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Nanotechnology Approaches for the Delivery of Exogenous siRNA for HIV Therapy

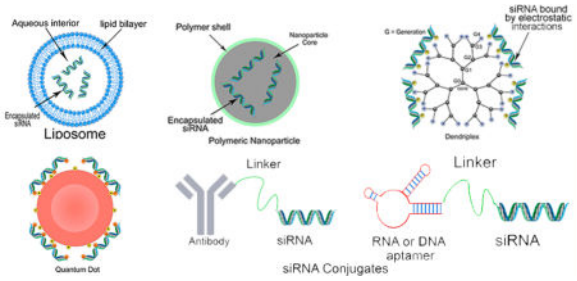
Simeon K. Adesina* and **Emmanuel O. Akala**

Department of Pharmaceutical Sciences, Howard University, Washington, DC 20059, United States

Abstract

RNA interference (RNAi) is triggered by oligonucleotides that are about 21–23 nucleotides long and are capable of inducing the destruction of complementary mRNA. The RNAi technique has been successfully utilized to target HIV replication; however, the main limitation to the successful utilization of this technique *in vivo* is the inability of naked siRNA to cross the cell membrane by diffusion due to its strong anionic charge and large molecular weight. This review describes current nonviral nanotechnological approaches to deliver anti-HIV siRNAs for the treatment of HIV infection.

Graphical abstract



Keywords

RNA interference (RNAi); liposomes; polymeric nanoparticles; dendrimers; small interfering RNA

INTRODUCTION

Combination therapy, also known as Highly Active Anti-Retroviral Therapy (HAART), is the current mainstay of HIV-1 therapy.[1] HAART involves drugs from different classes of antiretroviral drugs that inhibit various enzymes in the HIV replication cycle.[1–3] While HAART has been very successful in managing HIV/AIDS, the high mutation rate of HIV leading to the development of multiple resistant HIV strains, the existence of viral reservoir

***Corresponding Author**, Tel: 202-864-0401. Fax: 202-806-7805. simeon.adesina@howard.edu.

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sites inaccessible to current drug delivery methods, low oral bioavailability, poor drug-regimen compliance, poor drug pharmacokinetics, long-term drug therapy, increased side effects as a consequence of the high and frequent dosages, and high costs are some of the factors affecting HAART that result in failure of therapy.[2–6] These challenges with HAART have led investigators to seek alternative therapeutic options and strategies against HIV-1, which need to be exploited for HIV-1 to be successfully treated.

Nucleic acid-based therapeutics that involve the use of antisense oligonucleotides, aptamers, ribozymes, miRNAs, and RNA interference (RNAi), among others, for therapeutic purposes have emerged as an alternative to HAART. The use of these various agents either as single agents or in combination with other nucleic acids or chemical antiviral agents holds the potential to overcome some of the challenges associated with HAART and show greater efficacy in blocking viral replication and decreasing the incidence of resistant strains. As one of the most potent nucleic acid-based approaches, RNAi has become popular due to its potential to mediate sequence-specific post-transcriptional gene silencing and represents a viable option to currently available anti-HIV therapy.[7] While the high rate of HIV mutation has been identified as a constraint to effective treatment, RNAi holds the potential to overcome this constraint by targeting highly conserved regions on viral genes that are important for viral replication.[8,9] The RNAi mechanism is triggered by small interfering RNA (siRNA) that are about 21–23 nucleotides long and are capable of inducing the selective degradation and destruction of complementary mRNA.[6,10]

The HIV-1 life cycle is a complex series of steps that is regulated by both viral and cellular proteins.[11,12] The reader is directed to detailed reviews on these viral and cellular proteins and their roles in HIV replication.[12,13] The RNAi technique has been utilized to inhibit HIV replication by its effect on these HIV-1 or cellular factors in cell culture and animal models.[6,7,14,15] Furthermore, all HIV-1 encoded genes such as *tat, rev, gag, pol, nef, vif, env, vpr*, and the long terminal repeat are susceptible to the RNAi mechanism. Host cellular genes such as the chemokine receptor CCR5 have also been targeted using RNAi.[7]

Limitations to the successful utilization of RNAi *in vivo* can generally be divided into two categories: (i) the inability of unprotected naked siRNA to cross the cell membrane by passive diffusion (due to its strong anionic charge and large molecular weight) and (ii) the lack of methods and delivery systems to safely and efficiently deliver siRNA molecules into target cells and induce the RNAi response.[6,14,35,36] Other problems include rapid degradation of siRNA by endogenous nucleases, nonspecific distribution, low endosomal escape efficiency, removal by glomerular filtration, and development of RNAi viral mutants due to the high mutation rate of HIV-1.[37–39]

To encourage the use of therapeutic siRNAs *in vivo*, various attempts have been made to deliver siRNAs using two different categories of vectors, namely, viral and nonviral vectors.[36] Also worthy of mention is the delivery of genes and siRNA using recombinant proteins and physical methods such as electroporation, microinjection, and biolistic particle delivery (gene gun).[40]

VIRAL VECTORS

Viral carriers for exogenous siRNA delivery have been relatively successful in delivering genes and siRNA to cells. The use of viral carriers is based on the ability of viruses to infect cells.[40] Viral carriers are made up of viral particles consisting of three parts, namely, nucleic acid or the genetic material, the capsid protein, and an outer envelope structure.[41] The genomes of the viruses used as carriers have been modified by deleting one or more viral structural genes. This prevents viral replication, reduces cytotoxicity toward host cells, and generally makes the viral vectors safer to use.[41,42] Advantages of viral vectors include the following: (a) they contain strong promoters to facilitate a high level of transgene expression, (b) some viral vectors bear tissue-specific promoters to ensure expression only in target tissue and inhibit off-target effects, (c) specific recognition sequences can be introduced in the outer envelope structure to facilitate site-specific infection, and (d) gene retargeting.[41,42] Disadvantages associated with the use of viral carriers include the following: (a) strong immunogenicity, (b) toxin production, (c) low transgene capacity, (d) induction of inflammation, (e) carcinogenicity, and (f) risk of recombination.[40,42] The viral vectors that have been reported include retroviral vectors, adenoviral vectors, adeno-associated vectors (which are safer than adenoviral vectors as a result of deficiencies in their replication and pathogenicity), herpes simplex virus, lentiviruses, poxvirus vectors, Epstein–Barr virus, alphaviruses, and baculoviruses.[41,42] Viral vectors have been the subject of several articles and are not covered in this review. The reader is directed to the review by Nayerossadat et al. (2012) for a good review of the viral vectors.[42]

NONVIRAL CARRIERS FOR EXOGENOUS SIRNA DELIVERY FOR HIV THERAPY

Delivery methods using nonviral carriers employ synthetic and/ or natural compounds to deliver nucleic acids such as siRNA or DNA into cells. Compared to viral vectors, nonviral nucleic acid carriers exhibit considerably reduced transfection efficiencies and nonviral methods are generally considered to be less effective than viral methods.[43,44] However, unlike viral methods, the materials used in the fabrication of these carriers are less toxic and less immunogenic compared to viral vectors. Other advantages of nonviral carriers include biocompatibility, the potential for targeting or site-specific drug delivery, the ease of production of these carriers, especially in laboratory-scale formulations, and the potential for repeat administration without stimulating the immune system.[43] Nanotechnological nonviral approaches for exogenous siRNA delivery for HIV/ AIDS include the use of liposomes, polymeric nanoparticles, dendrimers, quantum rods, carbon nanotubes, and inorganic nanoparticles. The ideal delivery system must bind or encapsulate the siRNA in a reversible manner to facilitate siRNA delivery and release to the cell cytoplasm, protect the siRNA from degradation in circulation and in endosomes, be biocompatible and biodegradable, and also prevent clearance by the liver and kidney.[14,45] Discussed below are the various carrier-based, nonviral approaches described in the literature for the delivery of exogenous, therapeutic anti-HIV siRNA for HIV treatment (summarized in Table 1). Other approaches, such as targeted delivery of siRNA using conjugates of siRNA with aptamers without the use

of a carrier system,[46,47] have been the subject of several excellent articles[48,49] and are generally not covered in this review.

Liposomes

Liposomes are self-assembled nano- or micro-particles or colloidal carriers that spontaneously form when certain lipids are hydrated in aqueous media.[50,51] They consist of an aqueous volume enclosed by a membrane of one or more bilayers of natural and/or synthetic lipids (Figure 1).[52] Drugs may be encapsulated in the aqueous core or intercalated in the bilayer via passive mechanisms (i.e., drug encapsulation occurs during liposome formation) or actively (i.e., after liposome formation).[50,53]

Liposomes may be classified based on their size and number of bilayers into two categories, namely, multilamellar vesicles (MLV) (concentric phospholipid spheres separated by layers of water) and unilamellar vesicles (a single spherical phospholipid bilayer enclosing an aqueous core).[53] The unilamellar vesicles can be further classified into large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV). The structure formed is important because the liposome size and the number of bilayers impact the circulation half-life and the drug loading, respectively.

The main constituent of conventional liposomes is phospholipids, which are also the main components of the cell membrane; thus, phospholipids and liposomes have excellent biocompatibility.[54] For a great discussion of the extensive list of phospholipids and other substances used in liposome preparation, their properties, and liposome and micellar formation characteristics and classification, the reader is referred to a very recent review on phospholipids and their applications in drug delivery systems.[54]

Methods of Preparation of Liposomes—Liposomes can be prepared using a variety of methods such as physicochemical dispersion methods (examples include ultrasonication, membrane extrusion methods, etc.), solvent injection or dispersion methods (examples include ethanol injection method, reverse-phase evaporation methods, etc.), detergent removal methods (examples include dialysis method, column chromatography methods), and other miscellaneous methods. For in-depth discussions of liposome preparation methods, the reader is referred to comprehensive reviews of this topic.[52,53,55]

Numerous advantages and some disadvantages of the liposome as a drug carrier have been reported.[51–53,56] These are provided in Table 2.

To improve liposomal properties and increase the half-life, surface modification has been done with poly(ethylene glycol) and other agents to prevent their rapid clearance from circulation by cells of the mononuclear phagocyte system (MPS) to produce long-circulating liposomes.[54,56] More recently, multifunctional liposomes have been designed to selectively target cells or tissues of interest using antibodies, aptamers, or other ligands as targeting moieties.[51]

Cationic lipids are positively charged amphiphilic molecules that have a cationic polar headgroup responsible for interaction with negatively charged nucleic acids, among other

constituents.[57] They are used in the preparation of cationic liposomes for delivery of nucleic acids to cells. Cationic liposomes protect nucleic acids from enzymatic degradation in blood and also probably facilitate cell internalization by interactions with the negatively charged cell membrane.[57] For siRNA delivery in HIV therapy, Kim et al. (2010) have described a novel integrin-targeted and stabilized nanoparticle (I-tsNP) formulation targeted to human lymphocytes and monocytes.[16] The system uses a human lymphocyte function-associated antigen-1 (LFA-1) integrin-targeted antibody for the delivery of siRNA *in vivo*. They reported the silencing of leukocyte-specific HIV coreceptor CCR5 expression in bone marrow liver thymic (BLT) mice transplanted with human fetal hepatic CD34+ cells. This formulation uses neutral phospholipids and thus avoids the potential toxicity ascribed to cationic lipids and polymers used for siRNA delivery. They reported that surface-modified, LFA-1 I-tsNP-mediated siRNA delivery using liposomes of about 100 nm in diameter silenced CCR5 expression and protected mice from HIV challenge *in vivo*. This report suggests that the technique can be developed as an intracellular immunization strategy for clinical application.

The preparation of small unilamellar vesicles (SUV) that form a complex with negatively charged siRNA, termed the Neutraplex delivery system (Nx), has been described.[17] Positively and negatively charged lipoplexes (consisting of complexes of the Nx formulation and anti-CXCR4 siRNA) were prepared with diameters ranging from <100 nm to around 350 nm, depending on the surface charge of the formulation. The tested formulations include $Nx_{+}12$, $Nx_{+}20$, or Nx_40, which correspond to 12, 20, or 40 μg siRNA, respectively. The potential of targeting HIV coreceptor CXCR4 was evaluated in MAGI-CCR5 cells, which are a clone of HeLa cells that express human CD4 and CXCR4 and CCR5 receptors, and compared to that of a lipid-based, commercially available product, Lipofectamine RNAiMax reagent (Invitrogen Canada, Burlington, ON). Flow cytometry used for cellular uptake studies showed that the cells treated with the $Nx_{+}12$ formulation showed the highest cellular uptake. Live microscopy also revealed that the $Nx_{+}12$ or Nx_40 lipoplexes allowed better release of siRNA in the cytoplasm. Assessment of efficacy of the lipoplexes to knockdown CXCR4 mRNA expression carried out using qPCR analysis showed that the $Nx_{+}12$ lipoplex reduced mRNA expression by 70%, whereas the others reduced expression by at least 47% compared to RNAiMax, with 83% reduction. Similarly, knockdown of CXCR4 protein expression was evaluated using flow cytometry, and the data obtained corroborated qPCR analysis data. However, $Nx_{+}12$ reduced target protein expression to a similar extent when compared with that using RNAiMax. Thus, their data showed that, even though cationic lipoplexes were the most efficient, anionic formulations are capable of delivering siRNAs with a lower toxicity profile.

Polymeric Nanoparticles

Polymeric nanoparticles (Figure 2) are defined as sub-micron-sized colloidal systems (1–1000 nm) that can be fabricated from a variety of natural or synthetic polymers (biodegradable or nonbiodegradable) in various compositions.[58,59] Polymeric nanoparticles can be broadly classified into two categories based on their method of preparation: nanocapsules, which are reservoir or vesicular systems in which a liquid or semisolid drug-loaded core is surrounded by a polymeric membrane, or nanospheres, which are matrix

systems in which the drug is uniformly dispersed throughout a solid polymer matrix.[58,60] Depending on the method of preparation, the drug may be entrapped, dissolved, dispersed, encapsulated, or attached to the nanoparticle matrix.[61]

Polymers used in the fabrication of nanoparticles can be broadly classified into biodegradable and nonbiodegradable.[58,62] The ideal polymer for use in the preparation of nanoparticles must be biodegradable and must be completely eliminated from the body in a short time such that uncontrolled accumulation leading to lysosome and cellular overloading is avoided, allowing it to be repeatedly administered safely.[60,63] Other requirements of an ideal polymer include the following: (a) the polymer and its degradation products must be nontoxic and nonimmunogenic, (b) drug compatibility, and (c) suitable mechanical properties and ease of processing.[60,64] To date, only a few of the polymers available are approved for drug delivery and use *in vivo*.

The polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL), and their copolymers such as poly(lactide)-*co*-(glycolide) (PLGA), are the most widely used polymers because of their biocompatibility, nonimmunogenic, and nontoxic characteristics.[65–68] They are approved for use *in vivo* by the Food and Drug Administration (FDA).[64,69] Their biodegradation by hydrolysis of the ester bond leads to pharmacologically inactive and harmless degradation products, such as lactic acid, glycolic acid, and 6-hydroxycaproic acid from PLA, PGA, and PCL, respectively.[67,70–72] These degradation products enter the tricarboxylic acid cycle and are ultimately metabolized to water and carbon dioxide, and as such, their removal is not necessary after implantation.[65,67,73–75] Other polymers such as natural biopolymers categorized into polysaccharides (e.g., chitosan, alginate) and proteins (e.g., collagen) have been the subject of intense research for the delivery of genes and nucleic acids.[57]

Methods of Preparation of Nanoparticles—Nanoparticles can generally be prepared by two main methods: dispersion of preformed polymers and *in situ* polymerization of monomers.[61,68,76,77]

Nanoparticle preparation by dispersion of preformed polymers is commonly used in the preparation of biodegradable nanoparticles using FDA approved polymers such as PLA, PGA, and PLGA.[78] Commonly used methods include nanoprecipitation,[79] solvent evaporation,[80,81] spontaneous emulsification or solvent diffusion,[61] double emulsion,[82,83] microphase inversion,[67] and salting out.[60,76] Other techniques used for nanoparticle fabrication include gelation of the emulsion droplets by ions, by a variation in temperature or pH, or by suitable gelling agents. In this method, only polymers displaying gelling properties can be used.[60] The *in situ* polymerization reactions are heterogeneous polymerization processes, resulting in particle formation. The main heterogeneous polymerization reactions are suspension polymerization,[84] emulsion polymerization,[77] precipitation polymerization,[84] and dispersion polymerization.[85] The review by Vauthier and Bouchemal (2009) gives a description of each method, and the reader is referred to that article.[60] The advantages and shortcomings of polymeric nanoparticles in drug delivery have been reported, and some of these are provided in Table 3.[78,86,87]

The achievement of a long blood circulation half-life is dependent on two primary factors: particle size and surface characteristics.[88,89] The particle should be small enough to avoid the mechanical spleen or lung filtering effects. Also, cells of the mononuclear phagocyte system (MPS) recognize and rapidly clear nanoparticles from circulation by phagocytosis. It has been reported that MPS uptake increases with particle size; therefore, a particle size of <200 nm is advocated for a long circulating carrier.[63,88]

Modifications of surface characteristics to achieve MPS-avoiding characteristics and long-circulation half-lives have also been an intense area of research. It has been reported that adsorption or chemical attachment of hydrophilic, neutral molecules to the surface of nanoparticles enables the particles to evade recognition by the macrophages of the MPS and prolong blood circulation half-lives.[63,89,90] Poly(ethylene glycol) (PEG) and its copolymers are the most widely used polymers for surface modification due to their nontoxic properties, hydrophilicity, biocompatibility, and flexibility.[63,88,91,92] PEG is not biodegradable; therefore, it does not form toxic metabolic products. Also, PEG molecules with molecular weight below 40 kDa are readily excretable from the body through glomerular filteration.[93] It is also approved for internal use in human by the U.S. FDA.[63,92]

An advantage of using polymeric nanoparticles is the development of novel strategies to confer multiple functionalities to the nanoparticle delivery system. Multifunctional nanoparticles are systems that include any combination of a targeting agent (e.g., antibody or aptamer), imaging or contrast agent (quantum dots or superparamagnetic iron oxide nanoparticles (SPIONs)), a cell-penetrating agent, a stimulus-sensitive agent for drug release, a therapeutic agent, and surface modification for prolonged blood circulation half-lives.[78]

Polymeric nanoparticles have been used to deliver anti-HIV siRNA. Cationic polymers that bear positive charges are widely used in gene delivery systems and can interact with the negative charge of nucleic acids to form polyplexes.[57] In a recent article, the preparation of a novel biodegradable film formulation for the targeted delivery of siRNA-loaded nanoparticles to HLA-DR+ immune cells of the vaginal mucosa was described.[18] The nanoparticles were fabricated from a PEG–PLGA copolymer using the double emulsion method and loaded with polyethylenimine (PEI)–siRNA complexes. The nanoparticles were targeted to HLA-DR+ cells by conjugating the nano-particle surface to an anti-HLA-DR antibody for site-specific delivery. Their data showed that the targeted nanoparticles were transported across the vaginal epithelial layer and internalized by mKG-1 cells at significantly higher concentrations compared to those with the untargeted systems, leading to knockdown of SNAP-23 gene/protein expression. They concluded that the nanoparticle system has the potential for use for vaginal pre-exposure prophylaxis for the prevention of HIV infection. In another report, a novel siRNA delivery technology was described.[19] Using a single siRNA nanocapsule delivery technology achieved by forming a degradable polymer shell around a single siRNA molecule, they were able to deliver siRNA to target cells and demonstrate the release of the siRNA. Using siRNA that downregulates the expression of CCR5 (CCR5-siRNA), the primary HIV-1 coreceptor essential for HIV-1 infection, they demonstrated that the nanospheres with an average diameter of 20 nm did not show obvious cytotoxicity at an siRNA concentration below 200 nM. At 300 nM, cell viability was

reduced to about 75%. In addition, siRNA complexed with Lipofectamine and siRNA encapsulated in nanocapsules were compared for sensitivity to nucleases and human serum. Results showed that the nanocapsule formulation maintained the integrity of the encapsulated siRNA, whereas the siRNA either in the native state or formulated with Lipofectamine was degraded. Furthermore, their data showed that the CCR5-siRNA nanocapsules were taken up by 293T cells, leading to the successful downregulation of the CCR5 RNA fused with mCherry reporter RNA both in the absence and presence of serum.

The delivery of siRNA to T lymphocytes using PEGylated polyethyleneimine (PEG–PEI) was reported by Weber et al. (2012).[20] They synthesized PEGylated block copolymers PEI25k-PEG $(20k)_1$ and PEI25k $(2k)_{10}$, which formed complexes (polyplexes) with particles sizes between 180 and 300 nm after complexation with siNEF siRNA. They reported that the degree of PEG engraftment appears to hinder the condensing capability of PEI, leading to larger size particles for the PEI25k $(2k)_{10}$/siNEF particles. Cytotoxicity studies of the polyplexes in immortalized lymphocytic cell line SupT1, primary peripheral blood mononuclear cells (PBMC), and erythrocytes were carried out using assays for membrane rupture. It was reported that, at polymer concentrations for delivering 0.5 μM siRNA, the non-PEGylated PEI polymer was more cytotoxic than the PEGylated copolymers in SupT1 and primary PBMC. In addition, cytotoxicity studies in erythrocytes showed that the non-PEGylated PEI displayed appreciable toxicity compared to that of the PEGylated copolymers. To determine transfection efficiency, flow cytometry assays were done in SupT1 and primary PBMC using fluorochrome-labeled siRNA, siP24-R, after 3 and 24 h of treatment. At both time points, better transfection efficiency was seen with increased polymer concentration and constant siRNA. In addition, significantly higher transfection efficiency was reported for the PEGylated copolymers but not the non-PEGylated polymer at 24 h compared to 3 h. This was adduced to rapid internalization of PEI polyplexes by adsorptive endocytosis, a process delayed or hindered by PEGylation. They also reported that delivery of siRNA targeted to the HIV gene *nef* (siNEF) by PEG $(20k)_1$ achieved the greatest amount of HIV inhibition in SupT1 cells, similar to that with azidothymidine (AZT), over a 15 day period.

Dendrimers

Dendrimers are monodisperse, polybranched, usually highly symmetrical synthetic three-dimensional polymers in the nano range (Figure 3).[14,57,94] Dendrimers have well-defined chemical structures that can be differentiated into three parts, comprising an initiator or central core, interior layers, also referred to as generations (which are branches radiating from the core and made up of repeating units), and the exterior part that terminates the outermost interior generation and plays an important role in complexation of nucleic acids or dendrimer drug carrying ability.[95,96]

Dendrimer synthesis has generally been carried out using two main strategies; however, other approaches have been reported. The first is described as the divergent approach. In this method, the growth of dendrimers (or increase in generations) originates from a core site, whereas in the second method, referred to as the convergent approach, several dendrons are reacted with a multifunctional core to obtain a dendrimer product. The most widely used

dendrimers in biomedicine and drug delivery (PAMAM and polypropylenimine (PPI) dendrimers) are synthesized using the divergent method.[97] The reader is referred to excellent reviews on the synthesis, classification, and types of dendrimers and properties of dendrimers by Florence and Hussain (2001), Gillies and Frechet (2005), Dufes et al. (2005), Cheng et al. (2008), Nanjwade et al. (2009), Marvaniya et al. (2010), and Yavuz et al. (2013).[95–101]

Dendrimers have been used for the delivery of drugs in transdermal, oral, ocular, and pulmonary drug delivery systems.[102] In this regard, the drugs can be encapsulated in the dendrimer via noncovalent mechanisms such as incorporation in the dendrimer's core (dendritic boxes and unimolecular micelles) or complexation to charged functional groups for delivery of nucleic acids.[99–103] Covalent attachment of drugs to the dendrimer's periphery via degradable linkers (covalent dendrimer–drug conjugates) is another strategy for drug delivery using dendrimers.

Dendrimers of cationic polymers are frequently used as carriers of nucleic acids. Cationic polymers with a branched backbone such as dendrimers bear protonable amine groups. The relative number and pK_a of these amine groups vary from one polymer to another.[104] It has been reported that the type of amine group may influence transfection efficiency.[57] The primary amine groups facilitate binding of nucleic acids (dendriplexes), whereas tertiary amino groups enhance the release of nucleic acids into the cytoplasm via proton sponge mechanisms. De Smedt et al. (2000) give an in-depth review of polyplexes (defined as cationic polymer–nucleic acid complexes), their physicochemical characteristics, and their transfection characteristics *in vitro* and *in vivo*, and the reader is referred to it.[104]

The advantages and shortcomings of dendrimer nanoparticles in drug delivery have been reported, and some of these are provided in Table 4.[94,95,97,99,100,105]

Weber et al. (2008) have reported the synthesis and characterization of second-generation ammonium-terminated carbosilane dendrimers containing 8 or 16 positive charges (referred to as 2G-NN8 and 2G-NN16, respectively), which bind siRNA by electrostatic interactions between the negatively charged siRNA and the positively charged functional group on dendrimer extremities, forming dendriplexes.[14] The size of the dendriplexes ranges from 300 to 370 nm. The interior of the carbosilane dendrimers contains carbon–silicon bonds that hydrolyze slowly in water, which causes the liberation of the exterior branches and the siRNA payload. The siRNAs incorporated include siP24 (siRNA targeted to HIV *p24* gene), siGAG1 (siRNA targeted to HIV *gag* gene), and siNEF (siRNA targeted to HIV *nef* gene). Their data showed that the siRNA–dendrimer complex was able to inhibit HIV using siRNA targeted to the *nef* gene and a cocktail mixture of the three siRNAs in HIV-infected peripheral blood mononuclear cells (PBMC) after 24 h incubation. In a related work, Shcharbin et al. (2011) reported the formation of a stable complex of 2G-NN8 and 2G-NN16 with siGAG1, an anti-HIV siRNA.[22] The characteristics of the complexes were studied using circular dichroism, and the particle size and zeta potential were also found to be similar to those in earlier reports by Weber et al. (2008).[14]

In another paper, the 2G-NN16 dendrimer was tested for its ability to transfect different cell types implicated in HIV-1 infection, such as primary peripheral mononuclear cells (PBMC), lymphocytes (SupT1 cells), astroglia (U87MG), dendritic cells, and primary macrophages (Gonzalo et al., 2010) using siRNA and antisense oligonucleotides.[9] In these cell cultures, about 90% transfection efficiency was reported. The HIV-1 inhibition assays performed using antisense oligonucleotides of HIV-1, such as anti-transactivation responsive gene (TAR) (essential for HIV-1 transcription activation) and GEM 91 (involved in viral entry and reverse transcription), involved the determination of HIV p24 antigen release in culture supernatants of HIV-infected MT-2 (human lymphocyte T-cell line) and PBMC. It was reported that the 2G-NN16/TAR/ GEM91 dendriplex decreased p24 antigen release by 60% in PBMC and by 40% in MT-2 cells. Furthermore, Wrobel et al. (2014) compared the interactions of the 2G-NN16 and BDBR0011 carbosilane dendrimers and dendriplexes (complexed with siGAG1) with red blood cells and cell membranes in an attempt to investigate toxicity and suitability for siRNA delivery.[106] They concluded that the 2G-NN16 dendrimers may be better suited for siRNA delivery based on lower toxicity and less propensity to form aggregates.

The interaction between anti-HIV siRNA (siGAG1)-bearing second-generation carbosilane dendrimers (CBD) and large unilamellar liposomes has been described.[23] The dendrimer is made up of stable carbon–silicon bonds (CBD-CS) and oxygen silicon bonds (CBD–OS), which are slowly hydrolyzed in water. The large unilamellar liposomes (LUVs) studied are made up of zwitterionic or negatively charged phospholipids, modeling the cell membrane. The liposomes were made of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), dipalmitoylphosphatidylglycerol (DPPG), or DMPC/DPPG in a 7:3 molar ratio and were prepared using the extrusion method. The particle size of the dendriplexes is about 700 nm. They showed that both siGAG1/CBD/CS and siGAG1/CBD/ OS (1:4 molar ratio) interact with LUVs, forming aggregates ranging from 800 to 1000 nm in diameter, and that the liposome's surface plays an important role in LUV–dendriplex interaction. Stronger interaction was reported between dendriplexes and negatively charged liposomes. Furthermore, they reported that both siGAG1/CBD/CS and siGAG1/CBD/ OS may be used for delivery of siRNA to target cells.

Perise-Barrios et al. (2014) described the synthesis of a new second-generation carbosilane dendrimer $[G_2O_3(SiONN)_{12}]^{+}_{24}$ referred to as 2G-03NN24 (1,3,5-trihydroxybenzene core with 12 branches and 24 positive charges) and compared the effects of its functional groups with those of the 2G-NN16 dendrimer (silicon atom core with 8 branches and, therefore, 16 positive charges) to determine the effects of the different dendrimer functional groups on their ability to release siRNA.[25] Both dendrimers showed no toxicity at a concentration of 5 μM in purified CD4 T lymphocytes and macrophages using the MTT assay. Their results showed that dendriplexes were formed between the cationic dendrimers and siRNA involved in the downregulation of *nef* expression (siNEF). In addition, using flow cytometry, they reported the cellular uptake of fluorochrome-labeled siNEF dendriplexes in purified CD4 T lymphocytes after 2 h of incubation. Furthermore, they determined the effects of the dendriplexes on HIV replication in HIV-1-infected CD4 T lymphocytes' culture supernantant. Their results showed 25 and 50% reductions in p24 antigen release by 2G-NN16/siNEF and 2G-03NN24 dendriplexes, respectively.

The synthesis of Janus-type ammonium-terminated amphiphilic dendrimers consisting of two types of dendrons based on the carbosilane moiety and a PEG scaffold, respectively, has been reported.[26] Complexation of this dendrimer with siNEF and treatment of PBMCs with the dendriplexes resulted in p24 inhibition of up to 50% by p24 ELISA assay. In addition, lower toxicity to PBMCs in an MTT assay data was adduced to the presence of the PEG moieties.

In a recent article, Serramia et al. (2015) reported the delivery of siRNA to the brain using a carbosilane dendrimer (2G-$(SNMe_3I)_{11}$-FITC) carrier.[27] They studied the cell viability and uptake of dendrimers and dendriplexes in NHA primary human astrocytes. Uptake of siRNA-NEF was studied by flow cytometry in NHA cultures treated with Cy5-siRNA-NEF, dendrimer, and the dendrimer–siRNA complex (dendriplexes) (1:3 ratio) for 24 h. Transfection efficiency of the dendriplex was greater than 65%. Viral activity in HIV-1-infected NHA cells monitored by measuring p24 levels in culture supernatant 72 h postinfection showed that the dendriplexes had no effect on p24 production. However, this result was adduced to the effect of the FITC molecule. In addition, high dendriplex fluorescent levels were found in the brain at 1 and 24 h postadministration when the 2G-$(SNMe_3I)_{11}$–FITC–siRNA-NEF dendriplex was administered by retro-orbital injection to BALB/c mice, demonstrating that the dendriplexes crossed the blood–brain barrier (BBB) and showed significant uptake in an *in vivo* model. In a similar article, Jimenez et al. (2010) investigated the capacity of the 2G-NN16 amino-terminated carbosilane dendrimer to deliver siRNA to HIV-infected human astrocytes and to cross the BBB via an *in vitro* transcytosis assay using bovine brain microvascular endothelial cells.[21] Their results show reduced HIV replication in cell culture supernatants of HIV-infected human astrocytes.

The development of a generation four phosphorus-containing polycationic dendrimer G4 $(NH^+Et_2Cl^-)_{96}$ has been described.[24] They reported the preparation and characterization of dendriplexes of the dendrimer complexed with siNEF. Cytotoxicity studies in Sup T1 or PBMC show very low cytotoxicity. The dendriplexes also significantly reduced viral replication in HIV-infected PBMC cells *in vitro*.

The Rossi group has described the use of flexible PAMAM dendrimers for the delivery of combinations of siRNA for the treatment of HIV-1 infection in humanized mice.[28] They reported the formation of stable complexes of about 100 nm diameter at a N/P ratio (ratio of terminal amines in cationic dendrimer to phosphates in the siRNA) of 5 with the use of generation five (G_5) triethanolamine-core PAMAM dendrimers. Their data showed that the stable G_5-dsiRNA nanoparticles protected the siRNA's from RNase degradation and also efficiently delivered Cy3-labeled siRNA complexes to human T-lymphoblast CCRF-CEM cells using fluorescence microscopy. Live-cell confocal microscopy using the Alexa 488-labeled complex in HeLa cells also revealed efficient internalization. Using siRNAs against Transportin-3 (TNPO3) and CD4, it was reported that the dendrimer-delivered anti-HIV siRNAs effectively downregulated cellular CD4 and TNPO3 targets. Furthermore, the dendrimer complexed with anti-tat/ rev siRNA or a combination of anti-tat/rev siRNA, anti-CD4, and anti-TNPO3 siRNAs administered by intravenous (IV) injection to HIV-infected humanized $Rag2^{-/-}\gamma c^{-/-}$ mice (RAG-hu) suppressed HIV-1 infection by several orders of magnitude and also protected against virus -induced $CD4^+$ T cell depletion. This knockdown

of both viral and cellular transcripts demonstrates the effectiveness of this approach and represents a promising method for treatment of HIV-1 infection.

Inorganic Nanoparticles and Carbon Nanotubes

Inorganic nanoparticles have been successfully used for delivery of nucleic acids. Some of the inorganic materials used for this purpose that have been reported include carbon nanotubes, quantum dots, gold, and silica, to mention a few.[40] Inorganic nanoparticles have been reported to have moderate transfection efficiencies; however, their nonsusceptibility to microbial attack, ease of preparation, and good storage stability are some of their advantages over organic nanoparticles.[40] The advantages and disadvantages of inorganic nanoparticles that have been used to deliver siRNA for HIV therapy are shown in Table 5.

Quantum dots are nanometer-sized crystals made up of a core of a semiconductor material within a shell of another semiconductor material with a larger spectral band gap (Figure 4).[114] They are usually made up of elements from groups II and VI or groups III and V, and they range in size from 2 to 10 nm.[114,108] As a result of their favorable unique optical and electronic properties, quantum dots are being studied and utilized primarily as probes for biomedical imaging; however, their use for transfection has also been reported.[40,108] Quantum dots are inherently toxic, and this toxicity may be ascribed to the presence of surface cations and formation of photoinitiated radicals.[40] A number of approaches have been used to reduce the inherent toxicity of quantum dots and to make them biocompatible for biomedical use. Silanization, surface exchange with bifunctional molecules, and encapsulation in carriers and delivery systems have been employed to cap them and to make them hydrophilic.[114] In addition, the quantum dot surface can be modified to allow for site-specific delivery (targeting) by conjugation to a targeting moiety such as antibodies, peptides, small molecules, and aptamers.[107,114] For conjugation of biologically active molecules to the surface of quantum dots, several methods such as electrostatic attraction (for nucleic acids), covalent linkage, adsorption, mercapto (−SH) exchange, and other various chemistries are available.[107,114] For more information on the properties of quantum dots and their applications, the reader is referred to other reviews on these topics.[40,107,108,114]

The use of quantum dots for siRNA delivery has been reported by various authors.[115,116] For HIV therapy, a quantum rod–siRNA complex (QR–si510 siRNA complex) was synthesized and characterized.[6] The si510 HIV-1 siRNA targets the TAR/poly A region of the HIV-1 LTR, thereby suppressing HIV-1 viral replication. The quantum rods (QRs) terminated with carboxyl groups consist of a cadmium–selenium nanocrystal core and a thin zinc sulfide shell, which is less than 1.5 nm, grown over the nanocrystal core. For attachment of the carboxy termini, the core–shell quantum rods were coated with mercaptosuccinic acid (MSA). It was reported that the QRs have good colloidal stability with an average diameter of 15 nm. The effect of the nanoplexes on THP-1 cell viability was studied using the MTT assay. Results show that the nanoplexes did not produce appreciable toxicity. Cellular uptake of the nanoplexes was confirmed using confocal microscopy. Antiviral activity of the nanoplexes was measured using the p24 ELISA assay in culture supernatants of THP-1 cells as well as HIV-1 LTR quantification from RNA extracted from THP-1 cells using realtime

PCR. The ELISA assay showed a significant reduction in HIV-1 p24 production compared to that in cells transfected with Lipofectamine (Invitrogen, Carlsbad, CA) and also when compared to the untransfected control.

Gold nanoparticles such as nanospheres, nanocages, and nanorods (classified by their different shapes) have also been used as contrast agents as a result of their size and optical properties.[86]

In addition, gold nanoparticles (usually, 10–20 nm) are also used for delivery of bioactive agents to target organelles via covalent conjugation (e.g., using thiols) or other noncovalent mechanisms. For the delivery of nucleic acids, gold nanoparticles functionalized with positively charged quaternary ammonium or branched PEI[110] or coated with a cationic lipid bilayer have been reported.[94] The attachment of oligonucleotides to the surface of gold nanoparticles has also been reported. For a review of gold nanoparticles for nucleic acid delivery, the reader is referred to the review by Ding et al.[111]

In their paper, Reynolds et al. (2012) reported the use of gold nanorods for the delivery of anti-HIV siRNA. Galectin-1, implicated in HIV-1 adsorption, is an adhesion molecule expressed in macrophages.[29] Using human monocyte derived macrophages (MDM), it was shown that methamphetamine potentiates galectin-1 gene production and protein expression and facilitates HIV-1 infection. Gold nanorods complexed to galectin-1 siRNA (galectin-1 siRNA/GNR nanoplexes) were produced that reduced methamphetamine-potentiated galectin-1 expression and partially prevented the effects of methamphetamine on p24 antigen production and gene expression for HIV-1 LTR-R/U5 in MDM, leading to decreased HIV-1 infectivity.[29]

Carbon nanotubes have been described as highly elongated tubular nanostructures of graphene sheets with outstanding physical, mechanical, and chemical properties.[113,117] They are generally classified by their structure into two groups (depending on the number of graphene layers), namely, single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs).[118] SWCNTs have a smaller diameter and show more flexibility and improved photoluminescence compared to MWCNTs, which have an increased surface area for functionalization, leading to improved loading of bioactive agents.[113] Because of their nanometer-sized needle structure, they have the potential to cross the plasma membrane easily and accumulate in the cytoplasm of target cells via a diffusion-like, endocytosis-independent mechanism without causing cell death.[112,119]

Conventional carbon nanotubes are poorly soluble in most solvents; thus, to facilitate their use in the delivery of bioactive agents, carbon nanotubes are chemically modified or functionalized either covalently or via other noncovalent mechanisms (e.g., introduction of polar or charged surface groups) to improve their solubility and also to conjugate them to bioactive molecules.[112,119] Functionalization has also been reported to reduce the cytotoxic effect of carbon nanotubes and to improve their biocompatibility.[118] A number of excellent articles have been published that show and describe the use of carbon nanotubes for the delivery of small molecule drugs and nucleic acids.[113,117] For HIV therapy, SWCNTs with average length of 200 nm and 1–3 nm in diameter have also been shown to deliver siRNA

into human T cells and primary cells for the efficient degradation of CXCR4 and CD4 mRNAs. Knocking down these mRNAs gives rise to the depletion of CXCR4 and CD4 receptors on human T cells and peripheral blood mononuclear cells.[30] It was reported that the efficiency of the RNAi process using nanotubes as a delivery system exceeds that of existing delivery systems, such as different liposomal formulations. A 90% knockdown efficiency was observed when a solution of SWNT–siRNA$_{CXCR4}$ was incubated with H9, Sup-T1, and CEM cells for 3 days.

Other Carrier Systems

Eszterhas et al. (2011) reported a nanoparticle system of about 45–60 nm in diameter containing anti-CD4 and -CCR5 siRNAs.[31] Their goal was to prevent HIV-1 infection in the human female reproductive tract by knocking down the CD4 and CCR5 receptors, which are essential for HIV infection. The carrier used is INTERFERin (Genesee Scientific, San Diego, CA, USA), which is a nonliposomal cationic amphiphile transfection reagent. The siRNA-containing nanoparticles were applied to explants prepared from the endometrium (EM), endocervix (CX), and ectocervix (ECX) of hysterectomy tissues from HIV-1 seronegative women, after which the nanoparticle-treated explants were exposed to HIV-1. Their data showed that the nanoparticles reduced the expression of CD4 and CCR5 receptors and also inhibited HIV-1 infection.

The conjugation of a single-chain antibody (scFv) to nona-D-arginine (9R) peptide (scFvCD7-9R) for targeted delivery of siRNA into T cells via the T cell protein CD7 has been reported.[8] The delivery of siRNA to T cells *in vivo* was studied by intravenous (i.v.) injection of siCD4 and siLuc complexes of scFvCD7–9R in a NOD/SCIDIL2r$\gamma^{-/-}$ Hu-PBL mouse model. CD4 expression on peripheral blood T cells of siCD4-treated mice examined 60 h after injection showed a specific, significant reduction compared to that of siLuc-treated (control) mice (7.5 ± 0.7% and 59.5 ± 10.7%, respectively). In addition, PBMCs from scFvCD7-9R/siCD4-treated mice were infected with T cell-tropic HIV$_{IIIB}$ *ex vivo*, and p24 levels were determined. It was reported that p24 levels were significantly reduced in culture supernatants, showing enhanced inhibition of HIV infection. Furthermore, the efficacy of systemic delivery of a combination of siRNAs to Hu-PBL mice was assessed by determination of CD4 levels and p24 antigen levels. siRNAs blocking viral entry, preventing viral spread, and inhibiting viral replication by targeting conserved sequences in the viral *Vif* and *Tat* genes were administered as complexes to scFvCD7-9R. Their data showed that CD4 cells remained normal and that p24 levels were undetectable in 3 of 4 treated mice.

The design, synthesis, and characterization of dual-functional RNA nanoparticles (Figure 5) for site-specific delivery and HIV-1 inhibition have been described.[32] The delivery system consists of two parts: the Ba′ pRNA-gp120 aptamer portion to selectively target HIV infected cells and the Ab′ pRNA siRNA portion with a 2′-F modified sense strand to efficiently transport anti-HIV siRNAs. Overall, the dual-functional RNA nanoparticle was designed to block HIV-1 infectivity (to serve as a potential HIV-1 inhibitor) and to efficiently deliver anti-HIV siRNAs targeting the *tat/rev* common exon of HIV-1 specifically to HIV-infected cells for systemic anti-HIV therapy. Their results showed that the aptamer chimera inhibited HIV-1 p24 production after incubation with HIV-1-infected primary

human PBMCs. In addition, analyses by flow cytometry also revealed that the pRNA-siRNA chimera is delivered to the gp160-expressing cells by the pRNA–aptamer via dimerization. In a similar work, the authors reported the design and testing in a humanized mouse model of an aptamer–siRNA chimera that suppressed HIV-1 replication and prevented viral-induced helper $CD4^+$ T cell decline.[33] They reported that the aptamer acts as an HIV-neutralizing agent and as a siRNA delivery vehicle and thus provides a nontoxic delivery system for HIV therapy.

Song et al. (2005) reported the delivery of *gag* siRNA to HIV-infected CD4 T cells using a Fab antibody (F105) fragment directed against the HIV-1 envelope fused to protamine (F105P).[34] Efficacy was determined by evaluation of viral replication. It was reported that the proportion of infected cells was reduced from 85% in untreated cultures to 36% when treated with 1 nM *gag* siRNA. In addition, measurement of p24 *gag* antigen in culture supernatants by enzyme-linked immunosorbent assay (ELISA) as a measure of the release of viral particles from F105P and *gag* siRNA-treated primary cells using ≥100 pmol of siRNA revealed that release of viral particles was reduced from 170 ng/mL to <40 ng/mL. Furthermore, infection was reduced by about 30% when cells were treated with F105 or F105P antibody alone due to the neutralizing activity of the antibody.

CONCLUSIONS

In the vast majority of the approaches described, the electrostatic complexation of negatively charged nucleic acids by positively charged cationic amines has been exploited to achieve nanoplex formation and suitable nucleic acid loading within carrier systems. This ensures protection of therapeutic siRNA from degradation in circulation. Furthermore, the functionalization of these nanocarriers with targeting molecules with specificity for HIV-bearing tissues or cellular reservoirs to achieve site-specific delivery and the potential to deliver many chemotherapeutic agents, nucleic acid therapeutics, or a combination of chemotherapeutics and RNA therapeutics make the use of these nanoplatforms a versatile approach to treat HIV/AIDS and to reduce the incidence of adverse effects. While HAART has been successful in significantly reducing morbidity and mortality due to HIV-1 infection, problems exist that prevent the eradication of HIV. Reservoir sites protect the virus from biological elimination pathways, the immune response, and/or antiretroviral drugs, making it impossible to eradicate the virus and achieve a cure with current therapy. RNAi has emerged as a robust tool in medicine for the treatment of various disorders, and its prospects for being clinically utilized are bright. With the ever increasing arsenal of biological tools to study cellular processes and the sustained interest in nanotechnology for new medical therapies that has the capacity to specifically target cells and anatomical locations bearing the virus, we expect that nanoformulations of anti-HIV siRNAs will soon be clinically available to augment the ever increasing arsenal in the fight against HIV.

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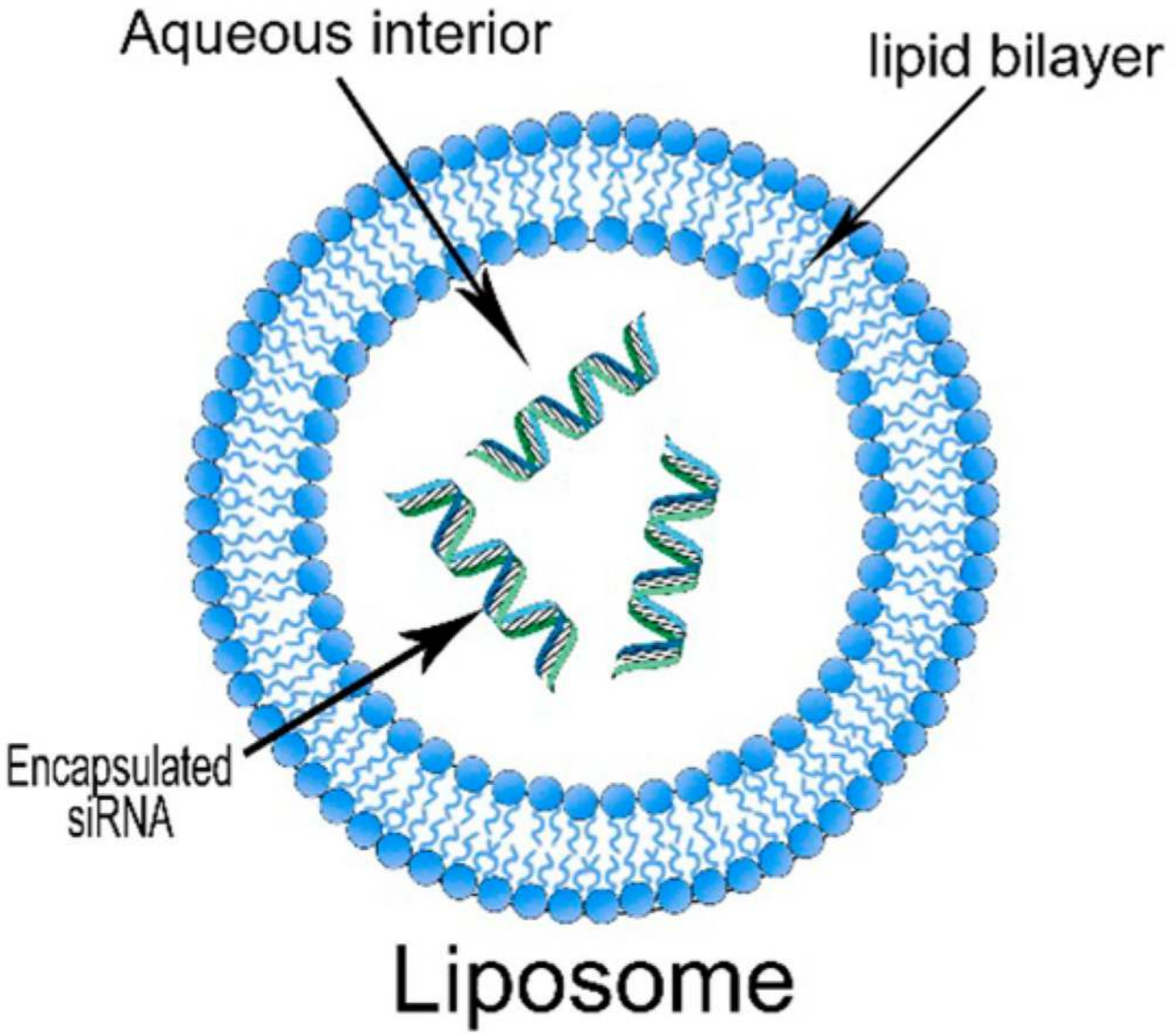

Figure 1.
Representative structure of a liposome.

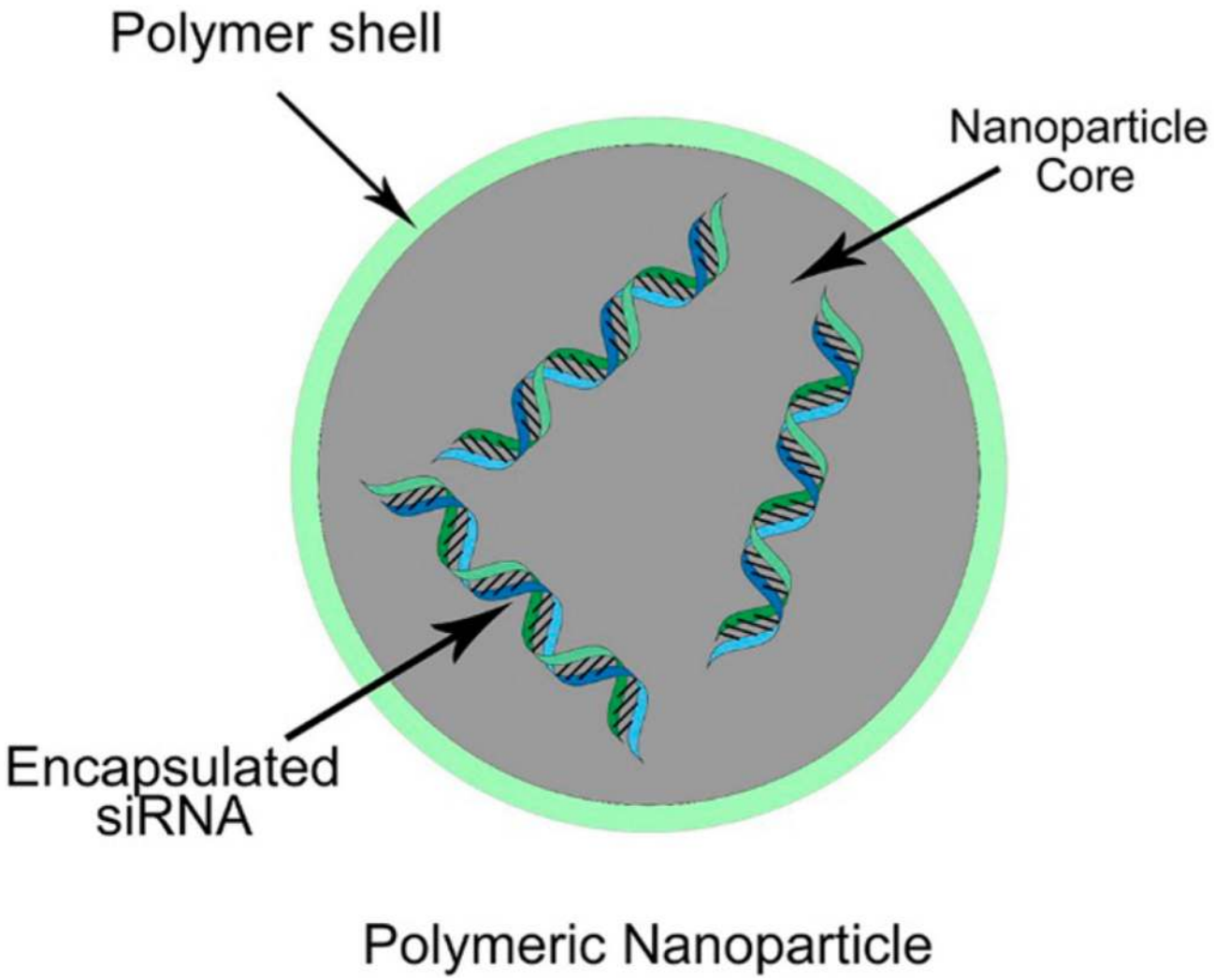

Figure 2.
Representative structure of polymeric nanoparticles.

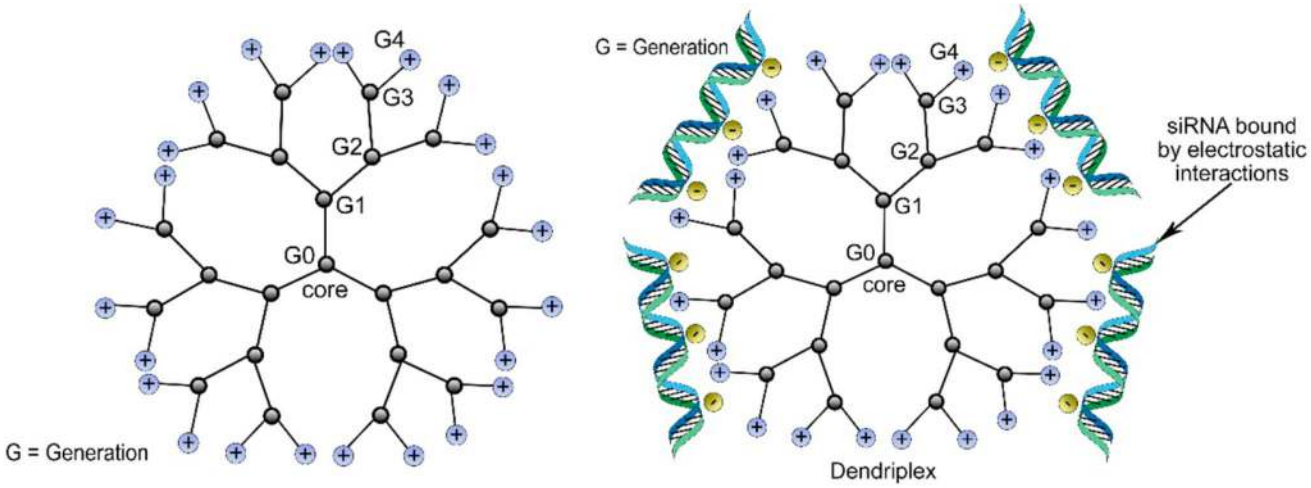

Figure 3.

Representative structure of a fourth-generation dendrimer (left) and dendriplex (right). Positive charges represent terminal ammonium groups.

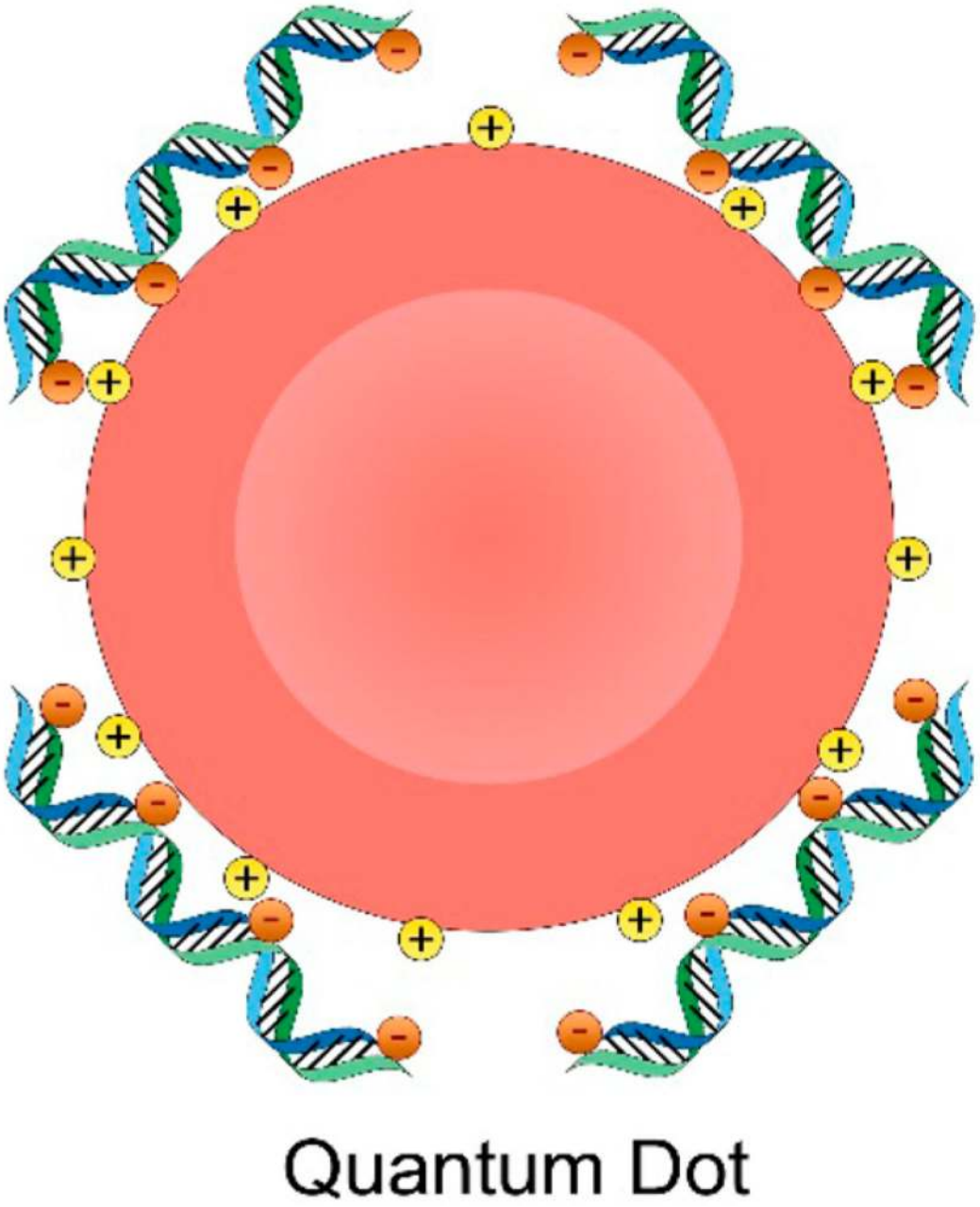

Figure 4.
Representative structure of a quantum dot complexed with siRNA.

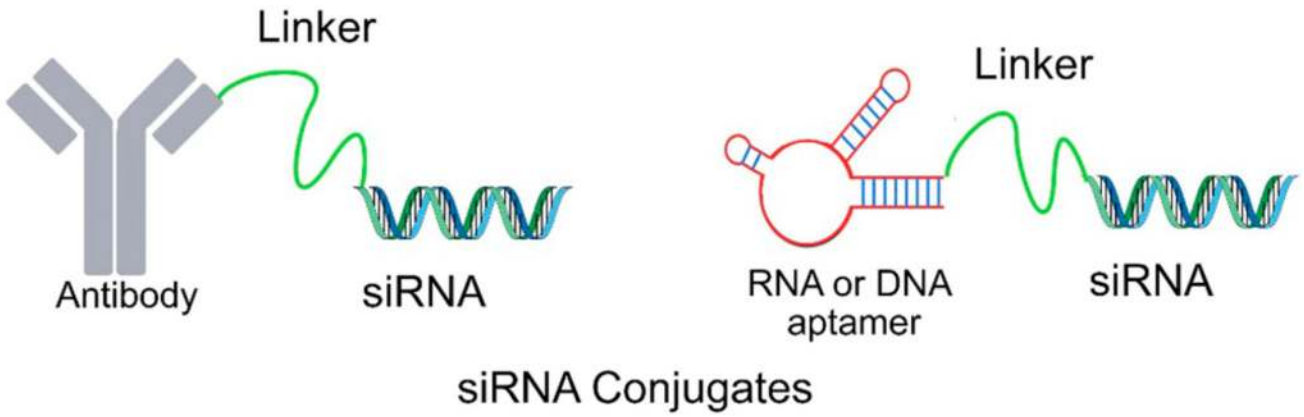

Figure 5.
Representative siRNA conjugates. Antibody–siRNA conjugate (left) and aptamer–siRNA conjugate (right).

Table 1

Summary of siRNA Targets and Nonviral Carrier Systems

	siRNA target	carrier system	results	ref
1	CCR5	liposome	inhibition of CCR5 expression; potential for development as immunization strategy	16
2	CXCR4	liposome	reduced CXCR4 mRNA expression by 70%	17
3	SNAP-23	polymeric nanoparticle	inhibition of SNAP-23 expression; potential for vaginal pre-exposure prophylaxis for HIV-1 infection	18
4	CCR5	polymeric nanoparticle	downregulation of CCR5	19
5	*nef*	polymeric nanoparticle	inhibition of HIV-1 replication	20
6	*p24, gag1, nef*	dendrimer	inhibition of viral replication	14,21–27
7	Transportin-3 (TNPO3), CD4, *tat, rev*	dendrimer	downregulation of targets, protection against $CD4^+$ T-cell depletion	28
8	TAR/poly A of LTR	quantum dot	significant reduction in p24 production	6
9	Galectin-1	gold nanorod	decreased HIV-1 infectivity	29
10	CXCR4, CD4	carbon nanotube	90% knockdown efficiency; decreased HIV-1 infectivity	30
11	CD4, CCR5	INTERFERin (Genesee Scientific)	reduction in CD4 and CCR5 expression	31
12	CD4, *vif, tat*	peptide carrier	reduction in CD4 expression and inhibition of HIV-1 replication	8
13	*tat/rev*	RNA nanoparticles	decreased HIV-1 infectivity; suppression of HIV-1 replication	32, 33
14	*gag*	protamine	decreased HIV-1 infectivity; suppression of HIV-1 replication	34

Table 2

Advantages and Disadvantages of Liposomes as Drug Delivery Vehicles

	advantages	disadvantages
1	biodegradable, biocompatible, and flexible	low stability and short half-life
2	can encapsulate and deliver both aqueous and lipid-soluble drugs	the carriers are leaky and agglomerate by fusion
3	provides sustained release and site-specific drug delivery	carrier lipids undergo oxidation and hydrolysis
4	protects entrapped drug from premature degradation in hostile environments	some liposomal constituents may stimulate allergic reactions
5	modulates the pharmacokinetics and pharmacodynamics of encapsulated drugs	accumulation in cells outside the target tissues (liver macrophages)

Table 3

Advantages and Disadvantages of Polymeric Nanoparticles as Drug Delivery Vehicles

	advantages	disadvantages
1	better stability compared to that of liposomes	nanotoxicity issues, especially with nonbiodegradable polymers
2	facilitates controlled drug release and can be targeted for site-specific release	small number of polymers approved for clinical use
3	multifunctional design and capability; suitable for targeted drug delivery and imaging	rapid clearance of unmodified nanoparticles by cells of the MPS
4	accumulates in tumors via the enhanced permeability and retention effect (EPR)	agglomeration due to large surface area makes physical handling difficult
5	can be administered by different routes	
6	modulates the pharmacokinetic properties of encapsulated drugs	

Table 4

Advantages and Disadvantages of Dendrimers as Drug Delivery Vehicles

	advantages	disadvantages
1	narrow polydispersity giving rise to reproducible pharmacokinetic behavior	nonspecific cytotoxicity and liver accumulation as a result of their high cationic charge density
2	water-soluble and biocompatible	rapid clearance, especially with low molecular weight dendrimers
3	displays multiple surface groups that can be functionalized for targeting or covalently conjugated for drug delivery	poor control over release of the encapsulated drug
4	binds to nucleic acids via electrostatic interactions, forming dendriplexes that protect nucleic acids from degradation	
5	ability to encapsulate a wide range of drugs; can encapsulate hydrophobic drug molecules via host–guest chemistry	
6	can facilitate passive targeting via the EPR effect	

Table 5

Advantages and Disadvantages of Inorganic Nanoparticles Used as Drug Delivery Vehicles

	advantages	disadvantages
	Quantum Dots[86,105,107,108]	
1	high photochemical stability compared to that of organic dyes	toxicity (including immune response and genotoxicity)
2	tunable spectrum by varying size	colloidal instability
3	potential for theranostic applications	nonspecific organ uptake and uptake by cells of the MPS
4	resistant to photobleaching, which enables extended dynamic imaging	lack of data on reproducibility and quantification
	Gold Nanoparticles[105,109–111]	
1	simple synthesis and relatively easy scale-up	not biodegradable
2	biocompatible	high cost of large-scale production
3	ease of conjugation of bioactive agents	nanoparticle aggregation
4	multifunctional monolayers can be created, allowing conjugation of multiple bioactive agents	
5	modulation of cytotoxicity, biodistribution, and *in vivo* excretion properties by regulation of particle size and surface functionality	
	Carbon Nanotubes[40,112,113]	
1	large modifiable surface area	poor solubility in water
2	scale-up for industrial production is relatively easy	not biodegradable
3	can be loaded with a broad spectrum of bioactive agents such as nucleic acids, drugs, and proteins	unfavorable pharmacokinetics and poor distribution
4	chemical inertness with the capacity for functionalization	toxicity
5	biomolecules can be loaded inside the tubular structure	
6	protection of oligonucleotides from degradation in circulation	