treatment of cancer [6,7], cardiovascular disease [8] and the use of genetic vaccines [9]. Additionally, nucleic acid delivery plays a crucial role in cellular engineering and basic biomedical research through the ability to knock-in and knockdown genes and proteins in the laboratory, as well as in the creation of induced pluripotent stem cells via viral methods [10,11] and investigations into the induction of induced pluripotent stem cells via nonviral [12] methods. The central challenge for effective therapy using nucleic acids is finding a safe and effective delivery system [13]. Since viral gene therapy can have serious safety concerns [14], recent efforts have focused on nonviral methods.

Nonviral methods can be used to deliver various nucleic acids (Table 1), including DNA [15], **siRNA** [16–18] for **RNAi** [19], **isRNA** [20], **shRNA** [21], **agRNA and saRNA** [22,23]. The choice of nucleic acid to deliver may influence where the nanocarrier needs to deliver its cargo (Figure 1). For example, to target Toll-like receptors (TLRs) such as

[†]Author for correspondence: green@jhu.edu.

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TLR-3, -7 and -8, isRNA should be targeted to the endosome itself [20]. siRNA needs to get into the cytoplasm; therefore, vectors that carry these cargoes, if they are trafficked through the endosome, need some method to escape it. Finally, DNA, shRNA-encoding plasmids, agRNA and saRNA all need to be further transported from the cytoplasm into the nucleus to be expressed, to interfere with, or to promote gene expression.

There are several barriers to cellular entry and delivery of the nucleic acid cargo that challenge the development of an effective delivery vehicle (Figure 1). The vehicle needs to form a stable complex with its nucleic acid cargo, protect it from degradation extracellularly, arrive at the cell of interest, get internalized (typically via either receptor-mediated endocytosis and/or nonspecific endocytic pathways), escape endolysosomal degradation, release its cargo and harmlessly degrade or otherwise be eliminated.

After escaping the endosomal compartment and making it into the cytoplasm, nucleic acids, such as DNA and agRNA, need to make it to the nucleus. This is among the largest challenges remaining for nonviral gene delivery. Simply getting the plasmid into the cytoplasm of the cell is not sufficient; in order to achieve the same level of **transfection**, delivery of up to 100-fold more DNA to the cytoplasm is required compared with direct delivery of DNA to the nucleus [24]. Dividing cells are more easily transfected due to the breakdown of the nuclear membrane that occurs during mitosis. While this breakdown can enhance localization of plasmids to the nucleus and transfection efficiency, cell division is not a requirement for successful transfection. Plasmids can also enter the nucleus through nuclear pore complexes (NPCs) when they are coupled to nuclear localization signals (NLSs; i.e., PKKKRKV), but this process is not as efficient [25].

Here we review current progress in nonviral nucleic acid delivery, with a focus on cationic polymers and inorganic nanoparticles (as well as their hybrids). Lipid-based materials for nucleic acid delivery are outside the scope of this article and are well-described elsewhere for siRNA [26] and gene delivery [27]. General properties and biomedical applications of polymeric and inorganic materials are described first. This is then followed by a discussion of new approaches to solve barriers to nonviral delivery when using these materials. Subsequently, an overview of past and present nonviral gene-therapy clinical trials is discussed.

Materials/general properties

Cationic polymers

Various cationic polymer systems have been utilized for nucleic acid delivery. A wide range of structures have been explored, including linear and branched nondegradable polycations as well as biodegradable and bioreducible polycations and oligosacharides. Some of the most commonly used polymer structures are shown in Figure 2. All of the cationic polymers have primary amine groups that are protonated at neutral pH, which enables electrostatic interaction with the anionic nucleic acid.

Poly-L-lysine (PLL) was one of the first polymeric gene transfection agents developed, and was shown to condense DNA into small complexes with rod (25–50 nm) or toroidal (40–80

nm) structures [28]. PLL can be synthesized by several-step polymerization of ε,*N*-benzyloxycarbonyl-α,*N*-carboxy-L-lysine anhydride [29]. PLL is limited for intracellular delivery by its lack of an endosomal escape mechanism, and endosomolytic groups such as histidine [30], have been used to improve delivery. To reduce serum interaction and increase cell uptake, a variety of other molecules, such as poly(ethylene glycol) (PEG) [31], and targeting ligands such as asialoorosomucoid [32], transferrin [33], galactose [34,35], lactose [36] and folate [37], have been conjugated to PLL. Poly(ethylenimine) (PEI) was the second polymeric transfection agent developed [38]. Branched PEI (b-PEI) can be synthesized from aziridine monomers under acidic conditions, and linear PEI can be synthesized by the hydrolysis of poly(2-proplyl-2-oxazoline)[39], or by polymerization of aziridine monomers at lower temperatures [40]. Compared to later generation nucleic acid delivery agents, PEI is cytotoxic, leading to necrosis and apoptosis [41]. The high proportion of nitrogen atoms provides for a strong buffering effect (`the proton sponge effect'), which is advantageous for endosomal escape, as described later. 25 kDa b-PEI has been shown to be an efficient transfection reagent with reduced toxicity as compared with higher molecular weight b-PEI [42]. For delivery of shorter nucleic acids (e.g., mRNA) low molecular weight PEI (2 kDa or less) leads to enhanced biological effect, as complexes with higher molecular weight PEI are more stable and do not release the nucleic acid as efficiently into the cytoplasm [43]. Standard PEI has also been modified by deacylation to boost delivery of DNA and siRNA by orders of magnitude *in vitro* and *in vivo* [44].

Poly(lactide-co-glycolide) (PLGA) microspheres have been used in nucleic acid delivery for their relative biocompatibility and biodegradability. PLGA is synthesized by copolymerization of cyclic dimers of glycolic acid and lactic acid with various catalysts. Microparticles can be formed from premade polymers by emulsion evaporation, emulsion diffusion, solvent displacement and salting-out techniques, and particle size depends on the formulation conditions and molecular weight of the starting material [45]. Both the polymer and its degradation products are well tolerated in animal studies [46,47]. PLGA has recently been used to deliver siRNA *in vivo* and achieved sustained gene silencing when delivered to the vaginal mucosa [48].

Poly(β-amino ester)s (PBAE) are synthesized by Michael addition of either primary or bis(secondary) aliphatic amines to diacrylate compounds [49], and their simple chemistry leads them naturally to a combinatorial approach to synthesis and screening of polymer libraries [50–54]. They are hydrolytically degradable at the backbone ester linkages, which allows for release of nucleic acid cargoes and reduced cytotoxicity.

As opposed to mostly linear, crosslinked or other branched systems, dendrimers such as poly(amido amine) (PAMAM) are synthesized iteratively to produce nanoscale structures characterized by dendritic connectivity and radial symmetry. Advantages of dendrimeric systems include precise, nanoscale, structural control, dense and tunable surface chemistry (for addition of targeting ligands, modification of surface charge and so on), and high-charge density for complexation and buffering. PAMAM dendrimers were first synthesized in the mid-1980s [55]. Typically, ethylenediamine or ammonia are used as cores and allowed to undergo repeating two-step reactions whereby methyl acrylate is added by Michael addition to all the primary amines, and then the ester groups are amidated by a large excess of

ethylenediamine to produce primary amine termini. They have been extensively studied for gene delivery [56,57] as well as oligonucleotide delivery [58–61]. Interestingly, thermal degradation of the dendrimers was shown to increase transfection efficacy [62]. Dendrons, rather than full dendrimers, have also been used for successful gene delivery [63]. Mannose–PEG–PAMAM linear-dendritic hybrid polymers successfully delivered the luciferase gene to P388D1 murine macrophages bearing the mannose receptor, and demonstrated a 1.6–1.8-fold more efficient transfection of these cells than PEI with no the presence of serum; this boosted transfection was shown to be targeting-ligand dependant [63].

Oligosaccharides

Sugars are crucial in a wide variety of biological applications. They are hydrophilic molecules composed predominantly of carbon, hydrogen and oxygen, and exist both in ring form as well as in extended conformations. Every extra cellular protein in the human body is glycosylated (addition of oligosaccharides to proteins). The ABO blood group antigens are oligosaccharides, and oligosaccharides play a crucial role in tethering and rolling via the interaction of selectins to sialyl-lewis X [64]. Glycosylation is a crucial consideration in the production of monoclonal antibodies (mAb) for therapeutics in terms of optimization of biological activity [65] and improved pharmacological profile [66].

Due to the hydrophilic nature of oligosaccharides and the fact that sugars are relatively well tolerated by the body, cationic polysaccharides have been explored for gene and nucleic acid delivery. Cyclodextrins (CD) are produced by the degradation of starch by the enzyme glucosyl transferase. This generates natural cyclic oligosaccharides composed of 6, 7 or 8 d(+)-glucose units known as α, β and γ CDs, respectively. CDs are of particular interest because, in addition to having low toxicity and good biocompatibility, they can form inclusion complexes with small, hydrophobic compounds. This ability allows for modification of the surface of the CD-based particles without interfering with polycation–nucleic acid interactions and particle morphology [67]. Polycationic CDs have been shown to transfect cells in serum in a comparable level to 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) [68]. Grafting of CDs onto a PEI polymer leads to reduced transfection efficacy depending on the extent of modification (increased modification leads to further decreases in transfection efficacy), but also leads to significantly reduced toxicity [67]. Interestingly, however, grafting of CDs onto PAMAM dendrimers increased their transfection efficacy (100× that of the dendrimer alone and comparable to Lipofectin® and TransFast™); the optimal formulation used α-CD [69].

One excellent recent example of utilizing CDs for nucleic acid delivery currently in clinical trials is CALAA-01. CALAA-01 is a transferrin-targeted, PEGylated CD-containing polymer for siRNA delivery [70,71]. The CD-containing polymers are synthesized by condensing two difunctionalized comonomers. In addition, imidazole end-group modifications were added to enhance endosomal escape [72]. Interestingly, separate *in vivo* studies using earlier constructs for DNA and DNAzyme delivery demonstrated that nanoparticle localization to the tumors was independent of the targeting ligand, but addition of the targeting ligand increased tumor-cell uptake [73,74]. CALAA was later formulated for siRNA delivery, and after animal studies was used to treat the first patient in a Phase I

clinical trial in May 2008 [72]; this trial was able to provide evidence of inducing an RNAi mechanism of action in a human from the delivered siRNA for the first time [71].

Another sugar used significantly in drug and nucleic acid delivery is chitosan. Chitosan is formed by the deacetylation of chitin. It is mucoadhesive and biodegradable, with a reasonable toxicity profile, and has been specifically useful in transmucosal drug delivery [75]. Oral administration of chitosan and *Arah2* (a peanut allergy gene) was shown to significantly decrease IgE levels in response to anaphylaxis induction [76]. *N*-alkylated chitosan was investigated for gene delivery, and lengthening the side chains to eight carbons was found to improve gene delivery [77]. The optimal trimethyl chitosan–cysteine conjugate showed 1.5-fold higher *in vitro* and 4.1-fold higher *in vivo* transgene expression when compared with Lipofectamine 2000 [78]. Imidazole-modified chitosan siRNA delivery demonstrated good gene knockdown in the lungs and the liver [79]. Chitosan hydroxybenzotriazole showed approximately 60% knockdown of the control-enhanced green fluorescent (GFP) protein gene expression [80].

Inorganic materials

Many types of inorganic nanoparticles are in use today for gene therapy and have properties that can be exploited for multifunctional use (i.e., theranostics – therapy and diagnostics). A large portion of these are gold nanoparticles (AuNP; Figure 3A–C) [81–97], carbon nanotubes (CNTs; Figure 3D & E) [98–103], silica (Figure 3F) [98,104–108], quantum dots (QDs; Figure 3G) [109–116], superparamagnetic iron oxide nanoparticles (SPIONs; Figure 3H) [115,117–120], and layered double hydroxide nanoparticles (LDHNPs; Figure 3I) [121].

AuNPs

Gold nanoparticles' structures can be solid spheres (Figure 3A), rods (Figure 3B) or shells (Figure 3D). Many investigators synthesize AuNP spheres by dissolving tetrachloroauric acid ($HAuCl_4$) in purified water and then adding a reducing agent (i.e., sodium borohydride or sodium citrate) converting Au(III) to its neutral and solid form [81,84–86,122]. To make gold nanorods (AuNR), a seed solution and a growth solution can be prepared separately and mixed. A possible seed solution uses cetyltrimethyl ammonium bromide (CTAB), $HAuCl_4$ and $NaBH_4$, and a possible growth solution uses CTAB, $AgNO_3$, $HAuCl_4$ and ascorbic acid [87]. When AuNP shells are synthesized, a shell forms around a core. Investigators have used the reverse-micelle method [88] or the Stöber method [89] to synthesize AuNP shells. AuNP shells have encapsulated SiO_2 [90], Fe [88,91], Pt [83] and other materials. Other ways to synthesize AuNPs are by citrate reduction or the Brust-Schiffrin method as reported by Daniel *et al*. [92]. The timing and the relative amounts of reagents used can be varied to tune the size of the AuNPs [92]. AuNPs are known to have low cytotoxicity, can be synthesized with decent monodispersity, and can be conjugated at high densities with a wide range of organic molecules [93,94]. AuNPs can be functionalized with antibodies for the detection of molecules. The detection is made possible because AuNPs have surface plasmon resonance effects that scatter light at various intensities and absorb light at different wavelengths depending on their size and degree of aggregation [95]. For example, the high expression of a surface receptor (i.e., folic acid receptor [107], EGF receptor [95] or HER2

[89]) due to the presence of a cancer can cause an increase in AuNP–antibody local concentration, which causes a shift in optical properties and detection. In addition, AuNPs do not undergo photobleaching [95].

Furthermore, AuNPs can be used to localize photothermal cancer therapy. Spatially localizing the thermal therapy minimizes collateral damage. The AuNPs can be excited at near-infrared wavelengths to produce heat in an aspect ratio-dependent manner [82,89,96]. The near-infrared light can be applied externally as biological tissue does not attenuate the energy significantly, which allows for control over timing of the thermotherapy.

Fullerenes

Fullerenes are carbon-only nanostructures (i.e., spherical, cylindrical and ellipsoidal). The two most common nanostructures applied to nanomedicine are CNTs and spherical fullerenes (C-60 buckyballs) as in the following section.

CNTs

Carbon nanotubes are highly ordered and hollow [97]. They are single atom-thick cylinders of sp^2-bonded carbon atoms [97]. CNTs can be synthesized by a variety of methods including laser ablation, arc discharge, thermal chemical deposition and plasma-enhanced chemical vapor deposition [99] (for an in depth discussion of methods (see [100]). Post-synthesis, CNTs can be sonicated to a desired length with some size restraints [101]. Single-walled and multiwalled CNT diameters can be 1–2 nm and 2–25 nm, respectively [101] (Figure 3D & E). Spherical, hydrophobic carbon–nanostructures can be cationically functionalized via amine groups to become soluble and to enable ionic complexation with nucleic acids [102] for gene-delivery applications. Carbon nanostructures can be conjugated to antibodies to increase specificity [123]. The single-walled CNTs have Raman signal to improve cancer detection and near-infrared absorption for photothermal applications [123] similar to AuNPs. CNTs have unprecedented high-tensile strength [99] and CNTs' electrical and thermal conductivities make them useful for biosensor applications [99]. CNTs also have a high surface area for dense loading of cargo [50,124]. Their needle-like shape can also enable the penetration of cell membranes with greater ease [124]; however, their structural similarity to asbestos warrants further research [125]. Nanotoxicology of these materials and other nanomedicines is important to consider prior to any clinical experimentation [116].

Carbon nanotubes are cytotoxic as there is lipid membrane peroxidation due to residual metal catalysts. Owing to this toxicity, CNTs are known to downregulate adhesive proteins and increase cell death (aspect ratio dependent), but can be nontoxic to primary immune cells when functionalized appropriately [126]. PAMAM dendrimers can be used to coat multi-walled CNTs to improve biocompatibility and cellular uptake [126]. Glycodendrimers can be used to coat single-walled CNTs to lower cytoxicity as well [127]. DNA-CNT complexes have been demonstrated to be nontoxic and nonmitogenic to activated or nonactivated lymphocytes [128]. Larger carbon nanomaterials (i.e., fibers and flakes) are less toxic than single-walled or multiwalled CNTs, possibly owing to their different interaction with the cellular membrane [129]. Aggregation of CNTs can influence their

toxicity due to the alteration of their physical properties [130]. Fullerenes can be stabilized via functional groups to decrease cell death. For example, functionalization using single-walled nanotubes in 1% Pluronic® F108 at 2 μg/ml, di-carboxylation with a carbon:functional group ratio of 23:1 at 2 mg/ml, SO_3H with a carbon functional group ratio of 80:1, 41:1 and 18:1 at 2 mg/ml resulted in approximately 65, 50, 40, 15 and nearly 0% cell death, respectively [131,132].

Spherical fullerenes

Carbon-60 spherical fullerenes (buckyballs) are approximately 1 nm in diameter. They have a propensity to aggregate, and are hydrophobic but can be made to be hydrophilic by the addition of functional groups (i.e., amine and carboxyl). Hydrophilic buckyballs mainly localize in the liver and have slow metabolism [133]. Buckyballs can function as an antioxidant for neuroprotective applications [103], an antiviral agent, such as HIV, a gene delivery carrier (particularly amine-derivatized fullerenes) and as a photosensitizer for photodynamic therapy applications [133]. Buckyballs can carry an unstable atom (i.e., Gd^{3+}, ^{99m}Tc, which mainly localizes in macrophages of the bone marrow, liver and spleen), which is not released into the biological system and can then be used as an MRI contrast agent, x-ray imaging agent or radiopharmaceutical [133].

Silica nanoparticles

Mesoporous silica nanoparticles (Figure 3F) used for gene therapy are commonly synthesized using tetraethyl orthosilicate and CTAB under basic conditions using sodium hydroxide [105,106] or aqueous ammonia [134] at approximately 80°C. By varying the amount of CTAB added, the nanoparticles size can be modified [134]. Mesoporous silica nanoparticles are used in gene delivery because they have relatively large surface areas for dense conjugation, tunable pore sizes for cargo encapsulation and are surface modifiable [105]. A few nonexhaustive applications of silica nanoparticles include the delivery of nucleic acids (siRNA) by functionalization of PEI for ionic complexation [105], the delivery of GFP to osteoblasts by ionic complexation of conjugated Ca–silica [108] and the delivery of genes (luciferase) to the Achilles tendon [97]. Silica nanoparticles can activate macrophages and produce proinflammatory cytokines and reactive oxygen species [135]. Positively charged silica nanotubes are significantly more toxic than their bare counterparts. Toxicity was also significantly greater for positively charged silica nanotubes that were 200 versus 500 nm for a given mass in both HUVEC and MDA-MB-231 cell lines [136]. It was reported that the smaller particles have a greater extent of interaction with cells and, therefore, increased cytotoxicity [136].

QDs

Quantum dots are composed in pairs of semiconductor elements (i.e., ZnS, CdS, CdSe, InP, CdTe, PbS and PbTe). For example, CdSe QDs can be synthesized by adding cadmium oxide to tetradecylphosphonic acid and trioctylphosphine oxide at 300°C under Ar flow to dissolve the Cd. At 270°C, a Se solution of tri-*N*-octylphosphine is injected. The QDs can be grown at 250°C for different lengths of time to control the size. This solution can then be injected into chloroform and the CdSe will then precipitate out of solution [109]. For

biological applications, QDs are sometimes synthesized in a core-shell fashion. The shell is chosen such that the band gap is wider than the core. This improves fluorescence properties, passivates the core, and prevents leaching (i.e., of toxic Cd ions) [110]. Heavy metal ions (i.e., Cd^{2+}) are toxic at low concentrations (0.65 μM); however, when using silica shells and silane-PEGylation (methoxy[polyethyleneoxy] propyltrimethoxysilane [Gelest P/N SIM6492.7; MW = 450–600]), there was no toxicity observed at QD concentrations up to 30 μM [137].

Quantum dots' photoluminescence spectra are resistant to photobleaching [111] and emission is narrow, symmetrical and tunable as a function of core size [110]. Figure 3G shows the color dependence of different radii of CdSe core QDs [104]. The intensity of the fluorescence typically has a half-life of 27 h, which is many-fold greater than other fluorescence agents (i.e., Alexaflour, R-phycoerythrin and FITC) [110]. QDs can be carboxy lated to conjugate peptidyl amine residues or aminated by 2-aminoethane thiol hydrochloride for maleimide derivatization. Thiolated and polyhistidine-conjugated biologicals can also directly interact and self-assemble on the surface of QDs, respectively [110]. QDs have been encapsulated in chitosan nanoparticles to track and monitor siRNA delivery and transfection of SKBR3 breast cancer cells [114]. QDs have been used to quantify and monitor changes in transgene expression of two similar prostate cancer cell lines (PC3 and PC3-PSMA) due to changes in microtubule dynamics [113]. QDs have been used for multiplex fluorescence imaging, tumor cell extravasation tracking and real-time *in vivo* imaging [111].

SPIONs

Superparamagnetism relates to the stochastic magnetization changes of nanoparticles. In the absence of a magnetic field, the nanoparticles have an average magnetic state of zero. However, under an external magnetic field, magnetism is induced and their magnetic susceptibility is stronger to that of paramagnets [138]. SPION are commonly synthesized by coprecipitation in water [118]. In one example, SPIONs can be synthesized by precipitation in a reverse water-in-oil microemulsion system of water/SDS and 1-butanol/cyclohexane [119]. They can also be synthesized in an aqueous solution by coprecipitation of ferric and ferrous chlorides in alkaline medium [115]. SPIONs constitute a hydrophobic crystalline iron-oxide core that can be coated with relatively hydrophilic and/or biocompatible materials (i.e., dextran, starch, polyol derivatives, phospholipids, silica or amphiliphilic polymers) [116,117]. SPIONs can be conjugated with targeting moieties and gene-delivery carriers and Figure 3H shows doxorubicin-loaded SPIONs.

SPIONS are rapidly cleared by the reticuloendothelial system (RES; also known as the mononuclear phagocyte system). Many are primarily cleared in the liver by Kupffer cells in a nanoparticle size-dependent manner [139]. In one example (AMI-25), there is 80% uptake of the initial dose by Kupffer cells with a half-life of 10 min [140]. SPIONs can be metabolized in the hepato-renal system and are capable of entering the endogenous iron reserves by means of hematopoiesis [141]. SPIONs can be used for delivery systems via magnetofection, contrast enhancers for magnetic resonance imaging for T2 and T2*-weighted imaging [116,142], tissue repair, immunoassays, detoxification and anticancer

magnetic hyperthermia [119,120]. Iron oxide nanoparticles associated with Metafectene® and dioleoylphosphatidyl-ethanolamine delivering anti-eGFP and firefly luciferase have been known to accomplish at least 90% knockdown at 48 h post-transfection with reasonable cell viability using the HeLa cell line [143].

LDHNPs

Layered double hydroxide nanoparticles are anionic clay materials that can be generally written as $[M^{II}_{n}M^{III}(OH)_{2+2n}]^{+}$ $(A^{m-})_{1/m} \times H_2O$ (n = 2–4) ($M^{II/III}$ = di/trivalent metal cation, A^{m-} = anion) [121]. They can be synthesized by dissolving 3:1 mols of $Mg(NO_3)_2$ and $Al(NO_3)_3$ in deionized water and quickly adding NaOH [121]. Inorganic crystals of controlled sizes can be synthesized in this manner. LDHNPs can be used in cellular drug and gene delivery, are relatively biocompatible, have large cargo capacities and can be tailored to have pH-controlled release of their cargo. In one example of siRNA delivery, there was a significant knockdown to the protein target, HEK293T, and viability was maintained at 94% [121].

Nucleic acid delivery via coprecipitating mineral solutions

Macromolecules such as DNA are capable of codepositing with inorganic minerals (biomineralization) to form bioactive nanocomposites. When in close proximity to cells, these nanocomposites can promote DNA uptake via controllable surface-mediated release [144]. In one example, an inorganic mineral solution constituting of $CaCl_2$, KH_2PO_4, NaCl, KCl, $MgSO_4$, $MgCl_2$, and $NaHCO_3$ was used to effectively coprecipitate and deliver β-galactosidase DNA (Figure 4) [144].

Physical requirements

When designing gene therapy vectors it is important to note that physical properties such as size, aspect ratio, molecular weight, surface area, shape, polydispersity and zeta potential can have an impact on cytotoxicity and delivery [124]. To meet certain barrier requirements for gene delivery, surface modifications can be used to modify the physical properties of the delivery system to improve circulation time and solubility (i.e., PEGylation) [145], localization (i.e., folic acid and RGD) [146], biostability (i.e., zeta potential: amine or carboxylic groups) [145], cyto toxicity (addition of carboxyl or hydroxyl groups) [126,131], internalization and inhibition of RES clearance [93,94].

Shape/surface morphology

Recently, manipulation of particle shape has come into focus as a new method for modulating drug delivery [147]. Local shape of the particle where it makes contact with the cell and not necessarily the overall shape dictate whether or not the particle is internalized by macrophages [148]. Elongated particles have been shown to circulate longer and avoid phagocytosis more effectively than spherical particles [149]; however, spherical particles are much more efficiently internalized into target tissues as compared with elongated particles [150].

Seeking to take advantage of this property, Yoo *et al*. has recently constructed PLGA-based, shape-shifting particles (one way, from ellipsoid to spherical) in response to temperature, pH or a chemical signal and demonstrated efficient uptake of the spheres as compared with the ellipsoids (Figure 5) [151]. It is also possible to complex anisotropic faces of AuNPs with DNA oligonucleotides to form sticky patches, which allows for complicated self assembly [152].

Other nanoparticle morphologies may prove worthwhile to investigate tuning cytotoxicity and nucleic acid delivery of potential vectors. Spherical silver nanoparticles can be induced via light to transform into triangular prisms with efficiency nearing 100%. This is accomplished by irradiating a solution of silver nanoparticles with a halogen lamp at 150 W for 5 h (bandpass filter at 550 nm) [153]. DeSimone and coworkers have elegantly used soft lithography using polydimethylsiloxane and perfluoropolyether to make molds enabling nanoparticle replication in a nonwetting template. Using this, nano particles with diverse shapes can be fabricated (i.e., 200 nm trapezoidal particles, 200 × 800 nm bar particles, 3 μm arrow particles and 2.5 × 1 μm^2 hexnut particles with 1 μm holes) [154].

Nanoparticle surface morphology has recently been shown to be another important aspect of controlling nanoparticle delivery [155]. Verma *et al*. demonstrated that AuNPs with surface `ribbon-like' domains of alternating hydrophobic and hydrophilic composition were able to enter the cell without the membrane disruption associated with cationic nanoparticle systems; control particles with random surface organization were unable to penetrate cells at all [156]. Cell-penetrating peptides appear to have similar functionality [156,157]. This property should reduce the toxicity usually associated with membrane disruption [156].

Size

Polymer nanoparticles have been developed with a wide variety of sizes for different purposes. Nanoparticles of approximately 100 nm show prolonged blood circulation and a relatively low rate of mononuclear-phagocyte system uptake [158]. Particles with a 1–5 μm diameter are likely to be trapped in the liver and phagocytosed by Kupffer cells [151]. Particles larger than 5 μm in diameter are likely to be trapped in capillary beds [151]. When NPs are greater than 200 nm they are likely to be filtered in the spleen, whereas the NPs less than 100 nm are likely to leave the blood vessels through fenestrations in the endothelial lining [151]. NPs that are approximately 50–200 nm diameter have been known to accumulate in tumors by the enhanced permeability and retention (EPR) effect (as a result of leaky vasculature and the absence of a draining lymphatic system) [117,159]. It has been suggested that particles must not exceed 300 nm to take advantage of the EPR effect [101]. Nanoparticles smaller than 50 nm are more likely to enter most cells, and those with sizes smaller than 20 nm can get out of the bloodstream and into tissues [142,159]. The glomerular apparatus' capillary wall has fenestrations of approximately 4–5 nm and it has been reported that nanoparticles >8 nm cannot be filtered through the glomerular filtration system [124], as a result this would increase circulation half-life [160].

Charge

To best avoid nonspecific electrostatic interactions and escape the RES, nanoparticles should be designed to have neutral or slightly negative zeta potentials [86]. On the other hand, a positive zeta potential enhances nanoparticle–cell contact and promotes uptake and internalization through stronger affinity for anionic proteoglycans on the cell surface [161]. For example, the zeta potentials of lysine-, arginine- and histidine-modified nanoemulsions were reported to be 50, 43 and 7 mV, with transfection efficiency decreasing with neutralization of the zeta potential [162]. Some nanoparticles may be more or less cytotoxic depending on their charge (i.e., AuNPs are less cytotoxic when anionic) [163]. It is important to consider zeta potentials when complexing nucleic acids via ionic interactions. The interaction must be strong enough to condense the nucleic acid to protect against restriction enzymes. It is important to note that zeta potentials of nanoparticles can switch signs when in the presence of serum and this should be considered in the design and testing process [51,164].

Biocompatibility

Biocompatibility is crucial for maintaining an appropriate host response during gene therapy. In-depth assessments and characterizations are required to elucidate the physicochemical differences responsible for low cytotoxicity and acceptable viability. PEI lacks degradable linkages and is too toxic for therapeutic applications, inducing both apoptosis and necrosis in an endothelial cell model [165]. As a result, a number of investigators have synthesized an array of degradable PEIs consisting of low molecular weight PEIs and degradable crosslinkers, in the hopes of achieving higher efficacy with the reduced cytotoxicity of low molecular weight PEIs [166,167]. Other groups have focused on developing new biodegradable polymers for nonviral gene delivery, which we will review here by method of degradation. Biodegradable polymers should be able to both reduce the cytotoxicity associated with the transfection reagent as well as potentially improve dissociation of the vector from its cargo to allow the cargo to be utilized intracellularly.

Multiple strategies have been formulated that use ester bonds to allow hydrolytic cleavage of the polymer. Amine-containing hydrolyzable polymers have been utilized, which are effective gene-delivery agents with significantly decreased cytotoxicity as compared with nondegradable polymers such as PEI [38] and PLL [28]. These structures include PLGA [168], hyperbranched poly(amino ester)s [169], poly(lactic acid) (PLA) [47] and linear poly(β-amino ester)s (PBAEs) [49] among others. Libraries of PBAEs have been developed for gene-vector screening [52,54]. Studies have shown that amine-terminated PBAEs are more effective at pDNA transfection than acrylate-terminated versions. Modification of the polymer ends with different amines can lead to virus-like efficacy in human primary cells *in vitro* (Figure 6) [170]. Tuning of polymer end group leads to significant differences in transfection efficacy, and the optimal end-group for each cell type appears to be cell-type specific [171,172]. PBAEs also have been shown to be nontoxic to human primary cells *in vitro* [173] and in mice *in vivo* [174,175].

`Stealth' properties

The binding of serum proteins to nanoparticle surfaces after intravenous injection causes the nanoparticles to be internalized by macrophages and removed from the blood [176]. Addition of hydrophilic moieties, such as PEG, poly(*N*-(2-hydroxypropyl-methacrylamide) (pHPMA) and various oligosaccharides, have been shown to increase solubility, prolong circulation time, neutralize zeta potential and reduce interactions with the environment within the bloodstream due to a higher tolerance against incubation with serum proteins [177,178]. One disadvantage of this approach is that while it may stabilize the polyplex in serum and reduce cytotoxicity, it may also interfere with complexation and reduce transfection efficiencies depending on the extent of addition [179]. Modification of the surface of preformed particles with PEG/pHPMA that can bind to exposed surface amino groups has been shown to alleviate this problem [177,180]. Recently, Yuan *et al*. demonstrated that adding PEG to PAMAM dendrimers via *bis*-aryl hydrazone bond linkages into the vector significantly enhanced the buffering capacity of the vector even with a high degree of PEGylation [181]. PEG can be added to a variety of nanoparticles and can be further modified to provide targeting [72,182–184], or can be attached by degradable bonds (such as matrix metaloproteinases [MMPs]) that can be cleaved to expose underlying functionalities [185–189]. Electrostatic coatings can also be used to improve the delivery properties of a charged particle without significantly altering the core particle [190,191].

Nucleic acid complexation

Many polymers for nucleic acid delivery rely on electrostatic interaction between a cationic polymer and the anionic phosphate backbone of nucleic acid substrate. For polymer–DNA complexes, requirements include condensation of the plasmid to an appropriate scale for internalization, neutralization of the negatively charged phosphate backbone of the DNA and protection of DNA from degradation both intra- and extra-cellularly [192,193]. Sufficient cationic charge is crucial to condense DNA, but it is also correlated with increased cytotoxicity, and higher DNA-binding affinity may lead to decreased DNA release and reduced transport through the cytoplasm [194]. AuNPs can also use electrostatic methods for complexation with nucleic acids. AuNP rods conjugated with cationic cetyltrimethylammonium bromide were electrostatically complexed with siRNA (*anti-DARPP-32* gene in dopaminergic neuronal cells) with 98% cell viability and 67% expression knockdown at 120 h post-transfection [86]. Alternatively, hydrolytically degradable nanoparticles can be formed through encapsulation of DNA by noncationic polymers such as PLGA. These particles degrade to release their nucleic acid cargo and the size of the particle can be controlled in the nanometer to micrometer range, depending on the method of particle formation used. Methods have been developed to protect the cargo from destruction during these processes [168], but are still limited by low encapsulation efficiency and potential DNA degradation in the hydrolyzing polymer core [195].

Nonviral vectors can encapsulate nucleic acids through other mechanisms as well. Chitosan can form nanoparticles through ionotropic gelation with polyanions such as sodium tripolyphosphate [196]. Chitosan has been optimized to allow for the encapsulation of both hydrophilic and hydrophobic drugs and has been utilized in nucleic acid delivery [78,197],

most successfully as hybrid copolymers with various polycations [198–201]. Inorganic nanoparticles can be complexed with nucleic acids via ionic complexation or a covalent bond. For example, AuNPs (4.1 nm) can be covalently attached to cationic *N*-dodecyl PEI (2 kDa) and complexed with β-gal pDNA. When this conjugated complex was delivered to COS-7 cells, there was 67% cell viability and 50% transfection efficacy, which compared favorably with regular PEI and PEI25 that had 4 and 8% transfection, respectively (~93% cell viability) [202]. Another nucleic acid complexation technique can involve 11-mercaptoundecanoic acid, which can be deposited on Au to bind oppositely charged polyelectrolyte solutions. In one example, deposited Au combined with PEI (23 kDa MW) and double-stranded 21-mer anti-enhanced GFP (EGFP) siRNA was delivered to CHO-K1 cells resulting in ~95% cell viability and ~72% knockdown of EGFP expression [203].

Cellular targeting

By utilizing a targeting moiety, smaller dosages can elicit comparable therapeutic responses while minimizing side effects and reducing the cost of therapy [204]. There are two types of targeting; passive and active. Passive targeting utilizes natural processes such as the EPR, in which the leaky tumor vasculature and lack of efficient lymphatic drainage in a solid tumor leads to passive accumulation of drugs or particles at the tumor site, given sufficient circulation time [205]. Active targeting consists of an additional ligand to assist in localization or internalization such as antibodies or their fragments (i.e., J591 against prostate-specific membrane antigens [206], anti-HER2 [trastu-zumab] [207]) [208], folic acid [209], sugars (i.e., galactose, mannose and lactose) [210], peptides (RGD) [211,212], transferrin [213] and nucleic acid aptamers [214]. Large targeting moieties, however, may hamper internalization and gene unpacking and having triggered removal of the target moiety at the cell surface may be worthwhile [204]. Targeting moieties are typically attached chemically but can be physically adsorbed to the delivery system as well [191]. Interestingly, it has been shown that biodistribution of cargo at the accumulation site can be independent of the presence of targeting ligands [159]. The reason for improved functionality when targeting ligands are used appears to be owing to an increase in cell internalization and specificity of the nanoparticles rather than tissue localization. Passively targeted nanoparticles have a propensity to end up in the extracellular space of tumors and in tumor-associated macrophages [159].

Cationic polymers have been modified with targeting ligands for various applications. For example, the addition of lung surfactant to ternary nanoparticles for aerosol-based gene therapy enhances gene delivery to the lung, resulting in 12-fold higher transfection compared with pure nanoparticles and 30-fold higher compared with polyethylenimine [215]. Insulin adsorption significantly increased gene expression of PEI–pDNA nanoparticles up to 16-fold on alveolar epithelial cells, but not on bronchial epithelial cells [216].

Gold nanoparticles can be complexed with PEG-NH_2 and folic acid via noncovalent interactions and can be taken up by cancer cells (OV167, OV202, OVCAR-5) proportional to the degree of folic acid receptors expressed on them. However, unintended delivery to the liver and kidneys can also occur due to overexpression of folic acid receptors there as well

[217,218]. AuNPs and the mAb CD11b have been targeted to RAW264.7 macrophages and resulted in 81% cell death post-30 J/cm^2 exposure as opposed to 0.9% cell death with nonlabeled cells [94].

Enhancing internalization

Gold nanoparticles can be internalized to a greater degree via electroporation, which causes membranes to become permeabilized by pulsed electric fields (several kV/cm amplitude and submicrosecond duration). Membrane pores occur momentarily as a result, allowing for easier passage of gene therapy systems. However, electroporation can also cause osmotic lysis of the cells. Kawano *et al*. has delivered AuNP-SS-mPEG-pDNA *in vivo* in combination with electrical pulses to the mouse liver and observed greater stability in circulation and a gene expression increase by tenfold in comparison to naked DNA [219].

Multiwalled CNTs combined with irradiation of microwaves for 8 s can aid gene delivery by creating transient nanochannels in the cell while maintaining cell viability at 100% [220]. Hexagonal LDHNPs are most likely taken up by clathrin-mediated endocytosis and localize to the perinuclear area of the cytoplasm (where siRNA/mRNA complexes can degrade). By contrast, rod-like LDHNPs concentrate in the nucleus [121].

Gene-associated magnetic nanoparticles (i.e., SPIONs) can be guided toward a particular region of the body via external magnetic fields. Application of external magnetic fields to aid traversing membrane barriers and enhancing cell contact is known as magnetofection [141]. CNTs with ferromagnetic nickel tips have been known to be able to align in an external magnetic field to spear cell membranes. This increases the shuttling efficiency of cargo by 107-fold and was demonstrated to increase transfection rates to approximately 100% to mammalian cells *in vitro* [221]. Ultrasound is noninvasive and safe at a broad range of frequencies and intensities, and can be used to enhance gene delivery. The main mechanism responsible for increased gene delivery is cavitation, where reversible nano-pores are formed (up to 100 nm with a half-life of a few seconds) by microbubble expansion and collapse [222]. Stride *et al*. endeavored to combine ultrasound-mediated gene delivery and magneto fection and showed significantly improved transfection over magnetofection and ultrasound alone [223].

Gene guns are biological ballistic hand-held delivery systems that physically propel nucleic acid-complexed nanoparticles (i.e., AuNPs) into cell cytoplasm and nuclei with a low pressured propellant (i.e., helium). Chitosan and poly-gglutamic acid (150–250 nm) nanoparticles have been used to encapsulate reporter genes and transfect liver cells via the gene-gun method, which increases delivery by 2-log orders of magnitude in terms of luciferase RLU/mg protein compared with naked DNA [224].

Endosomal escape

In early experiments with nonviral gene delivery, nondegradable polycations, including PLL and PEI were used. Compared with PLL, a major advantage of PEI is the `proton sponge' effect due to PEI's extensive buffering capacity. When PEI–DNA complexes gain entry to the endosome, the secondary and tertiary amines in PEI function to buffer acidification of

the endosome. This causes an influx of negatively charged chloride ions into the endosome to maintain electro-neutrality as protons are continually pumped into the endosome. Eventually, this leads to osmotic swelling, rupture of the endosomes and release of the vectors and cargo into the cytoplasm [225]. This mechanism has been widely explored for gene and siRNA delivery [38,226]. This concept has also been extended and heavily used in the design of next-generation biodegradable vectors that also have this buffering capacity. A widely used buffering moiety is the imidazole ring of histidine. It is a weak base (~6 pKa) capable of buffering the endosome. For example, a poly(phosphazene)-based polymer has been histidylated, and the resulting polymer showed improved transfection and reduced cytotoxicity when compared with the histidine-free polymer and branched PEI [227].

Newer methods for endosomal escape involve functionalizing a polymer with peptides that enhance endosomal release. Melittin enhances endosomal escape and nuclear transport due to the cationic C-terminal sequence lysine–arginine–lysine–arginine [228]. Modifying melittin either by reversible acetylation of a lysine residue in melittin [229] or replacement of two glutamines with glutamic acids, which get neutralized at acidic pH [230], takes advantage of the acidification of the endosome to induce membrane lysis only in the endosomal compartment and reduces the cytotoxicity associated with use. Functionalizing polylysine with PEG- and a pH-responsive melittin peptide was shown to be an efficient siRNA delivery agent [231]. In an alternate fusogenic mechanism, protonation of glutamate-containing peptides causes endosomal escape via spontaneous formation of a membrane-disprupting α-helical structure of the peptide [232–234]. Adding these peptides to polymeric vehicles was shown to enhance the endosomal escape rate constant by two orders of magnitude [235].

Release of cargo/degradation

Nucleic acids must be released from the vector to have an effect. This can be done by taking advantage of the redox potential gradient [236], acidic environment of the endosome [237], MMPs [138,238], photocatalysis [239] and hydrolytic degradation of the carrier [49,240,241]. It has been shown that plasmid unpacking can be a limiting step with regard to gene expression for sufficiently large polymer constructs [242].

Bioreducible polymers

Using bioreducible polymers via incorporation of disulfide linkages takes advantage of the relative reducing environment of the intracellular space. Intracellular reduction of the disulfide bond occurs via the glutathione (GSH) pathway. GSH is regenerated from its oxidized form by GSH reductase, is an important component in many cellular pathways and plays a major role in cellular defense against oxidative stress. Disulfide bonds are stable extra cellularly, preventing particle breakdown before the nano complex reaches the cell surface, whereas the reducing environment of the intracellular space allows for enhanced polymer breakdown and nucleic acid release [243–247]. Disulfide bonds have been shown to degrade intracellularly within 3 h [248]. When cell lines with different intrinsic GSH levels have been compared, increased cellular GSH levels give mixed delivery results. In some cases, there is improved delivery, while in other cases, no clear trend is observed with

GSH levels; in these cases, the cell line that demonstrated the best DNA transfection was the fastest dividing cell line [249].

Enhancing release of the pDNA cargo can lead to dramatic gains in transfection efficiency. Chen *et al*. synthesized a series of reducible hyperbranched PMAMs and found that reducible polymers were able to achieve nearly 200-fold higher transfection as compared with control polymers [250]. Combining hydrolyzable and bioreducible functional groups as a single polymer might also help further tune the release profile [251,252]. Reducible polymers have also been used to deliver siRNA. Histidine-containing reducible polycations based on $CH_6K_3H_6C$ monomers (His6 RPCs) were examined for their utility in delivering siRNA. Co-delivery of EGFP siRNA with EGFP DNA reduced reporter gene expression by 85%. Interestingly, as with most polymer systems, while larger polymer size correlated with increased DNA transfection efficiency, effective delivery of siRNA was only possible with smaller polymers (36–80 kDa) [253].

Low molecular weight PEI has also been cross-linked via disulfide linkages to show reduced cytotoxicity and equivalent DNA transfection efficacy to higher molecular weight PEI [254]. In one study, reducible poly(amido ethylenimine) was synthesized by addition copolymerization of triethylenetetramine and cystamine bis-acrylamide (poly[TETA/CBA]) and used as a carrier for siRNA. Under normal conditions there was significantly higher suppression of VEGF with poly(TETA/CBA) than with linear PEI. The addition of dl-buthionine sulfoxamine, which reduces intracellular levels of reduced glutathione, reduced the RNAi activity level of poly(TETA/CBA) formulation to that of linear PEI, showing that reduction of the polymer was crucial to gene knockdown [255]. Jere *et al*. used a reducible polyspermine carrier composed of multiple spermine units with disulfide linkages and demonstrated improved efficacy in gene delivery and gene knockdown compared with 25K PEI. Reductable polyspermine delivered anti-Akt1 sh/si/ssiRNA and altered the cancer-cell survival, proliferation and metastasis to different extents depending on the nature of siRNA treatment [256].

Acid-labile linkages

Acid-labile linkages linkages would also be useful for endosomal escape and for enhanced cargo release into the cytoplasm, as they take advantage of the acidification of the endosome to allow for release of the cargo. Acid-labile acetal and ketal bond-bearing polycations were recently developed for this purpose. Oligo-ethylenimines (OEI) linked by either acid-degradable ketal or acetal linkers in a copolymer with 5 kDa PEG formed complexes with half-lives of 3 min at pH 5.0, and 5 h (OEI-MK) or 3.5 h (OEI-BAA) at physiological pH 7.4 [187]. Using acylhydrazides or pyridylhydrazines to link a PEG shield to the polymer backbone enhanced transfection by two (*in vitro*) or one (*in vivo*) order(s) of magnitude compared with complexes whose PEG shield was not acid-hydrolyzable [185].

Irradiation release

Irradiation can be used for controlled release. In one example, an Nd:YAG laser was used to release DNA from a 44 nm spherical AuNP complex conjugated to PEG-orthopyridyl-disulfide. The Nd:YAG laser irradiation was applied at 80 mJ/pulse (~10 ns, 6 mm

diameter). DNA was released without any degradation seen [239]. When EGFP DNA-SS-AuNR was delivered to HeLa cells and was controlled remotely using femtosecond near-infra red (NIR), a shape change from rod to sphere was observed. It was proposed that this transformation induces DNA release from its conjugate [257]. Also, using NIR, AuNR-EGFP-DNA conjugates were delivered to HeLA cells and there was expression detected at the irradiated spots of NIR exposure (79 uJ/pulse for 1 min). There was 80% cell viability observed and the NIR irradiation induced plasmid release without structural degradation [258].

Gold nanoparticles of different aspect ratios can be melted selectively at their unique longitudinal plasmon resonance by morphing to a sphere to release DNA oligonucleotides. Aspect ratios at 4.0 and 5.4 will have a longitudinal plasmon resonance at 800 and 1100 nm, respectively. By irradiating a combined sample of the aspect ratios at one wavelength, 50–60% of the intended oligonucleotides can be released and are still functional whereas the unintended oligonucleotides from the other aspect ratio released <10% of its cargo [259].

Nuclear translocation

Diffusion of DNA longer than 250 base pairs in length is significantly reduced in the cytoplasm compared with water due to the involvement of the cytoskeleton [260]. The NPC forms a selective permeability barrier, allowing free diffusion of molecules (e.g., ions, small proteins and metabolites) with a mass/size less than ~40 kDa/10 nm [261]. Macromolecules greater than ~40 kDa are transported actively across the nuclear envelope through the NPCs using soluble transport factors or carrier molecules (β-karyopherins) that cycle between the cytoplasm and nucleus [262]. In the classical case, NLS are recognized by importin-α, which then binds to importin-β, and this complex is allowed through the NPC (Figure 7). Once inside the nucleus, the importin-β-binding domain is released by binding to RanGTP and the cargo is released [262]. By utilizing electron microscopy and AuNPs complexed to NLSs, Panté and Kann were able to show that the largest rigid particle to achieve nuclear entry through NPCs was ~39 nm, in diameter including NLSs [263].

Strategies for obtaining access to the nucleus include diffusion of DNA through the cytoplasm, nuclear breakdown during mitosis and use of NLS. Numerous groups have complexed synthetic or naturally occurring NLS peptides to DNA, with variable efficacy, and transfection enhancement may be due to the NLS peptides inducing improved nanoparticle complexation rather than improved nuclear import [25]. A single NLS has been shown to be sufficient to carry the DNA through the nucleus [264]. However, the addition of many NLS sequences to a plasmid can lead to no nuclear localization of the plasmid at all, perhaps as multiple NLS sites might lead to cellular machinery attempting to pull a single plasmid in multiple directions at the same time [25,264].

Multifunctional nucleic acid carriers

Multifunctionalized constructs are typically a hybrid of materials that are intended to accomplish different objectives simultaneously, such as gene therapy and diagnostics (i.e., imaging), commonly referred to as theranostics. Multifunctional hybrid vectors could also incorporate components to overcome the multiple entry barriers discussed above: nucleic

acid complexation, physical requirements (i.e., charge, biocompatibility), targeting, internalization, endosomal escape, cargo release, biodegradation and nuclear translocation.

Hybrid AuNPs/siRNA/PBAE system

Thiol-modified siRNA can be combined with PBAEs and complexed with thiol-modified AuNPs by disulfide linkages for multiple functionality (AuNP for sensing, siRNA for silencing and reduction-triggered release of cargo) (Figure 8) [93]. PEG can be used as a spacer between the disulfide bond and the AuNP surface as the Au can induce release of the cargo. This system has high stability and low aggregation of the ~100 nm particles [93]. There was no significant cytotoxicity reported and the system resulted in ~95% gene knockdown of luciferase expression in HeLa cells [93].

Multifunctional QDs

ZnO QDs have been reported to have dual functionality (pDNA delivery and cell labeling) when capped with poly(2-(dimethylamino)ethyl methacrylate). This system was capable of condensing pDNA into nanoplexes and delivering DNA to COS-7 cells with real-time imaging of gene transfection under UV [265].

Mesoporous silicon

Mesoporous silica nanoparticles have been tri-functionalized (imaging, targeting and therapy). In one example, optical agent ATTO 647N, cRGDyK peptides to target $\alpha_v\beta_3$ integrins over-expressed in tumor metastatic and endothelial cells, and an oxygen-sensing porphyrin-based photosensitizer were used to create such a system [266]. *In vitro* experiments using MCF-7 human breast cancer cells and U87-MG human glioblastoma cells demonstrated that there was excellent specificity, minimal collateral damage, and potent photodynamic effects [266]. Mesoporous silica can also be used in other ways for combination/theranostic use [267].

Nonviral gene therapy clinical trials

As of June 2010, according to the *Journal of Gene Medicine* website [301], there have been a total of 1644 gene therapy clinical trials approved worldwide. The leading diseases being treated in these clinical trials are cancer (64.5%), cardiovascular diseases (8.7%) and monogenic diseases (8.2%). The majority (60.5%) of the clinical trials are in Phase I study. The most common gene types used have been antigens (19.8%), cytokines (18.4%), tumor suppressors (10.5%) and growth factors (7.7%). Table 2 is a summary of nonviral gene therapy clinical trials detailing the type, the current or last clinical phase, the nucleic acid being delivered and the disease target. These data highlight that the majority of clinical trial thus far have been with viral vectors (~75%), and the leading nonviral approaches in clinical trials are `naked' free DNA (18%) followed by lipofection (7%). Polymeric and inorganic vectors for gene delivery, although promising for the future, are, for the most part, still in preclinical stages of development. However, one recent approach in polymeric gene delivery to reach clinical trials is Mark Davis' and Calando Pharmaceutical's work with CD-based polymers [70,71]. In this work it has been demonstrated that biocompatible polymeric

nanoparticles can reach solid tumors following systemic administration in humans and within these tumors the particles can cause siRNA-specific knockdown of a target gene.

Inorganic gene delivery systems used in clinical trials to date have so far been limited to the gene gun [302–303]. However, several promising technologies are on the horizon and may prove successful in the near-coming years. For example, AuNPs (27 nm) were recently conjugated with recombinant human TNF (rhTNF) and thiolated PEG (CYT-6091) in a Phase I clinical trial [268]. The results showed that previously toxic concentrations of rhTNF were no longer toxic by systemic administration and that CYT-6091 may target tumors [269].

Conclusion

Polymeric and inorganic-based vectors for nucleic acid delivery need to overcome many crucial barriers in the delivery process, and a variety of novel approaches have been investigated to overcome these challenges. A wide array of materials have been investigated for their potential in this area, including degradable and nondegradable cationic polymers, oligo- and polysaccharides, fullerenes, CNTs, QDs, gold, silver, silica, layered-double hydroxide and iron-oxide nanoparticles. Each has unique properties and potential advantages.

For effective delivery, the vector first must be stably complexed to the nucleic acid cargo and needs to stay compacted until cellular entry. The size, shape, surface charge and surface functionality of the gene delivery particles are critical to efficient delivery, increased circulation time and specific cellular entry. Size is a crucial parameter in determining the passive biodistribution of a nanoparticle delivery system and charge shielding/PEGylation has been shown to improve circulation time and increase accumulation at tumor sites as a result of the EPR effect. Particles can be fabricated in a variety of different shapes and shape-shifting particles whose shape change can be triggered by pH, heat and light are also possible.

A majority of delivery systems achieve cellular entry via endocytosis. The desired delivery compartment within the cell is dependent on the type of nucleic acid being delivered. For delivery of isRNA, interaction with TLR7 in the endosome is the end-goal, so particles should be designed to target and then remain in the endo-some. For siRNA and all DNA-based systems, there needs to be a mechanism for endosomal escape. Mechanisms employed by nonviral vectors for endosomal escape include the proton-sponge effect, endosomolytic peptide-based lysis, and acid-triggered hydrophobic residue exposure. Hydrolysis, bioreduction and photolysis have been utilized to reduce toxicity and promote unpacking of nucleic acid cargo intracellularly. Finally, for cargo such as DNA that needs to localize to the nucleus, particles and nucleic acids can make use of endogenous cell machinery and NLS sequences to allow nuclear import via the NPC.

Future perspective

While nonviral nucleic acid delivery remains less efficient than viral delivery, recent advances offer the promise that soon there will be significant clinical effect from these

approaches. CD-based polymers have found early clinical successes and additional biocompatible polymers are likely to soon follow suit. Incorporation of inorganic materials into such particles can also enable multimodality and theranostic applications. Several new directions are evolving, which offer approaches to achieve the goal of targeted, efficient, nonviral nucleic acid delivery.

Mesenchymal stem cells (MSCs) and neural stem/progenitor cells (NSPCs) are capable of migration toward pathological sites such as tumors and associated metastases. MSCs can be used to carry cargo while evading the immune system, as they are hypoimmunogenic and can then engraft into the stroma after arrival [117,270]. For these reasons, MSCs are a very promising avenue for nonviral targeted gene delivery. Recently, it was shown that virally transduced NSPCs could be implanted intracranially as an anticancer therapy. The NSPCs were transduced to stably express an enzyme that activates a 5-fluorocytosine prodrug, and following systemic 5-fluorocytosine treatment there was a significant (71%) reduction in tumor burden [271]. Another group has modified human neural stem cells to secrete anti-HER2 immunoglobulin molecules as a tool to target and attack metastatic breast cancer in the brain. Researchers were able to show that anti-HER2-secreting NSCs exhibit preferential tropism to tumor cells and can deliver antibodies to human breast cancer xenografts in mice [269]. Potential safety complications with viral transduction of these cells could be alleviated with a nonviral approach. Remaining issues with nonviral delivery include timing of gene expression, differentiation of NSPCs/MSCs, and the possibility that these cells become tumorigenic. It is critical that all potential safety concerns with this approach are thoroughly investigated in nonhuman primates before clinical trials commence.

Translocation of pDNA to the nucleus and nuclear import remain critical barriers for gene delivery. This is because in many ways, nonviral gene delivery research has focused on transporting pDNA safely and effectively into the cell, but has not focused as directly on its sub cellular location. Moreover, many biomaterials are designed to release naked DNA to the cytoplasm even though nuclear import is known to be inefficient. Enhancing nuclear import by other modalities in addition to NLS sequences and simple diffusion would be of great interest. It is known that dynein enables transport along microtubules in the direction of the nucleus [272] and that viruses, such as HIV, are able to exploit the cytoskeleton for directed movement towards the nucleus [273]. With further characterization of microtubule-associated transport, synthetic particles could similarly exploit endogenous cell machinery to enhance active transport to the nucleus and nuclear uptake.

Finally, addition of targeting moieties is a widely used and important technique in the field. In addition to targeting ligands to cell surface receptors, a complementary approach is targeting specific enzymes located at a specific microenvironment such as MMPs. MMPs are upregulated during tumor growth (i.e., MMP-3, -7 and -13) and play a role in cell growth, death, malignant conversion and tumor-associated angiogenesis [138]. siRNA has been used to downregulate MMP-9 and was shown to aid the inhibition of invasion and migration of prostate cancer cells, leading to apoptosis both *in vitro* and *in vivo* [274]. In one approach, QDs have been conjugated to folic acid, which is sterically shielded from the environment by MMP-7 cleavable PEG (exhaustively cleaved at 5 nM) [275]. This work combines the passive targeting of the EPR effect with MMP-sensitive release of cargo to

take a twofold approach for the targeted delivery of the nanoparticles. The cancer type and stage are important in determining which MMP should be used to cleave cargo or be a target itself [138]. Compared with conventional cancer chemotherapies, gene therapy can enable a much wider therapeutic window due to increased specificity. Nanoparticles can be passively targeted by the EPR effect, targeted to a microenvironment through enzyme activity, targeted to a cell receptor through a ligand interaction, and targeted to a cell-type through biomaterial optimization [172]. Once DNA is delivered, it can then be transcriptionally targeted to the cell type of interest and the gene product itself could also be specific to that cell type. Thus, many layers of targeting can be enabled in a nonviral gene-delivery system and particles that use multiple methods of targeting will likely become more widespread in the future. The directions sketched herein and other innovations in biology, bioengineering, materials science and nanotechnology will continue to guide the field of nonviral gene delivery.

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Key Terms

siRNA	Short, 20–25 base pair dsRNA that directs the target of RNAi. Active siRNA can be produced by the cleavage of longer dsRNA by the enzyme Dicer or siRNA can be used directly for therapeutic application.
RNAi	The process whereby short dsRNA is used for directed cleavage of mRNA leading to decreased expression of the protein of interest.
isRNA	Short dsRNA has also been shown to have immunostimulatory properties that are unrelated to their effects in the RNAi pathway; these RNAs interact with Toll-like receptors (TLR) 3,7, and 8; Type I interferon induction by synthetic isRNA requires TLR7 and is sequence dependent.
shRNA	These are single stranded RNA (ssRNA) that form a `hairpin' secondary structure and have been shown to suppress the expression of desired genes. This method allows delivery of a plasmid that produces shRNA and leads to knockdown of gene expression.
agRNA and saRNA	Newly recognized classes of RNA that target the promoter region of certain genes to induce or inhibit gene expression.
Transfection	The successful introduction and expression of exogenous nucleic acid in a cell.

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■ of interest

■■ of considerable interest

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Executive summary

- Key barriers to nucleic acid delivery include stable vector–cargo complexation, protection from extracellular degradation, transport to the cell of interest, cellular internalization, endo–lysosomal escape, cargo release, intracellular transport and vector degradation.
- A wide array of materials have been investigated for their potential for delivery, including degradable and nondegradable cationic polymers, oligo- and polysaccharides, fullerenes, carbon nanotubes, quantum dots, gold, silver, silica, layered-double hydroxide and iron-oxide nanoparticles.
- The desired delivery compartment within the cell is dependent on the type of nucleic acid being delivered.
- Size is a crucial parameter in determining the passive biodistribution of a nanoparticle delivery system and charge shielding/PEGylation has been shown to improve circulation time and increase accumulation at tumor sites as a result of the enhanced permeability and retention effect. Particles can be fabricated in a variety of different shapes and shape-shifting particles whose shape-change can be triggered by pH, heat and light are also possible.
- Electroporation, magnetofection, irradiation and ultrasound can be combined with nanoparticle delivery systems in ways that enhance delivery and the therapeutic effect at target cells.
- Mechanisms employed by nonviral vectors for endosomal escape include the proton-sponge effect, endosomolytic peptide-based lysis, and acid-triggered hydrophobic residue exposure.
- Approaches to reduce toxicity and promote intracellular unpacking include hydrolysis, bioreduction and photolysis.
- Multifunctional constructs have been designed that accomplish different objectives simultaneously, such as gene therapy and diagnostics.
- Most current clinical trials for gene therapy are primarily accomplished via viral vectors. Most nonviral vectors currently used for clinical trials involve lipofection and naked nucleic acids, but more polymeric and inorganic methods are expected to be used for clinical trials in the near-coming years.

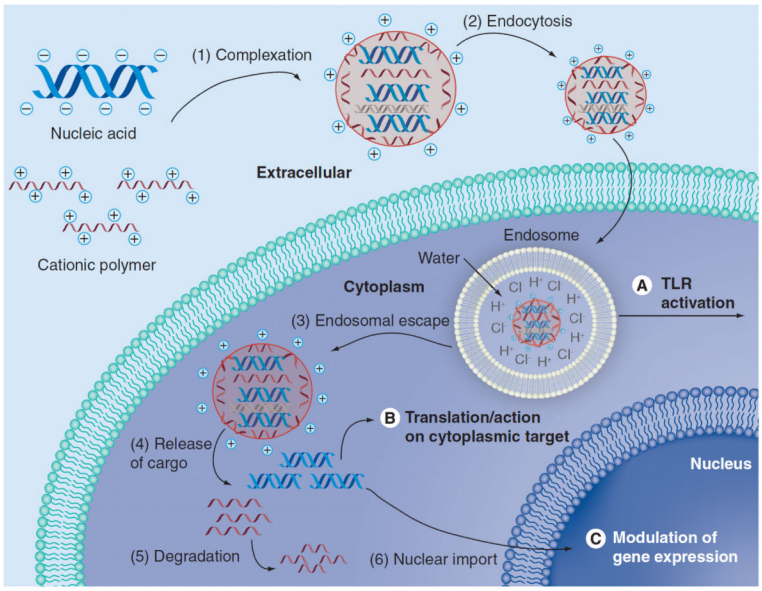

Figure 1. Barriers to intracellular nucleic acid delivery

(1) Nucleic acid must be complexed to the nanocarrier and protected from degradation as it makes its way to the target cell. (2) The nanocarrier and cargo must be internalized successfully. **(A)** TLR7 is localized to the endosome; for isRNA activity, endosomal escape is not required. For other nucleic acid, (3) endosomal escape is required. **(B)** (4) For cytoplasmic activity, nucleic acid must be released intracellularly. (5) Nanocarrier degradation is not required, but is useful for reduced toxicity. **(C)** (6) For DNA, shRNA-encoding plasmids, and agRNA, nuclear import is required for successful effect.

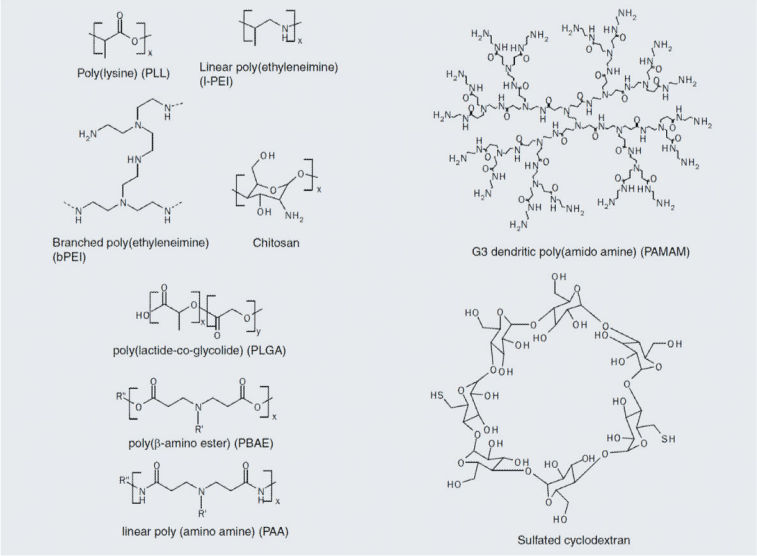

Figure 2.

Commonly used cationic polymers and polysaccharides used in gene delivery.

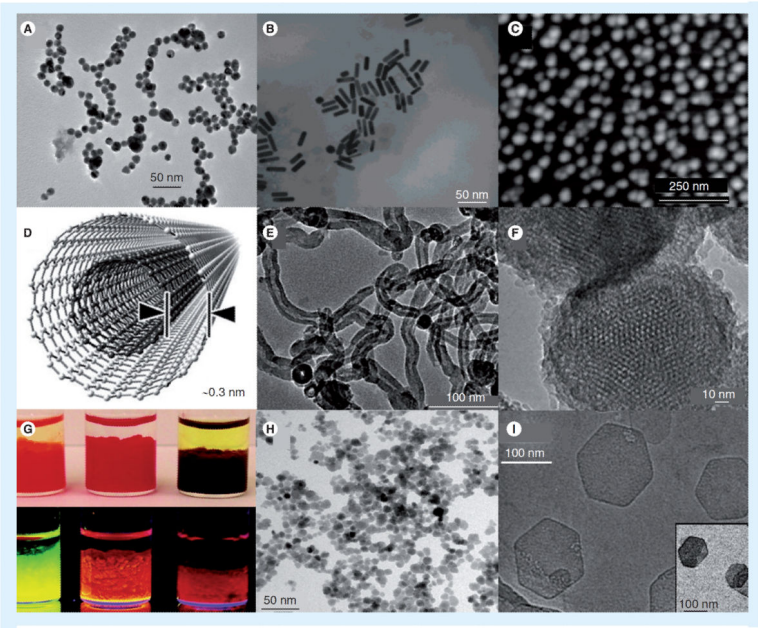

Figure 3. Typical inorganic nanoparticles being investigated for nucleic acid delivery

(A) Transmission electron microscopy (TEM) of gold nanoparticle (AuNP) spheres, adapted from [81]; **(B)** TEM of AuNP rods, adapted from [82]; **(C)** AFM topography of AuNP shells coating platinum, adapted from [83]; **(D)** Multiwalled carbon nanotubes adapted from [276]. **(E)** Representative TEM of carbon samples produced by catalysis, adapted from [98]; **(F)** Mesoporous silica nanoparticles, adapted from [134]; **(G)** Quantum dots - top and bottom row are illuminated under visible and UV, respectively, adapted from [104]; **(H)** Doxorubicin-loaded superparamagnetic iron oxide nanoparticles with a diameter of 8 ± 2 nm, adapted from [115]; **(I)** TEM of pristine-layered, double-hydroxide NPs of $Mg_2Al(OH)_6NO_3$ - inset NPs are associated with siRNA, adapted from [121].

Figures adapted with permission.

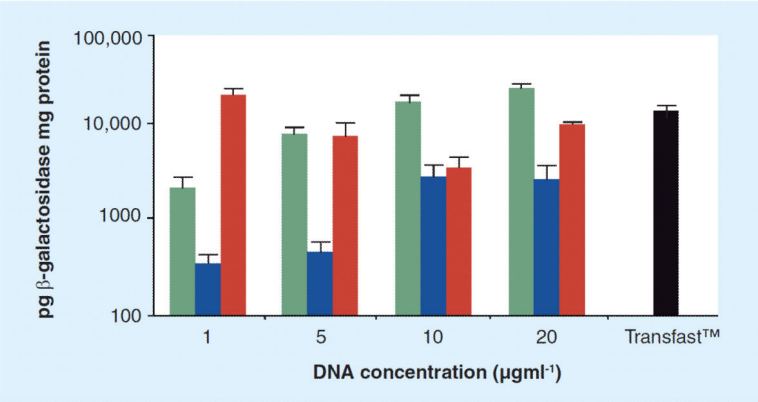

Figure 4. β-galactosidase delivered to MG-63 cells with acceptable viability (>90%) and greater transfection efficacy than Transfast™ (1 \g=m\g/ml) using inorganic nanocomposites formed from mineral solutions containing various amounts of $CaCl_2$, KH_2PO_4, NaCl, KCl, $MgSO_4$, $MgCl_2$ and $NaHCO_3$

Adapted with permission from [144].

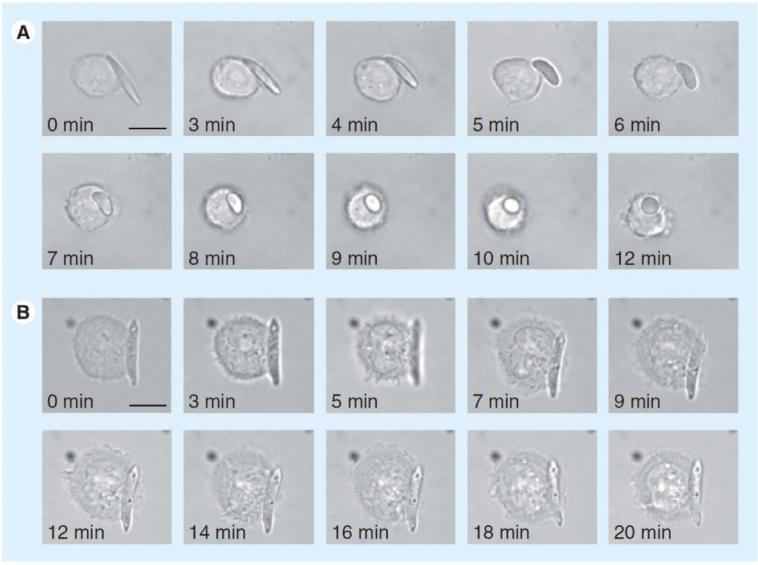

Figure 5. Time-lapse video microscopy stills of shape-dependent phagocytosis by macrophage

(A) Shape-switching poly(lactide-co-glycolide)- ester elliptical disk allows macrophage internalization. **(B)** Poly(lactide-coglycolide)-ester elliptical disk that does not switch shape prevents internalization. Scale bar: 10 μm. Reproduced with permission from [151].

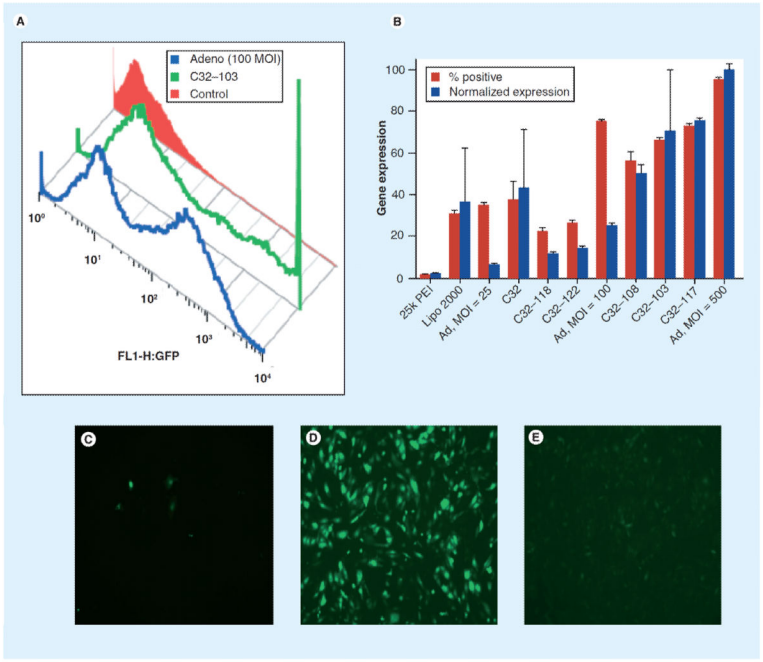

Figure 6. Gene expression of poly(β-amino ester)s compared with adenovirus

(A) Gene expression histogram comparing adenovirus, PBAE and negative control. **(B)** Comparison of various poly(β-amino ester) formulations with adenovirus with respect to % positive cells and normalized expression. Images of GFP^+ cells 24 h post-transfection with **(C)** PEI, **(D)** C32–103 and **(E)** 500 MOI adenovirus.

GFP: Green fluorescent protein; MOI: Multiplicity of infection; PEI: Poly(ethylenimine).

Reproduced with permission from [170].

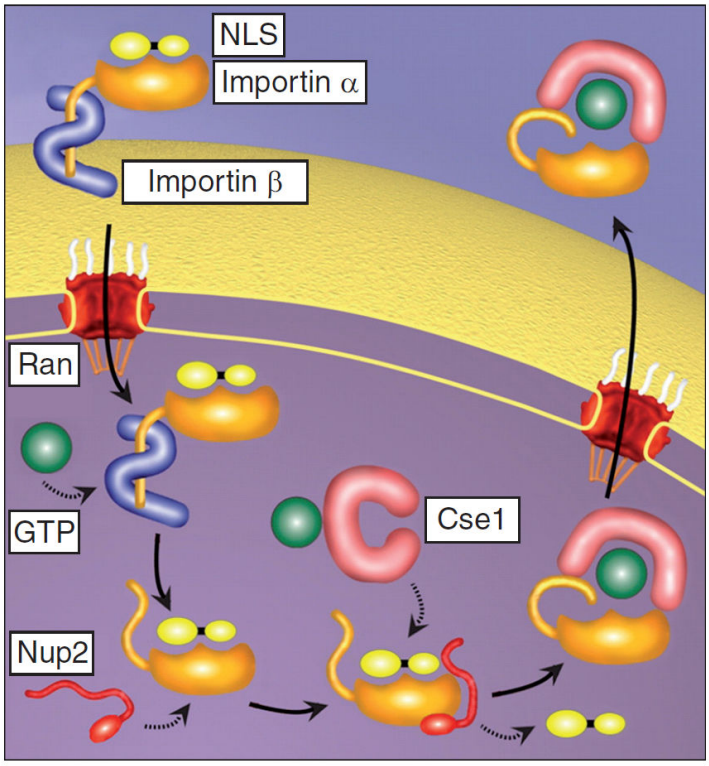

Figure 7. Nuclear import through the nuclear pore complex
Adapted with permission from [262].

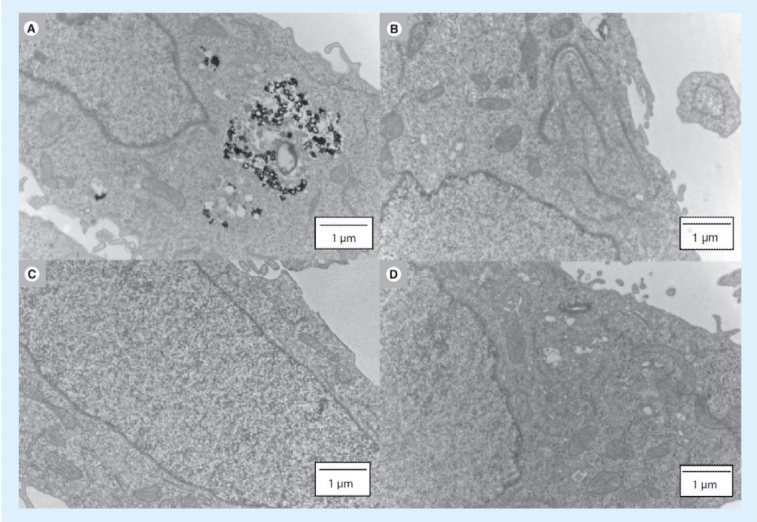

Figure 8. Transmission electron microscopy images of HeLa cells

(A) PBAE-siRNA-AuNPs; **(B)** siRNA-AuNPs without PBAE; **(C)** unmodified AuNPs; **(D)** no nanoparticles (control).

AuNPs: Gold nanoparticles; PBAE: Poly(b-amino ester).

Adapted with permission from [93].

Table 1

Summary of results of various polymeric and inorganic vectors for delivering genes.

Materials used & size	Cargo (DNA)	Target	Cell viability	Transfection efficacy	Ref.
Folate PLL, chloroquine	Luc	KB cells	Not reported in detail	6-times higher than w/o folate at 24 hpt	[37]
Partially histidylated PLL	Luc	HepG2	No cytotoxicity 4–24 h incubation	~5 log orders of higher RLU than PLL at 48 hpt	[30]
Galactosylated PLL (Gal_{13}-PLL_{13000}; ~179 nm	CAAT	Human epitome cell line HepG2	Not reported in detail	~850 mU/mg at 48 hpt	[34]
800 kDa PEI (nitrogen to DNA base ratio 9:1)	Luc	3T3 cells	Only toxicity above concentration for optimal efficiency	4 log orders more efficient than PLL (light units/mg protein)	[38]
Low molecular weight PEI at 11.9 kDa; high molecular weight PEI at 1620 kDa	Luc	ECV304	MTT assay: low molecular weight, none up to 1 mg/ml; high molecular weight, IC_{50}: 35 μg/ml	Low molecular weight PEI (N:P = 66.66) RLU was 100-fold higher than high molecular weight PEI (N:P = 13.33)	[40]
Fully deAc linear PEI: 25 kDA (PEI25) and 87 kDa (PEI87)	β-Gal *in vitro*; Luc *in vivo* (mouse)	A549 cells *in vitro*; lungs *in vivo*	>80% viability with N:P ratio <20; increasing toxicity with increasing N:P; deAc-PEI was more toxic than PEI	deAc-PEI25 21x higher than Ac-PEI25 *in vitro*; 115x-higher expression vs PEI25 seen by deAc-PEI87 *in vitro*; 10 N:P deAc-PEI25 showed 5 log orders of higher RLU and 1500-fold enhancement in lung specificity vs PEI25 *in vivo*	[44]
PBAE (C32); 71 nm; 1.2:1 amine:acrylate ratio	Luc	COS-7	No cytotoxicity observable	Better than Lipofectamine™ 2000	[50]
PBAE (C32–117); <200 nm	Luc	HUVEC	2 orders of magnitude lower than 25 kDa PEI	~adenovirus and lentivirus; 2 log orders greater than 25 kDa PEI	[51]
PBAE (C32–117); ~200 nm	GFP and RFP	hESC	70% at 24 hpt	50% at 24 hpt	[17]
(Mannose/galactose)-PEG-PAMAM linear-dendritic hybrid polymers (~150 nm)	Luc	P388D1 murine macrophages bearing man-receptor	60–80% for G5.0 and 50–70% for G6.0 when transfecting P388D1 cells	Man-PEG-G6.0 transfected P388D1 1.6–1.8-fold more efficiently than PEI with no serum and fourfold more efficiently in the presence of serum	[63]
Chitosan (~150 nm)	Arah2 (peanut allergy gene)	Oral administration	Not reported in detail	Decreased IgE levels in response to anaphylaxis induction	[76]
Biomineral solution ($CaCl_2$, KH_2PO_4, NaCl, KCl, $MgSO_4$, $MgCl_2$, $NaHCO_3$) (Figure 3)	β-Gal	MG-63	>90% at 24 h using concentrations ranging from 1 to 20 μg/ml	Transfection is greater than Transfast™ (1 μg/ml) using inorganic mineral solutions at 1, 10 and 20 μg/ml	[144]
Tetra(piperazino) fullerene epoxide	eGFP	C57/BL6 mice	No acute toxicity for liver or kidney	Increased plasma insulin levels and reduced blood	[102]

Materials used & size	Cargo (DNA)	Target	Cell viability	Transfection efficacy	Ref.
				glucose concentrations	
ZnO quantum dots with poly(2-[dimethylamino] ethyl methacrylate)	Luc	COS-7	90% at 50 μg/ml; however, at 100 μg/ml (experimental levels) viability was 18%, which is most likely due to quantum dot vectors	~1 log order lower than PEI(25 k) at 48 hpt	[265]
PLGA nanoparticles (slightly <200 nm) with spermidine or protamine used as a counter ion to the siRNA in the loading process	Anti-MAPK1 (ERK2) siRNA *in vitro*, anti-eGFP siRNA *in vivo*	Vaginal epithelium	*In vitro*: no observed decreased cell viability up to 10 mg/ml; *in vivo*: no histological changes (mice significant inflammation when treated with siRNA lipopolyplexes)	Spermidine improved loading by >40-fold; *in vitro*: ≥ gene silencing compared with Lipofectamine™ RNAiMax; *in vivo*: 50–60% knockdown in vaginal epithelium and submucosa	[48]
$Mg_2Al(OH)_6NO_3$; layered double hydroxide nanoparticles; ~100 nm	Anti-MAPK1 (ERK2) siRNA	HEK293T	>80% over 3-day period; IC_{50}: 0.125 mg ml^{-1}	RLU for nanoparticle alone and with siRNA: ~1.1 a.u. at 24 hpt and ~0.1 a.u. at 8 hpt	[121]
PBAE (C32–221)-siRNA-SS-PEG- AuNP; ~100 nm	Anti-Luc siRNA	HeLa	~90% at 24 hpt	~95% at 24 hpt	[93]
Mesoporous silica nanoparticles-PEI; ~100 nm	Anti-eGFP siRNA	PANC-1	Not reported in detail	61.7% at 72 hpt	[105]
AuNP, siRNA, MUA, 25 kDa PEI (LbL); 26.8 nm	Anti-eGFP siRNA	CHO-K1	~95% at 0.37 nM AuNP	~72% at 0.37 nM AuNP	[203]
Lipospheres, cationic lipid metafectene, dioleoylphosphatidyl-ethanolamine functionalized with SPION	Anti-eGFP and Anti-Luc siRNA	eGFP-HeLa, firefly Luc-HeLa	Anti-eGFP siRNA: not significantly affected; anti-Luc: 50% viability; likely due to transfection	90% at 48 hpt	[143]

It is important to note that direct comparisons are difficult as the experimental setups are likely different.

β-Gal: β-galactosidase; a.u.: Absorbance units; CAAT: Chloramphenicol acetyltransferase; deAc: Deacetylated; (e)GFP: (Enhanced) green fluorescent protein; hESC: Human embryonic stem cell; hpt: Hours post-transfection; HUVEC: Human umbilical vein endothelial cell; Luc: Luciferase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBAE: Poly(β-amino esters); PEG: Polyethylene glycol; PEI: Poly(ethylene imine); PLGA: Poly(lactide-co-glycolide); PLL: Poly(L-lysine); RFP: Red fluorescent protein; RLU: Relative light units SPION: Superparamagnetic iron oxide nanoparticle.

Table 2

Summary of clinical trials (some of which are ongoing) including naked plasmids, the gene gun, lipofection, siRNA, asDNA, gold nanoparticles and BD Accuspray™.

Type	Clinical Phase	Nucleic acid	Disease target	Ref.
Naked plasmid/DNA (intramyocardial)	II/III	VEGF	Angina	[277304]
Naked plasmid/DNA (lung injection)	II	heNOS	Hypertension	[305]
Naked plasmid/DNA (intramuscular)	II	FGF	PAOD	[306]
Gene gun	I/II	IL-7,12; GM-CSF	Malignant melanoma	[302]
Lipofection for direct gene transfer using Leuvectin™ (intratumoral)	II	IL-2	Renal cell cancer	[307]
Gene gun (intradermal)	I	GM-CSF	Malignant melanoma, sarcoma	[303]
Lipofection for direct gene transfer using Allovectin-7 (intratumoral)	II	HLA-B7/β 2 μg globulin	Malignant melanoma	[308]
Lipofection (E1A lipid complex); for cancer without overexpression of HER-2/neu (intraperitoneal)	II	E1A	Ovarian cancer	[309]
Lipofection using SGT-53 (intravenous)	I	P53	Solid tumors	[310]
Lipofection as a tumor cell vaccine (intradermal)	II	B7.1 (CD80), HLA-A1/2	Nonsmall cell lung cancer	[311]
siRNA (intravenous)	I	I5NP	Acute renal failure	[278312]
Naked DNA (corpus cavernosum injection)	I	hSlo DNA	Erectile dysfunction	[279]
siRNA (intravitreal)	III	Bevasiranib (anti-VEGF)	AMD	[280]
siRNA (inhalation)	IIa/b	Anti-RSV nucleocapsid gene	RSV	[281]
siRNA (systemic, lipid–ionic complexation)	I	Plasmid DNA ≥ 4 RNAi, inhibiting all viral genotypes	Hepatitis B virus	[313]
siRNA (topical)	II	Bevasiranib/Cand5 anti-VEGF	Diabetic macular edema	[314]
siRNA (injected into callus on foot)	I	TD101	Pachyonychia congenita	[315]
siRNA (intravitreal)	II	AGN211745	AMD/CNV	[316]
cDNA encoding two growth factor isoforms (intramuscular)	II	VM202 (HGF-723/728)	Critical limb ischemia	[317]
Anti-sense DNA (intratumoral), DC-Chol liposomes	I	EGF receptor	Head/neck cancer	[318]
Anti-sense DNA (intratumoral injection)	I/II	EGF receptor	Head/neck squamous cell carcinoma	[319]
siRNA via cyclodextrin-based polymer (intravenous)	I	Anti-M2 subunit of ribonucleotide reductase (R2)	Solid tumor	[320]
PEGylated AuNPs	I	rhTNF; not nucleic acid	Advanced-stage cancer patients	[268]
siRNA delivered with BD Accuspray™	II	ALN-RSV01 siRNA	Respiratory syncytial virus	[282]
siRNA delivered with stable nucleic acid–lipid particles	I	ALN-TTR01 siRNA	Transthyretin-mediated amyloidosis	[321]
Two siRNAs delivered via lipid nanoparticle formulation	I	Contains both kinesin spindle protein VEGF siRNAs (ALN-VSP02)	Liver cancer	[322]

AMD: Age-related macular degeneration; CNV: Choroidal neovascularization; DC-Chol: 3B[N-(iV',W-dimethylaminoet hane)-carbamoyl] cholesterol; heNOS: Human endothelial nitric oxide synthase; PAOD: Peripheral artery occlusive disease; rhTNF: Recombinant human TNF; RSV: Respiratory syncytial virus.