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Non-viral nucleic acid containing nanoparticles as cancer therapeutics

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Abstract

Introduction—The delivery of nucleic acids such as DNA and short interfering RNA (siRNA) is promising for the treatment of many diseases, including cancer, by enabling novel biological mechanisms of action. Non-viral nanoparticles are a promising class of nucleic acid carriers that can be designed to be safer and more versatile than traditional viral vectors.

Areas covered—In this review, recent advances in the intracellular delivery of DNA and siRNA are described with a focus on non-viral nanoparticle-based delivery methods. Material properties that have enabled successful delivery are discussed as well as applications that have directly been applied to cancer therapy. Strategies to co-deliver different nucleic acids are highlighted, as are novel targets for nucleic acid co-delivery.

Expert opinion—The treatment of complex genetically-based diseases such as cancer can be enabled by safe and effective intracellular delivery of multiple nucleic acids. Non-viral nanoparticles can be fabricated to deliver multiple nucleic acids to the same cell simultaneously to prevent tumor cells from easily compensating for the knockdown or overexpression of one genetic target. The continued innovation of new therapeutic modalities and non-viral nanotechnologies to provide target-specific and personalized forms of gene therapy hold promise for genetic medicine to treat diseases like cancer in the clinic.

Keywords

cancer; DNA; gene delivery; gene therapy; nanoparticle; polymer; siRNA

1. Introduction

The discovery that exogenous DNA introduced into isolated nuclei can be transcribed into mRNA [1] and lead to protein expression [2, 3] created the promise of gene therapy as a modality capable of treating myriad diseases. siRNA is a more recently discovered

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therapeutic modality and can be used to knock down gene expression through the RNA interference (RNAi) pathway. RNAi was initially discovered in *C. elegans* as a gene silencing pathway used as a natural mechanism for viral defense [4]. A strand of long, double-stranded RNA (dsRNA) is cleaved by the Dicer protein into 21–25 bp siRNAs [5]. siRNA is then incorporated into the RNA-induced silencing complex (RISC) where the sense strand is removed. The antisense strand is then used as a template for complementary mRNA. mRNA that pairs with siRNA-RISC is cleaved, thus preventing translation and thereby gene expression. (For review, see Hannon [6].)

Early delivery methods of these nucleic acids often involved introducing nucleic acids by mechanical disruption of the cell membrane or direct injection [7, 8]. However, these methods are laborious and not clinically translatable. Viral methods of DNA and siRNA delivery are effective [9], yet often induce immunogenicity or tumorigenicity and are therefore limited for clinical translation [10]. Non-viral nucleic acid delivery has traditionally been considered less effective [11], but can be designed to avoid tumorigenesis and immune stimulation. Recent advances in nanoparticle vectors for nucleic acid delivery have continued to improve delivery efficacy while minimizing toxicity, but several obstacles remain that make successful delivery an ongoing challenge.

1.1 Obstacles to intracellular delivery

Due to their size and negative charge, nucleic acids cannot readily pass through the cell membrane to their intracellular sites of action (Fig. 1). Nanocarriers can encapsulate nucleic acids to not only to promote successful delivery into cells, but also to protect them from degradation by extracellular nucleases. Nanocarriers for nucleic acid delivery include liposomes that hold DNA and siRNA within their aqueous interiors [12–14], cationic polymers that bind anionic nucleic acids to form polyplexes [15, 16] and solid nanoparticles that can carry nucleic acids via covalent linkages [17]. For nanocarriers that electrostatically bind to nucleic acids, special considerations must be taken for short oligonucleotides like siRNA, which are much shorter and stiffer than plasmid DNA, and are therefore often harder to complex into nanoparticles [18, 19]. To prevent unwanted non-specific interactions between nanoparticles and biomolecules and cells, nanoparticles are frequently coated with hydrophilic polymers, such as polyethylene glycol (PEG) [20].

For intracellular delivery, cells must take up the nucleic acid carrying nanoparticles. To enable cellular uptake, cell-penetrating peptides can be used to promote internalization directly through the cell membrane [21–23], or cationic nanoparticles can nonspecifically interact with the negatively charged cell surface to promote endosomal uptake [24–27]. If internalized via endosomes, the nanoparticle must escape the endosome to prevent degradation in lysosomes, prevent recycling out of the cell, and to promote cytosolic delivery. This can be achieved with hydrophobic or amphiphilic biomaterials that can destabilize the endosomal membrane [28, 29]. Endosomal escape can also be achieved using the proton sponge mechanism, in which a nanomaterial may act as a buffer against endosomal acidification and eventually result in endosomal lysis. Although this mechanism has been challenged [30], it is the most widely accepted hypothesis to explain successful transfection when utilizing nanomaterials with titratable amines [31, 32].

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For siRNA delivery, the nanocarrier must release its contents at the site of RNAi in the cytosol [33]. Several polymeric materials degrade hydrolytically and can thereby release siRNA as the polymer degrades [34, 35]. As the cytosol is approximately 1000 times more reducing than the extracellular space [36], nanomaterials may also employ bioreducible disulfide bonds to promote release targeted specifically to the cytosol. (For review, see Son *et al.*[37]) Nanocarriers delivering DNA may need to remain intact longer, as naked DNA is slow to diffuse in the cytosol and may be degraded by cytosolic nucleases on its way to the nucleus [38, 39]. Nuclear penetration is an additional major bottleneck to gene delivery. It has been shown that actively dividing cells are easier to transfect [40], and this can be an avenue to increase transfection in cancer cells compared to non-cancerous slower growing cells. In non-mitotic cells, attaching a nuclear localization signal peptide sequence to DNA is a strategy that improves nuclear penetration by using the cell's own nuclear import machinery [41]. Complexing DNA within a polymeric nanocarrier has been shown to increase nuclear association and permeability [42]. For all of these steps, nanomaterial properties are key in order to achieve intracellular nanoparticle-based DNA and siRNA delivery (Table 1). Table 1 also illustrates the evolution of nanomaterials used for non-viral gene delivery from readily available off-the-shelf chemicals to custom biomaterials designed specifically for intracellular nucleic acid delivery.

2. Nanoparticles for DNA and siRNA delivery

2.1 Liposomes and lipid-based materials

Lipid-based nanoparticles are the most commonly used non-viral chemical method of intracellular nucleic acid delivery. Several commercially available transfection reagents including Lipofectamine® 2000 [43], 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [44], RNAifect [44], TransIT-TKO and TransIT-siQuest [45] are all lipid-based. For DNA delivery, the molecular structure of the cationic lipid is an important factor in transfection efficacy as it determines how the liposome interacts with the cell membrane [46, 47]. These liposomes can be modified by conjugating targeting ligands on the surface [48] or adding cholesterol to improve cell binding and uptake [49]. In liposomes delivering siRNA, cholesterol is often added to formulations to increase membrane fluidity and thereby increase cell membrane fusion and cellular uptake [50, 51]. Other lipid-based approaches utilize modified structures of individual lipids to make it energetically easier for the liposome to leave the lamellar phase and disrupt the endosome, thus releasing nucleic acid cargo into the cytoplasm and preventing lysosomal degradation [24, 52, 53]. Liposomally delivered siRNA cargo simultaneously escapes the endosome and is released from its carrier into the cytosol, its site of action. Lipid hydrocarbon tail properties such as chain length and saturation have been shown to play a role in cell membrane fusogenicity and can be optimized to promote cellular uptake [54].

Factors other than efficacy must be considered when designing nanoparticle systems. For example, DOTAP forms stable nanoparticles that protect DNA from degradation [55] but has been shown to promote strong interferon responses in mice [44]. Chono *et al.* modified DOTAP with hyaluronic acid and were able to demonstrate reduced immunotoxicity as measured by inflammatory cytokine expression [56]. Functionalizing liposomes with a

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hydrophilic polymer poly(ethylene glycol) (PEG) has also been shown to reduce immune stimulation [57]. Semple *et al.* were able to show successful intravenous administration of siRNA using PEGylated lipids in non-human primates [58]. Interestingly, some groups have been able to take advantage of the immunogenic properties of DOTAP. Ott *et al.* showed that DOTAP nanoparticles in a DNA vaccine formulation induced greater antibody response compared to naked DNA [59]. Thus, DOTAP could potentially be used as an adjuvant as well as a carrier in DNA vaccines.

2.2 Inorganic nanoparticles

Calcium phosphate (CaP) nanoparticles enable DNA delivery via co-precipitation of CaP and DNA into nanoscale crystals [60–62]. Sokolova *et al.* synthesized nanoparticles with a CaP core and alternating DNA and CaP shells that protected DNA from degradation and improved transfection [63]. Methods to optimize CaP for siRNA delivery have often employed polymers. Polymethacrylate-PEG (PMA-PEG) block copolymers were coated onto the surface of CaP/siRNA nanoparticles and assisted in endosomal escape [64]. To improve loading of siRNA into CaP, Zhang *et al.* covalently functionalized siRNA with PEG and then co-precipitated siRNA and CaP [65].

Gold nanoparticles are advantageous for several types of gene delivery because they are safe, easy to chemically functionalize, and have the potential for diagnostic as well as therapeutic use [66, 67]. The particle surface can be modified by cationic groups such as quaternary ammonium salts to increase DNA binding [68]. Alternatively, anti-sense DNA oligonucleotides have been covalently linked to the surface of nanoparticles to induce gene knockdown [69]. Spherical nucleic acids, oligonucleotides arranged in a dense, oriented, and spherical configuration, have shown promise for intracellular delivery in multiple applications and are often designed by conjugation to an inorganic nanoparticle core, such as thiolated nucleic acids conjugated to gold nanoparticles [70, 71]. The covalent linker can also be modified to allow greater control of DNA release. Han *et al.* used a photoactive *o*-nitrobenzyl ester linker to control the spatial and temporal release of DNA by applying a near-UV light [72]. Alternative approaches to deliver siRNA using inorganic nanoparticles include combination with polymers. siRNA can be non-covalently layered onto gold nanoparticles by alternating layers of siRNA with the cationic polymer poly(ethylene imine) (PEI) [73]. A combinatorial approach was designed by Lee *et al.* in which siRNA was covalently linked to gold nanoparticles via disulfide bonds and then electrostatically coated with PBAEs in order to promote cell uptake and endosomal buffering [74].

Quantum dots such as CdSe/ZnS nanoparticles can be used as fluorophores as well as nucleic acid delivery vehicles [75–77]. DNA can be covalently conjugated onto the quantum dot using a peptide nucleic acid linker [78], or through non-covalent association with cationic polymers that are capped on the quantum dot surface [79]. Methods for siRNA delivery commonly employ covalently linking siRNA to the quantum dot surface, often with a polymeric spacer [80, 81].

Mesoporous silicas are solid materials that have a honeycomb-like porous structure with empty channels (mesopores) that can encapsulate bioactive molecules.[82] This unique porous structure provides an inner and an outer surface onto which cargo can adsorb. Silica

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has been shown to have high affinity for the head groups of phospholipids that promotes its association with the cell membrane, enhancing cellular uptake through physical concentration of mesoporous silica nanoparticles (MSNs) on the cell surface [83, 84]. MSNs for DNA delivery require surface modification with cationic groups for DNA binding [85]. Similarly, MSNs have been coated with cationic polymers such as PEI [86] and PAA [87] to facilitate siRNA binding. In addition to nucleic acid binding on the outside, the internal surfaces of MSN mesopores have been used to encapsulate fluorescent dyes for intracellular tracking [88] or anticancer drugs for multimodal therapies [87]. Li *et al.* designed an MSN modified with PEI and the fusogenic peptide KALA encapsulating siRNA targeting vascular endothelial growth factor receptor [89]. When these particles were injected intratumorally into mice that had been subcutaneously inoculated with human lung cancer cells, they significantly inhibited tumor growth through the suppression of tumor neovascularization. These results demonstrate the potential of MSNs delivering nucleic acids as powerful anti-cancer therapies.

2.3 Polymeric nanoparticles

Poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles are advantageous for several types of drug delivery due to their biodegradability and safety, and PLBA as a biomaterial has already been used in a number of FDA-approved devices. PLGA particles are typically synthesized via a simple emulsion-solvent evaporation process [90] and have a readily functionalizable surface chemistry that allows easy attachment of molecules to promote delivery functions like tissue homing and cellular uptake [91]. DNA can be encapsulated within PLGA particles through a double emulsion process or adsorbed onto the particle surface after surface treatment with bioadhesive agents such as Carbopol, a polyacrylic acid-based polymer [92]. Cationic polymers such as chitosan or spermidine can be blended into PLGA particles, the latter of which was used by Woodrow *et al.* for intravaginal siRNA delivery [93].

More commonly employed polymers for polymeric gene delivery nanoparticles are often cationic and electrostatically interact with nucleic acids to form polyplexes. Early gene delivery strategies often employed poly(*L*-lysine) (PLL) due to its cationic nature [15, 16]. PLL particles delivering DNA have been modified by adding PEG groups to prevent particle aggregation in serum [94] and Kim *et al.* created a terplex system with stearyl-PLL, low density lipoprotein, and DNA that increased particle compactness and improved DNA binding [95]. PEG-PLL block copolymers have also been used for siRNA delivery [96], but were often so stable that the siRNA cargo could not be released. Miyata *et al.* enabled PEG-PLL nanoparticles to undergo cytoplasmic siRNA release by crosslinking PLL end chains with bioreducible disulfides [97]. A similar delivery system was later modified with the RGD integrin recognition peptide to promote *in vivo* tissue targeting [98].

PLL nanoparticles often are unable to escape the endosome. This realization led to the exploration of other cationic polymers such as PEI, a polymer that contains primary, secondary, and tertiary amines to enable both nucleic acid binding and more efficient endosomal escape [99]. Godbey *et al.* used extensive confocal microscopy experiments to elucidate the intracellular fate of PEI-DNA nanoparticles. They found that PEI particles

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aggregate in discrete patches on the cell surface before being internalized through endocytic vesicles; some particles then escape through lysed endosomes and localize to the nucleus [100]. The polymer structure can be tuned to modulate gene delivery as low molecular weight PEI cannot condense DNA as well as its high molecular weight counterpart but is less toxic than higher molecular weight PEI [101]. Modifications with targeting ligands and PEGylation have also been shown to improve particle stability and *in vivo* transfection [102]. PEI analogs optimized for siRNA delivery frequently employ lower molecular weight linear PEIs linked with disulfide bonds to enable degradation and siRNA release, as PEI itself contains no biodegradable moieties [103].

Similar polymeric materials such as poly(amido amine)s (PAAs) and poly(amido ethyleneimine)s (PAEIs) have been designed that have buffering capacities in the endosomal pH range superior to PEI [104]. Partial degradation of PAA dendrimers by heat treatment increases the dendrimer flexibility and has been shown to lead to better transfection [105]. PEGylation further increases transfection efficacy and decreases toxicity [106]. PAA modifications for siRNA delivery employ disulfides linkages in the polymer backbone, and have shown superior siRNA delivery compared to PEI even in cells with comparable nanoparticle uptake [107–109]. This is likely due to the enhanced cytoplasmic siRNA release enabled by the inclusion of bioreducible disulfides. Modification of disulfide containing PAAs with PEG has shown a reduction in hemolysis and particle aggregation *in vivo*, but with reduced particle stability and decreases in gene knockdown [110].

Cyclodextrin-based nanoparticles are effective and have reached clinical trials. Cycldextrins are a class of water-soluble molecules of 6–9 glucose units that form a cone-shaped structure with a hydrophobic interior that can complex with various molecules, including nucleic acids [111]. Nanoparticles for DNA delivery can be made by conjugating cyclodextrin with polymers including PEI [112] and PAA [113], which condense DNA through electrostatic interactions. DNA can also be covalently linked to cyclodextrin via cationic adamantyl linkers [114]. For siRNA delivery, self-assembled nanoparticles can be made from cyclodextrin polymer, siRNA, and adamantane-PEG conjugates [115, 116]. These nanoparticles were used to deliver siRNA targeting the M2 subunit of ribonucleotide reductase in a Phase 1a/1b clinical trial which demonstrated siRNA activity in humans [116, 117].

Dendrimers are polymer structures that consist of a central core molecule from which highly branched arms extend out in an ordered and symmetric fashion. The stepwise method of dendrimer synthesis lends greater control of polymer size while the branching structure results in a higher density of terminal groups, offering unique surface characteristics and additional attachment sites for drugs or targeting moieties [118]. Two dendrimers that have been used for gene delivery are the PAAs mentioned above and polypropylenimine (PPI). PPI dendrimers with a butylenediamine (DAB) core have been shown to increase DNA binding with increasing dendrimer generations, with generation 2 providing the optimal balance between nucleic acid binding and toxicity [119]. Arginine has been conjugated to the terminal ends to increase membrane permeability and improve nuclear localization [120]. PPI nanoparticles for siRNA delivery have been modified with a disulfide crosslinking molecular cage on the surface to increase particle stability [121].

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2.4 Poly(beta-amino ester) (PBAE) nanoparticles for DNA and siRNA delivery to cancer

PBAEs are a class of polymer that contains tertiary amines and ester bonds along the polymer backbone. These chemical moieties provide positive charge for nucleic acid binding, buffering to promote endosomal release, and hydrolytic degradability for cargo release [122]. PBAEs have been well studied for DNA delivery, and have been designed to deliver DNA more efficiently and with less cytotoxicity than commercially available reagents such as PEI and Lipofectamine® 2000 in several cell types [123, 124]. By changing the chemical properties of the PBAE, it is possible to design nanoparticles that selectively deliver DNA to certain cell types, while avoiding delivery to others [124]. PBAEs can also be designed to deliver DNA to cancer while avoiding healthy cells, thereby allowing for the delivery of cell-killing genes to tumor cells without off-target effects [125–127]. Additionally, PBAE-DNA nanoparticles can be fabricated, dried, and stored as a powder for at least two years at −20°C without losing function, highlighting their translational potential [125, 127].

Various modifications to the PBAE backbone have been made to allow controlled DNA release from PBAE nanoparticles. A light-responsive 2-nitrobenzene moiety was added to the PBAE backbone to allow quick and controlled DNA release upon UV radiation (Fig. 2) [128]. Gu *et al.* electrostatically linked pH-sensitive carboxymethyl poly(L-histidine) groups to PBAE-DNA nanoparticles to neutralize the particle's positive surface charge and increase buffering capacity; this modification decreased erythrocyte agglutination and enhanced the particle's tumor targeting capabilities after intravenous injection [129].

Due to the physical differences between DNA and siRNA described above, PBAE-based siRNA delivery had initially been difficult without the addition of other delivery vectors such as gold nanoparticles [74]. Hong *et al.* took the approach of modifying the siRNA itself. They designed complementary DNA/siRNA strands that self-assembled to form a dendrimeric siRNA structure; these siRNA dendrimers had higher charge density and structural flexibility, which allowed them to form stable particles with PBAE formulations that had been optimized for DNA delivery [130]. Tzeng *et al.* modified the polymer structure by end-capping traditional PBAEs with a disulfide-containing small molecule and showed successful siRNA delivery to both cancer cells and mesenchymal stem cells [131, 132]. The addition of the degradable disulfide moiety enabled this polymer structure to form polyplexes at a higher polymer:siRNA mass ratio (wt/wt) without causing significant toxicity, even though the disulfide bonds were only at the polymer end-caps. Building on this work, Kozielski *et al.* designed a novel disulfide containing monomer to form disulfide bonds within every repeat unit [133]. This monomer, 2,2′-disulfanediylbis(ethane-2,1-diyl) diacrylate, was referred to as "BR6" as it was the reducible form of a well-established PBAE monomer known as "B6," hexane-1,6-diyl diacrylate [134]. PBAE nanoparticles made from BR6 were shown to bind siRNA with the same strength as particles made from its non-reducible analog but quickly released its siRNA cargo in a reducing environment, unlike the conventional non-bioreducible PBAEs. Furthermore, these particles achieved gene knockdown *in vitro* that was significantly higher than that achieved by Lipofectamine® 2000 (Fig. 3) and was shown to preferentially deliver siRNA to brain cancer cells while avoiding delivery to healthy brain cells.

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The ability to effectively bind and deliver both DNA and siRNA make PBAEs an attractive option for gene delivery in cancer therapies. PBAE nanoparticles delivering the p53 tumor suppressor gene inhibited tumor growth in a small cell lung cancer mouse model after intratumoral injection [135]. A nanoparticle with a PBAE-DNA core and a pullulan-methotrexate shell showed enhanced circulation time and targeted delivery to hepatoma cells *in vivo*, with high levels of particle accumulation and transfection in the tumor [136]. For more controlled long-term release, Segovia *et al.* encapsulated PBAE-siRNA particles in a PAA-dextran aldehyde hydrogel; when implanted in a breast cancer model, they saw a high level of knockdown even after 7 days [137]. Our group is particularly interested in the use of PBAE nanoparticles for the treatment of glioblastoma. Glioblastoma (GBM), a grade IV glioma, is one of the most deadly human cancers with a median survival of only 15 months following treatments such as tumor resection, chemotherapy and radiotherapy [138–140]. Polymeric nanoparticles for the intracellular delivery of nucleic acids enable new modalities of treatment for many cancer cell types, including GBM. By tuning the PBAE polymer structure, we were able to form PBAE nanoparticles that preferentially delivered nucleic acids to brain tumor initiating cells, a cell population that is believed to be responsible for tumor recurrence [127]. High levels of transfection were seen when PBAE particles delivering a GFP reporter gene were injected into an orthotopic GBM murine model [125]. In addition, these particles were able to achieve a therapeutic effect. PBAE nanoparticles delivering DNA encoding the herpes simplex virus-derived thymidine kinase (HSVtk) were injected intracranially in a rat GBM model while the ganciclovir pro-drug was administered systemically [126]. The PBAE nanoparticles penetrated through the whole brain tumor volume (a length of approximately 2 mm) and HSVtk catalyzed the phosphorylation of ganciclovir into its active form to enable killing of brain cancer cells, resulting in significant survival benefits [126].

Further modifications of PBAEs such as synthesis of dendrimeric versions of the polymers are interesting future directions for enhanced nucleic acid delivery. Cutlar *et al.* synthesized a highly branched PBAE that showed higher transfection efficacy when compared to linear counterparts as they could better condense their DNA cargo [141]. Zhou *et al.* synthesized a dendrimeric ester nanoparticle that successfully delivered microRNAs to a liver cancer model and achieved significant survival benefits [142]. The authors hypothesized that the increased nucleic acid binding capacity and degradability of these polyester dendrimers contributed to successful RNA delivery while maintaining low hepatotoxicity. Indeed, dendrimeric PBAEs may produce smaller, more compact nanoparticles that contain more polymer end groups, which could increase biomaterial-mediated cell specificity. This may be especially relevant for cancer therapy, including brain cancer therapy, where smaller particle sizes can increase particle penetration and transport.

2.5 Methods for DNA and RNA co-delivery

Despite the physical differences between DNA and RNA that present different challenges for their intracellular delivery, several strategies have been developed for co-delivery in order to achieve novel therapeutic goals. In designing nanoparticle formulations, it is particularly important to also ensure that each of the different nucleic acids to be delivered reaches the target cells at the desired ratios. To enable co-delivery to the same cell, loading different

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nucleic acids into the same particles (rather than delivering a combination of particles, each with its own cargo) has been show to increase the co-expression of delivered nucleic (Fig. 4) [143]. As polyplexes are formed through self-assembly between cationic polyelectrolytes and anionic polyelectrolytes, with larger more multivalent polyelectrolytes leading to enhanced stability, carrier DNA can be complexed into the same polyplexes as siRNA as a strategy to stabilize the particle for enhanced siRNA delivery [144]. This can be an effective way to achieve gene knockdown and expression in the same cell to achieve synergistic therapeutic effects [145, 146]. Another way to complex multiple nucleic acids in the same particle is through layer-by-layer (LbL) assembly. Elbakry *et al.* used LbL to synthesize a particle with a gold core, 11-mercaptoundecanoic acid coating, and PEI-siRNA layers to condense siRNA into a particle and achieve effective knockdown [73]. Bishop *et al.* adopted a similar strategy but added DNA, siRNA, and PBAE layers [147]. This strategy can be used to deliver multiple nucleic acid cargos as well as control their relative release times.

3. Conclusion

Non-viral nanoparticle technologies for DNA and siRNA delivery have advanced rapidly, with many complementary biomaterial and particle designs. Several promising delivery platforms involving lipid-based, inorganic, and polymeric nanocarriers have been developed with strong *in vivo* efficacies, some of which have entered clinical trials. The interest in these technologies is due to the large potential for gene delivery and siRNA-induced gene knockdown to treat diseases caused by aberrant gene expression, such as cancer, and the need to obtain safe and effective delivery methods. Non-viral nanoparticles have the potential to fulfill this promise. Continuing to investigate the barriers to intracellular delivery as well as to innovate the nanotechnologies capable of overcoming these barriers may one day allow genetic medicine to clinically treat genetically based diseases such as cancer.

4. Expert Opinion

As polymeric nanoparticle-based gene therapy shows increasing promise against cancer *in vitro* and for local administration *in vivo*, increasing attention is being turned towards strategies to allow the systemic delivery of these particles to treat metastatic cancer. A common method employs PEGylation, which shields the particles from interacting with serum proteins or off-target cells. For example, PEGylation of PLL, PEI, and PAA-based nanoparticles has been shown to enhance their circulation time and reduce hemolysis and serum-induced aggregation [110, 148]. Such a strategy could greatly enhance the ability of newer types of non-viral nanoparticles, such as PBAE-based nanoparticles, to enable them to circulate effectively and diffuse through tissue, improving their translational potential for use in cancer applications.

PEG can also be used as a linking molecule onto which targeting ligands may be conjugated to enable nanoparticle targeting to cellular receptors. Ligands that have successfully been conjugated to PEGylated nanocarriers for cancer targeting include the RGD peptide sequence targeting integrins in tumor vasculature [149] as well as folate [150] and transferrin [151], molecules whose receptors are overexpressed in many cancer cell types. This strategy takes advantage of PEG's ability to increase nanocarrier serum stability, reduce

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non-specific uptake, and better enable the display of targeting moieties on the nanoparticle surface, resulting in higher particle accumulation in the tumor. However, one potential concern with PEGylated electrostatic polyplex nanocarrier systems is that while the charge masking properties of PEG have been shown to increase nanoparticle colloidal stability in serum, they may also decrease particle complexation stability. Kichler *et al.* showed that in a PEI polymer covalently endcapped with high molecular weight PEG, the resulting nanoparticles could not protect their DNA cargo from nuclease degradation and resulted in poor transfection when compared to un-PEGylated PEI [152]. Similarly, Mao *et al.* showed that in PEI-PEG block copolymers, formulations with lower molecular weight PEG at higher substitution levels resulted in large, loosely structured particles that could not effectively condense siRNA and resulted in poor knockdown [153]. The charge shielding capability of PEG molecules protect cationic polymers from serum aggregation but also reduce their ability to electrostatically bind to nucleic acid cargos. To create a PEGylated polymer for successful nanoparticle formation, it is crucial to balance these opposing forces, such as through the addition of crosslinks or by adding non-PEGylated polymers to the co-complex to increase its stability.

Another strategy for systemic delivery is coating the particle with peptides to decrease toxicity, enhance circulation time, and enable particle targeting to specific organs or tumors [154]. Simberg *et al.* coated a peptide sequence to iron oxide particles that targeted clotted plasma proteins in leaky tumor vasculature [155]. These particles in turn induced more clots and amplified the effect. PBAE nanoparticles electrostatically coated with poly(glutamic acid) based peptide sequences reduced *in vivo* toxicity and could enable targeting to specific organs based on peptide sequence differences and nanoparticle properties [156]. Peptide coating can allow nanoparticles to remain in circulation for longer periods of time and can enhance particle targeting and uptake through ligand-mediated endocytosis. An important area for future research in the field is the investigation of new types of nanoparticle coatings that enable greater specific intracellular delivery to on-target cancer cells (perhaps in a manner specific to the receptors on a patient's particular tumor), while preventing intracellular delivery to off-target cells. In addition, such next generation coating must enable prolonged circulation times, including resistance to clearance by neutralizing antibodies, even after multiple previous treatments of the next-generation nanoparticles.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is a recently discovered genome editing tool with many potential therapeutic applications. The CRISPR/Cas9 system derives from a bacterial defense mechanism, where foreign DNA segments are transcribed into a dual-RNA complex and used to recognize and silence invading targets through double-stranded breaks (DSB) induced by the Cas9 endonuclease [157]. A single chimeric sgRNA was recently developed that can activate site-specific cleavage by Cas9 [158]. The resulting DSB can be repaired through error-prone non-homologous end joining, leading to indels that knock out gene function, or homology-directed repair upon introduction of a donor repair template [159]. The CRISPR/Cas9 system has been successfully used in human cells to introduce permanent changes in the genome [159, 160], making it a powerful tool for gene editing to treat diseases like cancer.

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Only a handful of studies have been done using non-viral methods to deliver the CRISPR/Cas9 system. Jinek *et al.* constructed a plasmid encoding Cas9 and the sgRNA and delivered it using commercial transfection agents. However, they were only able to achieve editing efficiencies in the range of 6–8% [161]. Sun *et al.* constructed DNA nanoclews by packing sgRNA and the Cas9 protein into DNA strands that are partially complementary to the sgRNA and coating the outside with PEI (Fig. 5); the DNA nanoclew system achieved a 36% editing efficiency [162]. The low editing efficiency seen by many groups may be due to poor delivery efficacy of the Cas9 plasmid or sgRNA. Co-delivery of the two using non-viral nanoparticles, such as biodegradable polymeric nanoparticles, is a potential way to increase expression and gene editing efficacy. Strategies such as a layer-by-layer approach can be used to package the Cas9 plasmid and sgRNA using polymers that are suitable for each and to control their intracellular temporal release.

Another interesting target for the co-delivery of DNA and siRNA is the TNF-related apoptosis-inducing ligand (TRAIL) system. TRAIL induces apoptosis in many transformed cell lines by binding to the death receptors DR4 and DR5 on the cell surface [163, 164]. Its apoptotic function is selective for transformed and tumor cells [165], and exhibits a bystander effect [166]. These properties make TRAIL an attractive delivery target for cancer treatment as it can produce a cancer-specific, self-amplifying apoptotic effect. However, it has been shown that many cancer cell types resist TRAIL action. One explanation for this phenomenon is the presence of decoy receptors DcR1 and DcR2, which lack functional intracellular death domains [167]. Studies have shown that DcR2 is upregulated in some TRAIL-resistant breast and prostate cancers [168, 169]. siRNA knockdown of DcR2 in these cells sensitized them to TRAIL-induced apoptosis. When TRAIL plasmids were delivered to the same cells, their tumorigenic potential was significantly reduced. This suggests that co-delivery of siRNA to knock down decoy receptors and DNA to upregulate TRAIL expression can work synergistically to cause cancer cell apoptosis and may be a promising target for polymeric nanoparticle delivery. Similarly, other siRNA and DNA co-delivery strategies may enable breakthroughs against cancer resistance and are enabled by non-viral nanoparticles.

Treatment of genetically-based diseases such as cancer often requires a combinatorial approach, as cells can often compensate for the knockdown or overexpression of one genetic target. For the proposed treatment strategies suggested herein, co-delivery of DNA and RNA is required to occur within the same cells, not simply within the bulk of a tissue or tumor. While the materials optimal for DNA and siRNA often vary, a treatment strategy requiring co-delivery would ideally require a material optimized to deliver both. As previously demonstrated [143], a blend of nanoparticles containing different cargos is less likely to co-deliver both cargos to the same cells. Conversely, particles containing the cargo blended within each nanoparticle results in high co-delivery rates. Temporal control of DNA and RNA release [147] is also imperative for systems which would require DNA transcription and siRNA-induced knockdown to occur in a non-simultaneous fashion. Future nanoparticle designs that would have the sophistication and control to combinatorially deliver multiple types of nucleic acids against multiple targets have the potential to address the heterogeneity and mutational capabilities of genetic diseases such as cancer.

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highlights

- Obstacles to intracellular nucleic acid delivery include rapid clearance from circulation, tissue and tumor targeting, cellular internalization, endosomal escape, and intracellular release.
- Lipid-based and inorganic materials protect nucleic acids from degradation and condense them into nanoparticles for improved cellular uptake.
- Cationic polymers self-assemble into polyplexes with nucleic acids via electrostatic interactions and possess functional groups to aid in improved cellular uptake, endosomal escape through endosomal buffering, and intracellular cargo release via biodegradable linkages.
- Nanoparticle formulations optimized for the co-delivery of multiple DNA or siRNA cargos can be used to reach novel synergistic cancer therapy targets.
- Therapeutic modalities such as DNA, siRNA, and CRISPR/Cas technology may benefit from non-viral nanoparticle delivery platforms for the treatment of complex genetically-based diseases.

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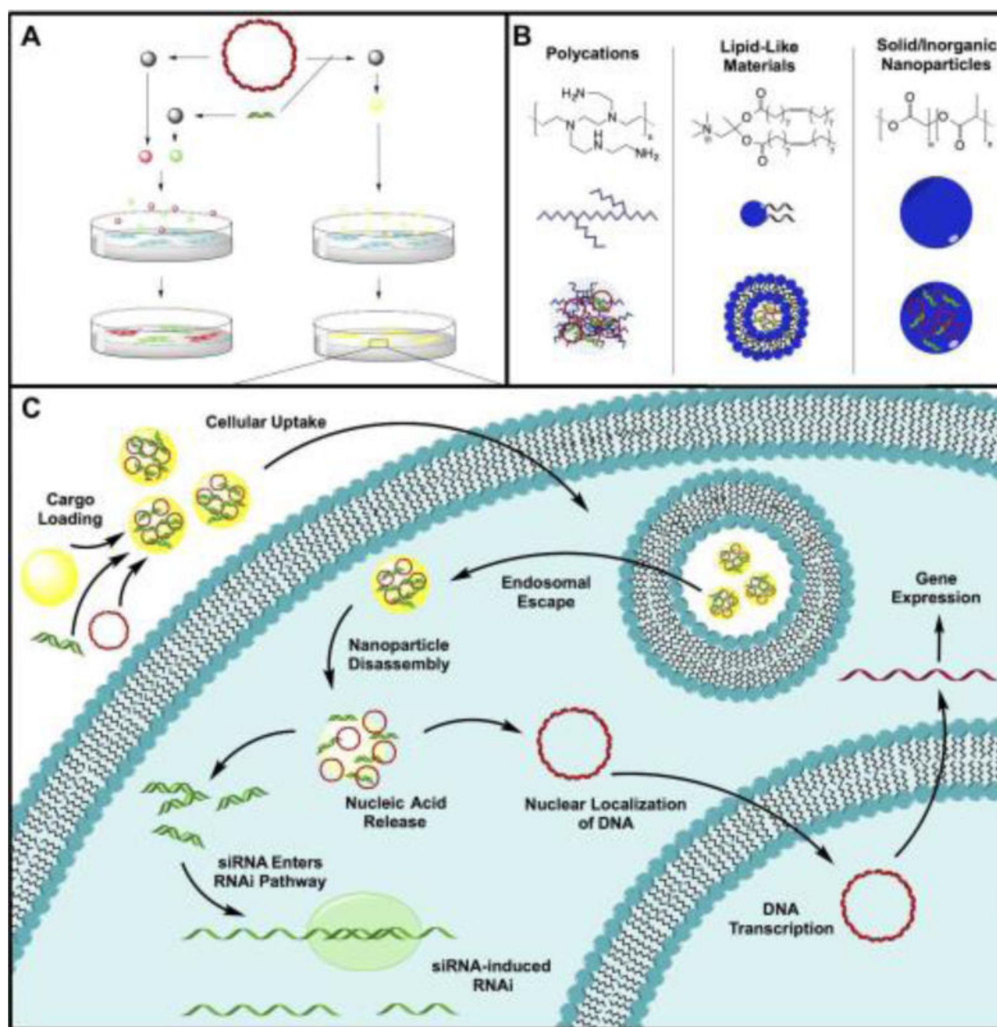

Figure 1.

Overview of nanoparticle codelivery of DNA and siRNA. **(A)** Nanoparticles carrying DNA and siRNA cargoes separately yield little codelivery, while nanoparticles carrying both nucleic acids optimize coexpression. **(B)** There are multiple classes of non-viral nanoparticles for gene delivery. Each class with its own representative chemical structure (top), nanoparticle structure (center), and method of carrying nucleic acid (bottom). **(C)** There are multiple steps for successfulintracellular delivery of DNA and siRNA.

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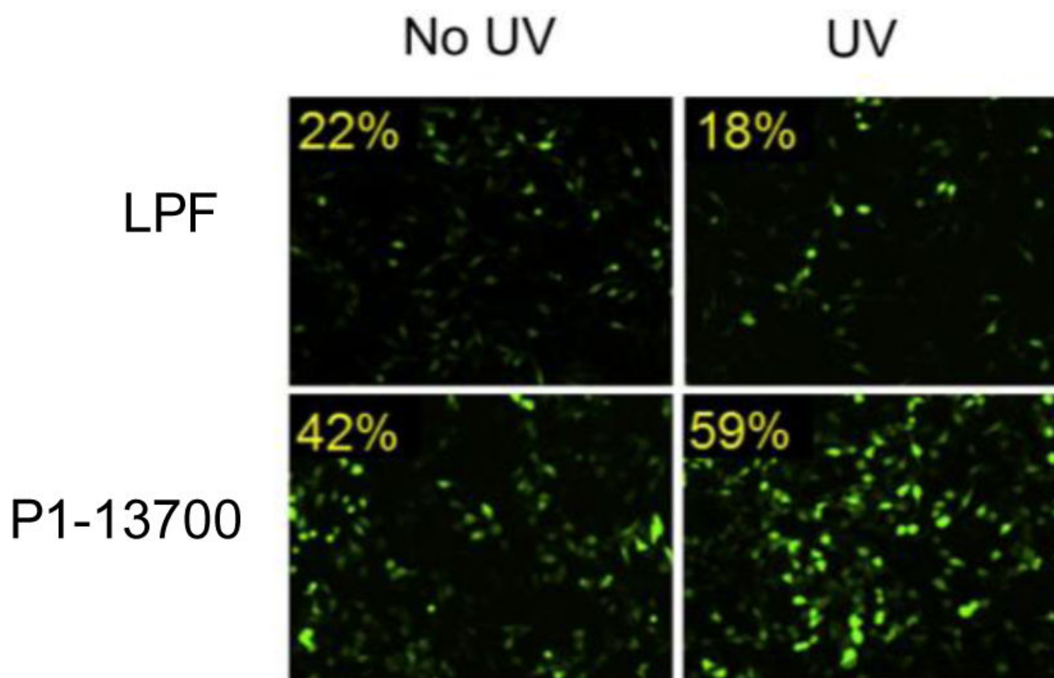

Figure 2.
Light-responsive PBAE nanoparticles (P1-13700) delivering an EGFP plasmid to Hela cells with or without 2 minutes of UV irradiation; Lipofectamine™ 2000 (LPF) was used as a control. PBAE transfection efficacy increased with UV treatment, which broke 2-nitrobenzene linkers in the polymer backbone and allowed controlled DNA release. Reproduced with permission [128].

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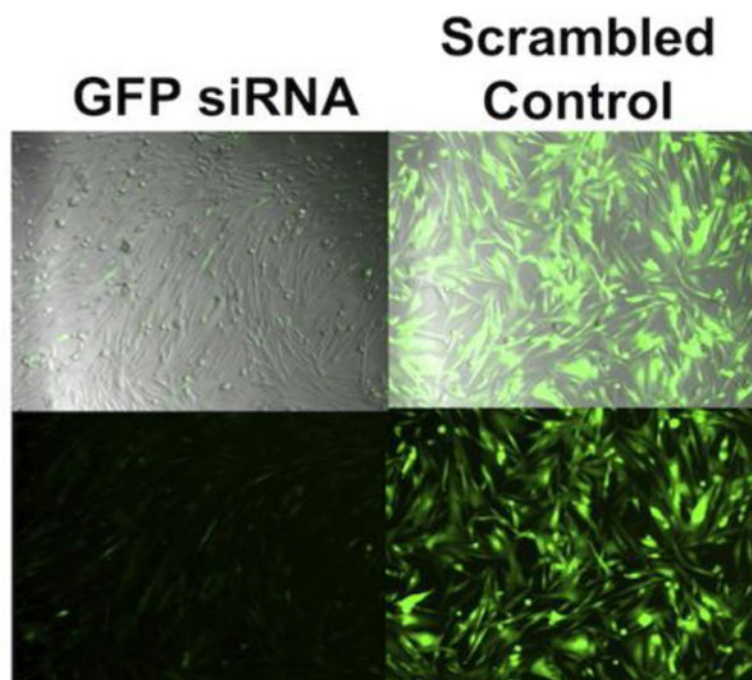

Figure 3.

Phase contrast (**top**) and fluorescence (**bottom**) images of GFP+ GBM cells with bioreducible PBAE (R647) nanoparticles delivering either an siRNA targeting GFP (**left**) or a scrambled control RNA (**right**) [170].

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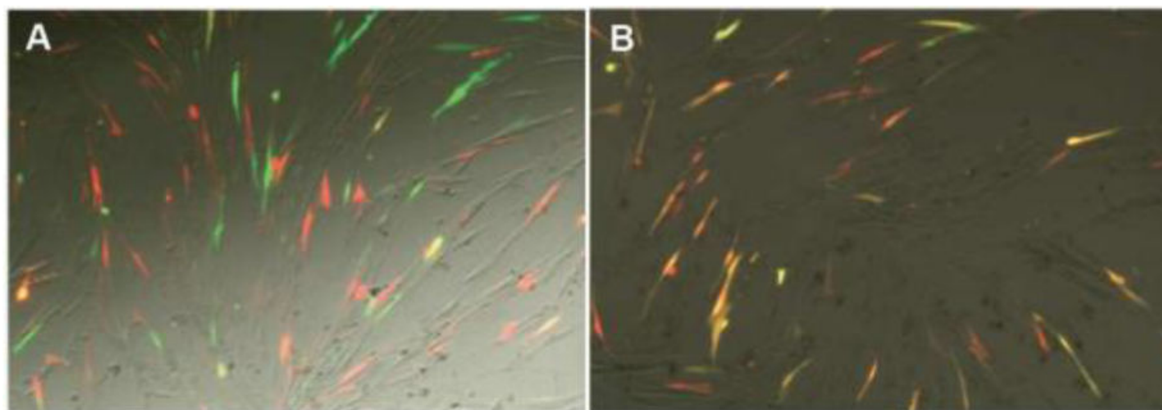

Figure 4.

(**A**) Nanoparticles carrying either GFP or DsRed plasmid DNA are blended following nanoparticle fabrication, resulting in particles containing only one type of plasmid. Transfection of IMR90 human fibroblasts with this nanoparticle combination yields little codelivery, as indicated by few cells coexpressing GFP and DsRed (yellow cells). (**B**) Nanoparticles formed using a blend of GFP and DsRed plasmids yield particles containing both plasmids, and coexpression is high. Reproduced with permission [143].

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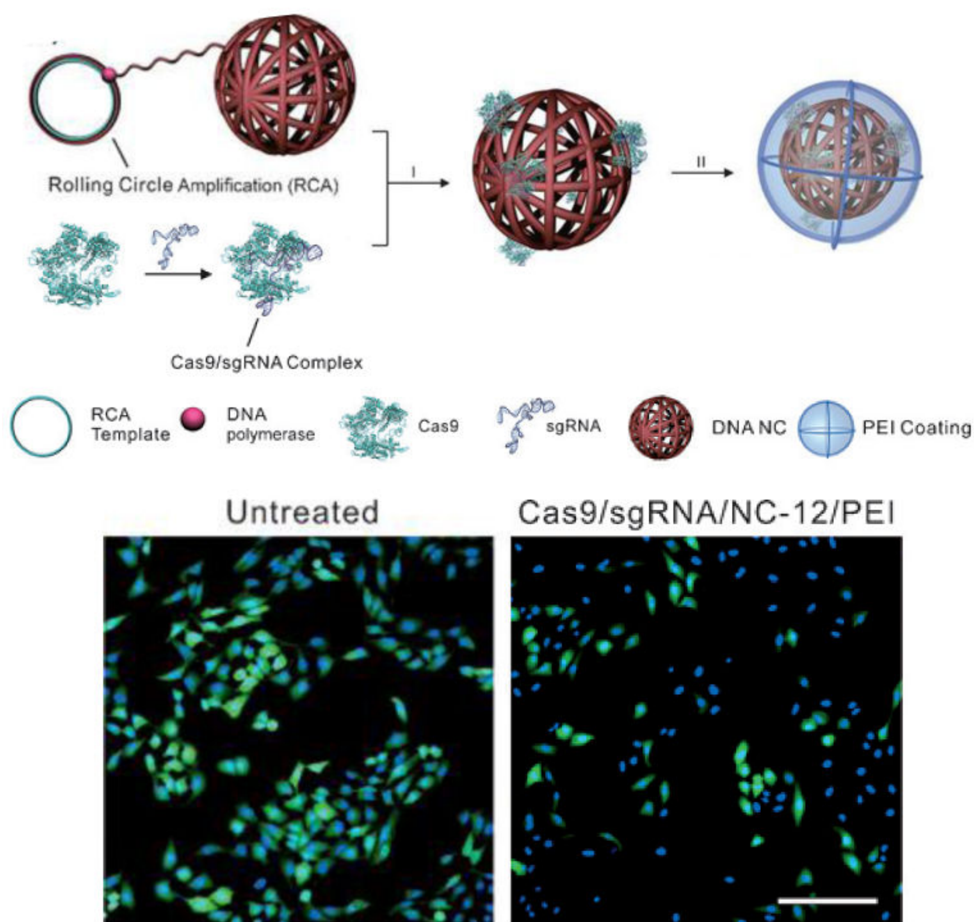

Figure 5.

(**Top**) Assembly schematic of DNA nanoclew (DNA-NC) carrying Cas9 and sgRNA. Cas9 and sgRNA were incubated together and complexed with the DNA-NC; a PEI coating was then applied to the outside to facilitate endosomal escape. (**Bottom**) Fluorescence microscopy images of EGFP+ U2OS cells with or without treatment with DNA-NC delivering Cas9 and EGFP-specific sgRNA. Cas9-induced DNA cleavage resulted in significant EGFP gene knockout. Reproduced with permission [162].

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Table 1

Key nanomaterials listed in chronological order of their initial investigation to illustrate the evolution of nanomaterials used for non-viral gene delivery. Representative cancer types that have been investigated using each material for therapeutic gene delivery are also listed.

Non-viral Vector	Key Characteristics	Early Investigation	Representative Cancer Types
Calcium phosphate	Co-precipitate nucleic acids with calcium phosphate to form nanocrystals	1973[171]	Melanoma and breast [172] cancer ; nasopharyngeal carcinoma[173]
Liposomes	Encapsulate nucleic acid cargo in aqueous interior	1980[174]	Colorectal cancer and breast cancer[175]; pancreatic islet cell tumors[176]; Lewis lung carcinoma[48]
PLL	Cationic polypeptide for nucleic acid binding	1987[16]	Lung cancer[177]; bladder cancer[178]
Gold	Chemically inert, easily functionalized; can be used for theranostic purposes	1990[179]	Breast cancer[180, 181]
Dendrimers	Highly branched polymers with greater shape control and end group density	1993 (PAA)[182]; 1999 (PPI)[183]	Breast cancer[184]; ovarian cancer[185]; epidermoid carcinoma and glioblastoma[186]
PEI	Titratable amines facilitate endosomal escape	1995[16, 99]	Neuroblatoma[149]; glioma and medulloblastoma[187]; glioma and hepatoma[150]
PLGA	Encapsulates nucleic acids through double emulsion process	1997[182, 183, 188]	Lung cancer[92]; prostate cancer[184–186, 189]
Cyclodextrin	Water-soluble polysaccharides that can complex with nucleic acids	1999[190]	Hepatoma[114]; leukemia[151]; breast and ovarian cancer[191]
Mesoporous silica	Solid material with porous structure allowing cargo adsorption on the outer surface and inside pores	2000[83]	Lung cancer[89]; ovarian cancer[87]; breast cancer[192]
PBAE	Contains hydrolizable ester bonds for greater biocompatability	2000[122]	Glioblastoma[125, 126, 170]; melanoma[129]; small cell lung cancer[135]; hepatoma[136]; prostate cancer[193]