Nanoparticle Delivery of Antisense Oligonucleotides and Their Application in the Exon Skipping Strategy for Duchenne Muscular Dystrophy

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Antisense therapy is a powerful tool for inducing post-transcriptional modifications and thereby regulating target genes associated with disease. There are several classes of antisense oligonucleotides (AONs) with therapeutic use, such as double-stranded RNAs (interfering RNAs, utilized for gene silencing, and single-stranded AONs with various chemistries, which are useful for antisense targeting of micro-RNAs and mRNAs. In particular, the use of AONs for exon skipping, by targeting pre-mRNA, is proving to be a highly promising therapy for some genetic disorders like Duchenne muscular dystrophy and spinal muscular atrophy. However, AONs are unable to cross the plasma membrane unaided, and several other obstacles still remain to be overcome, in particular their instability due to their nuclease sensitivity and their lack of tissue specificity. Various drug delivery systems have been explored to improve the bioavailability of nucleic acids, and nanoparticles (NPs) have been suggested as potential vectors for DNA/RNA. This review describes the recent progress in AON conjugation with natural and synthetic delivery systems, and provides an overview of the efficacy of NP-AON complexes as an exon-skipping treatment for Duchenne muscular dystrophy.

Antisense-Mediated Exon-Skipping Strategies

OVER THE LAST TWO DECADES, nucleic acids have emerged as versatile therapeutic agents, due to their ability to target and interfere with the flow of genetic information from DNA to protein. The most promising nucleic acids, which include antisense oligonucleotides, small interfering RNA (siRNA), aptamers, and ribozymes, demonstrate high structural diversity, and express many enzymatic activities with the ability to influence cell metabolism (Bedi et al., 2013). Indeed, DNA/RNA oligonucleotides are currently used to alter gene expression by several different approaches: (1) siRNA molecules to selectively degrade messenger RNA (mRNA); (2) cholesterol-conjugated single-stranded RNA analogues, termed “antagomirs,” to block microRNAs; (3) antisense oligoribonucleotides to either modulate alternative splicing or alter gene expression patterns; (4) decoy oligodeoxynucleotides (ODNs) to inhibit the binding and transcriptional action of several transcription factors; (5) synthetic ODN containing one or more CpGs to stimulate the immune system; and (6) triplex-forming oligonucleotides, composed of DNA, RNA, or synthetic base analogs, for targeted mutagenesis (Sazani and Kole, 2003;

Krutzfeldt et al., 2005; Castanotto and Rossi, 2009; Chin and Glazer, 2009; Vollmer and Krieg, 2009; Bennett and Swayze, 2010; Soldati et al., 2011; Juliano et al., 2012).

The literature reveals that antisense-mediated exon skipping represents a promising treatment strategy for several diseases via the restoration of normal splicing. Indeed, this approach has potential applications for: (1) cryptic splicing mutations, as in β -thalassemia, breast cancer, and cystic fibrosis; (2) switching between alternative splicing isoforms, with therapeutic potential for inflammatory diseases, myotonic dystrophy, anti-apoptosis, and cancers; (3) inducing exon inclusion, for cancers and spinal muscular atrophy; (4) correcting the reading frame to allow the production of internally deleted partially functional proteins in Duchenne muscular dystrophy and dystrophic epidermolysis bullosa; and (5) induction of reading-frame disruptions to achieve partial protein knockdown in, for example, atherosclerosis and cancers (van Roon-Mom and Aartsma-Rus, 2012).

The idea behind splicing therapy for Duchenne muscular dystrophy (DMD) is to convert the out-of-frame transcript into an in-frame transcript that codes for functional proteins, thereby transforming a severe phenotype (DMD) into a milder form (Becker muscular dystrophy) (van Ommen et al.,

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FIG. 1. Schematic representation of exon skipping. An out-of-frame deletion of exons 45 in the Duchenne muscular dystrophy *DMD* gene induce a frame-shift in the mRNA, generating a premature stop codon. Targeting exon 44 with antisense oligonucleotide (AON), which hides it from the splicing machinery, leads to exon-44 skipping and reframing, partially restoring protein function.

2008; Wilton and Fletcher, 2008; Aartsma-Rus, 2012; Douglas and Wood, 2013) (Fig. 1). Notably, this example of targeted antisense-mediated exon skipping therapy is already at the clinical trial stage.

Antisense Oligonucleotide Chemical Modifications

The progress of therapy based on nucleic acids is dependent upon advances in chemistry that improve the development of antisense oligonucleotide (AON) molecules with backbones able to confer them a nuclease resistance, without interfering with their ability to hybridize with the right target (Jarver et al., 2013), and that provide new carriers for their protection and systemic administration (Lu et al., 2011; Nimesh et al., 2011). The ideal AON should (1) bind to a specific sequence on RNA transcript, (2) be readily taken up by cells in target tissues, and (3) be active in the appropriate intracellular compartment. Furthermore, in a splicing strategy it is essential that AON molecules localize in the nucleus, whereas the siRNA approach depends upon AONs remaining in the cytoplasm, where the interfering RNA (iRNA) can act. AONs also need to possess good safety, pharmacokinetic, and pharmacodynamics profiles, as well as resistance to nuclease degradation (Douglas and Wood, 2013). Due to their stability to nucleolytic degradation and relative ease of synthesis, phosphorothioate oligonucleotides (PSs) have been the AONs' most widely studied to date. Vitravene™ (ISIS 2922, fomivirsen), the first antisense drug to be approved by the U.S. Food and Drug Administration (FDA), is a PS-AON designed for the treatment of cytomegalovirus-induced retinitis in AIDS patients (Geary et al., 2002).

In PS oligonucleotides, nuclease resistance is conferred by replacing the non-bridging oxygen atom of the RNA phosphate group with a sulfur atom (Fig. 2a), although this substitution does not, in itself, confer RNase H resistance, which also requires 2'-O-modification of the ribose residue (Deleavy and Damha, 2012). Another advantage of phosphorothioates is their solubility, arising from their negative charge. This also allows them to complex easily with cationic lipids and proteins (Bennett and Swayze, 2010), although they cannot be readily conjugated to peptides by covalent linkages (Douglas and Wood, 2013). PS-modified AONs are water soluble and have high protein-binding capacity, which prevents rapid renal excretion and facilitates uptake to tissues.

AONs can be altered in other ways to improve their therapeutic prospects. For instance, modification of their back-

bone can yield thiophosphoramidate analogs, which are more stable acid oligonucleotides with unchanged RNA binding properties. What is more, modification of the ribose moiety of AON can give rise to 2'-O-methyl (2'MeO) (Fig. 2b) or 2'-O-methoxyethyl (2'-MeOE) oligonucleotides, both of which possess increased nuclease resistance and target-RNA binding affinity.

Other products of ribose residue modification are the N3→P5 phosphoramidate oligonucleotides, in which the 3-oxygen is substituted by an amine group. Phosphoramidates are nuclease resistant and hybridize specifically to the complementary RNA with higher affinity than phosphodiester or phosphorothioate AONs, but do not activate RNase H. Other backbone modifications are represented by methylphosphonate, in which one of the non-bridging oxygen atoms is substituted by a methyl group, neutral in charge.

FIG. 2. Chemical structure of antisense oligoribonucleotides. (a) Phosphorothioate; (b) 2'-methoxy oligonucleotide; (c) morpholino oligonucleotide; (d) peptide nucleic acid (PNA).

Methylphosphonates have high nuclease resistance but are not able to activate RNase H. Moreover, high methylphosphonate content in an oligomer decreases solubility and affinity for the target RNA.

Another common class of AONs is the morpholino phosphorodiamidate oligomers (PMO), which have (1) a nonionic backbone at physiological pH, (2) a ribose sugar replaced by a 6-membered morpholine moiety, and (3) phosphorodiester intersubunit bonds with phosphorodiamidate linkages (Fig. 2c). PMOs are charge-neutral and refractory to biological degradation (Amantana and Iversen, 2005). Morpholinos do not activate RNase H but do block protein translation and other mechanisms such as splicing. Moreover, the nonionic properties of PMOs prevent potential nonspecific interactions with cellular components, and it is possible to conjugate PMOs to different delivery systems, such as cell penetrating peptides (CPPs). In addition, the modified chemistry of PMOs provides excellent resistance to nuclease and protease activity (Amantana and Iversen, 2005; Pan and Clawson, 2006; Bennett and Swayze, 2010; Douglas and Wood, 2013).

Peptide nucleic acids (PNAs) are another class of neutrally charged AONs that are resistant to nuclease and protease degradation. They possess a backbone of repeating N-(2-aminoethyl)glycine units, with nucleobases bound via carbonyl linkages (Nielsen, 2010) (Fig. 2d). PNAs are able to hybridize with DNA and RNA and have a mechanism based on translation inhibition and splicing modulation. However, due to their lack of charge, PNA molecules are characterized by low solubility and weak cellular uptake. For these reasons,

PNAs have been conjugated with short peptides or modified to bear charged amino acids in their backbone.

Nanoparticles for Antisense Oligonucleotide Delivery

All oligonucleotide types have to be delivered into tissues and cells in order to reach their target transcript and carry out their functions. Some naked oligonucleotides that feature both phosphorothioate linkages and sugar modifications can enter hepatocytes or kidney cells efficiently *in vivo*. However, their bioavailability is limited by poor cell trafficking and endosomal entrapment (Järver et al., 2012).

This has prompted a rigorous search for new delivery systems capable of delivering nucleic acids into target cells. An optimal delivery system needs to be target cell specific, controllable in terms of drug release, and able to protect nucleic acids (Bedi et al., 2013).

Liposomes, polymers, and cell-penetrating peptides have all been proposed as such vehicles, but their clinical applicability is plagued by a lack of cell specificity, not to mention difficulties in controlling drug release. Researchers have recently begun to focus on nanotechnology as a potential nonviral delivery system. These vehicles could deliver a variety of drugs and macromolecules, like proteins, oligonucleotides and plasmid DNA, via various administration routes, including intravenous, oral, intramuscular, pulmonary, intranasal, ocular, rectal, and intraperitoneal (Lamprecht et al., 2005; des Rieux et al., 2006; Diebold and Calonge, 2010; Cherif et al., 2011; Zhao and Castranova,

FIG. 3. Main devices used in nanomedicine.

2011; Raghuwanshi et al., 2012; Schairer et al., 2012; Wanakule et al., 2012; Wang et al., 2012).

Nanomaterials are classed according to their physicochemical features and size, both of which will affect their usefulness: liposomes (80–200 nm), nanoparticles (20–1000 nm), block copolymer micelles (50–200 nm), gold nanoparticles (5–50 nm), and nanosized drug crystals (100–1000 nm) have all been developed (Duncan and Gaspar, 2011) (Fig. 3). However, to be used successfully, certain issues still remain to be addressed: (1) the difficulty in stable nucleic acid/drug nanocomplex formation, (2) identification of a suitable administration route, (3) their ability/inability for systemic delivery, (4) reliable tissue targeting, (5) their limited intracellular uptake, and finally, (6) effective regulation of gene expression.

At this stage, several synthetic lipid- and polymer-based nanocarriers have been synthesized and tested as nucleic acid delivery systems. These nanoparticles have a better safety profile than viral vectors, can adapt to both nucleic acids and small molecule drugs, and readily lend themselves to chemical modification and functionalization (Li et al., 2013).

Overview of Nanomaterials

Organic nanomaterials

In 1989, the U.S. FDA approved Lupron Depot, the first parenteral sustained-release formulation based on biodegradable poly(d,l-lactide) (PLA). A copolymer of poly(ethylene glycol) (PEG) and poly(d,l-lactide-co-glycolide) (PLGA) named Genexol-PM, formulated with paclitaxel into nanoparticles (NPs), was used to treat breast cancer in phase 2 clinical trials in the United States. Parenteral PLGA nanoparticles have been used as carriers in various other applications in vaccination, cancer therapy, and the treatment of cerebral disorders (Danhier et al., 2012). For example, Woodrow and co-workers obtained efficient and sustained gene silencing in the mouse female reproductive tract through siRNA loaded into PLGA NPs (Woodrow et al., 2009).

PLGA nanoparticles do have a number of intrinsic drawbacks, indeed they are considered as a weak drug-delivery system. After an intravenous administration, they undergo rapid clearance before reaching the site of action and, moreover, PLGA NPs are not able to discriminate different cell types (Sah et al., 2013). However, research has shown that surface modifications can improve their functionality. Panyam and colleagues, for instance, have demonstrated that introducing the amine-rich cationic polymer poly(ethylene imine) (PEI) into the PLGA matrix can improve oligonu-

cleotide encapsulation, as well as siRNA retention and release. This is due to the fact that PEI is more hydrophilic than PLGA and hence more readily dissolves in aqueous buffers. PEI also contains a number of amino groups that can be protonated at physiological pH, thereby conferring the potential to combine with negatively charged nucleic acids effectively and prevent their biodegradation before they reach target cells. In addition, according to the “proton sponge effect” hypothesis (Akinc et al., 2005), PEI can assist its cargo to escape from the endosome into the cytoplasm. The incorporation of PEI into PLGA nanoparticles also affects their cellular uptake and increases their cellular accumulation.

Furthermore, tests in two different cell culture models suggest that siRNA-loaded PLGA–PEI NPs efficaciously reduce gene expression (Patil and Panyam, 2009). It is also possible to deliver a drug–nucleic acid combination by directly encapsulating both types of agents in biodegradable polyester particles. For instance, Panyam et al. described the simultaneous delivery of paclitaxel and P-glycoprotein siRNA using PLGA–PEI nanoparticles (Patil et al., 2010).

The cytotoxicity of PEI is a major limitation, but this can be overcome by using a low concentration PEI (Patil and Panyam, 2009; Yang et al., 2011). PEI's cationic toxicity can also be minimized by conjugating it with PEG to yield different sets of PEG–PEI copolymers, characterized by different sizes of both molecules. After coupling with PEI, the nonionic PEG polymer coating can provide dimensional shielding, improve the biological stability of the transfection complexes, and prolong their circulation times via reducing nonspecific protein binding in the serum (Neu et al., 2007). Coating the NP surface with hydrophilic polymers such as PEG is known to enhance colloidal stability in the extracellular environment and reduce recognition by mononuclear phagocyte system (Raemdonck et al., 2013). Indeed, a PEG coating provides PLGA nanoparticles with a hydrophilic corona (Fig. 4a), thereby overcoming a significant obstacle to the use of naked PLGA NPs, namely their hydrophobicity. Indeed, nanoparticles with negative surface charge are vulnerable to opsonization and rapid elimination from the blood after intravenous administration (Sheng et al., 2009). PEGylation therefore improves their aqueous solubility and stability, reducing intermolecular aggregation and immunogenicity, and prolonging their systemic circulation time (Sah et al., 2013).

Liang and coworkers have successfully used nanoscale particles formed from PEG–PEI and siRNA to silence the expression of Nogo receptor in the central nervous system (CNS). These NPs demonstrated high efficacy and low

FIG. 4. Schematic representation of poly(ethylene glycol)–poly(d,l-lactide-co-glycolide) PLGA–PEG block-copolymer (a) and polymerosomes (b).

toxicity, making them ideal candidates for mediating gene expression in CNS (Liang et al., 2012). Moreover, Williams and colleagues showed that dystrophin expression significantly increased when *mdx* mice were given intramuscular injections of 2'OMe-AON conjugated with PEI2K-PEG550 complexes. This set of PEG-PEI copolymers containing 2 kDa PEI and either 550 Da or 5 kDa PEG able to bind 2'OMe-AON with high affinity and form stable complexes with a low surface charge. To enhance biocompatibility and cellular uptake, PEI-PEG copolymers were also covalently conjugated with nanogold (NG) or adsorbed colloidal gold (CG), but no significant differences were found in dystrophin expression between nonfunctionalized PEI2K-PEG550 copolymers and the resulting NG-PEI2K-PEG550 or CG-PEI2K-PEG5K (Williams et al., 2008).

Formulations of PEG-PEI copolymer, functionalized with cell-penetrating peptide TAT (trans-activator of transcription), CG, or both TAT and CG, have also yielded promising results. Highest level of dystrophin were found in muscles from *mdx* mice intramuscularly injected with 2'OMe-AON conjugated with different PEG-PEI complexes and functionalized with TAT, with respect to *mdx* treated with 2'OMe-AON alone. Once again, adsorption of CG to either PEG-PEI or TAT-PEG-PEI copolymers failed to increase dystrophin expression further (Sirsi et al., 2008).

An alternative system for efficient delivery of AON and siRNA molecules is nonionic controlled-release polymer vesicles (polymersomes). Polymersomes are characterized by an aqueous lumen suitable for loading, and drug release occurs through either oxidation-sensitive or hydrolysis-sensitive copolymer amphiphiles. Polymersomes circulate *in vivo* for longer than lipid vesicles, and their degradation can produce surfactants that induce endolysosomal release (Kim et al., 2009) (Fig. 4b).

A novel class of molecules, termed lipid nanoparticles (LNPs), has been developed to deliver short RNAs to mammalian cells both *in vitro* and *in vivo*. LNPs contain ionizable amino lipids that self-assemble into nanoparticles (about 100 nm) when mixed with polyanionic oligonucleotides, playing a dual role in the drug delivery process. The electrostatic interaction of LNPs with polyanionic nucleic acids promotes the encapsulation of siRNA, which is then able to escape the endosomal compartment and access the cell cytoplasm following endocytosis of LNPs by target cells (Jayaraman et al., 2012). Lipid nanoparticles (LNPs) with different compositions of cationic lipids, PEG, and cholesterol, have been used to deliver luciferase siRNA to the liver via systemic (intravenous) administration in mice (Tao et al., 2010).

Another class of lipid-like delivery molecules, termed “lipidoids,” has also been used to efficiently deliver immunostimulatory RNA (isRNA) to Toll-like receptor-expressing cells by subcutaneous injection, stimulating innate and adaptive immune responses. A library of lipidoids has been synthesized, conjugated with isRNA, and shown to induce type-I interferon activation in human peripheral blood mononuclear cells (Nguyen et al., 2012).

Other vectors that have been developed to enhance cell delivery of oligonucleotides and siRNA are the CPPs—short cationic or amphipathic peptides with the capacity to enter into the cells and deliver different molecules across the cell membrane. Examples of natural CPPs are the Tat domain of HIV-1 and the penetrating domain of *Drosophila antennape-*

dia (Dupont et al., 2011). CPPs containing cationic amino acids, such as multiple arginines, can strongly enhance AON delivery due to their ability to carry associated cargoes across the plasma and endosomal membranes. Yin and coworkers have reported a promising new peptide PMO, named Pip5e-PMO, able to restore a high level of dystrophin synthesis all muscles from *mdx* mice after a single 25 mg/kg administration (Yin et al., 2011). Ezzat and collaborators have demonstrated that a new CPP, PepFect 14, binds siRNA through a non-covalent interaction. This is then able to induce efficient RNA interference response in different cell types (Ezzat et al., 2012).

Chitosan/pluronic F68-coated poly(isobutyl cyanoacrylate) (PIBCA) nanoparticles obtained by emulsion polymerization are also attractive carriers for *in vivo* delivery of oligonucleotides. The efficacy of intravenously injected siRNA-PIBCA complexes has been tested on mice with papillary thyroid carcinoma, resulting in a significant reduction of tumor growth (De Martimpres et al., 2010). Zandanel and Vauthier recently proposed a model for investigating how PIBCA NPs deliver siRNA to biological targets, both in cell cultures and *in vivo* (Zandanel and Vauthier, 2012). They incorporated a fluorescent probe into PIBCA nanoparticles by a covalent linkage, without modifying the surface properties of the latter.

Inorganic nanomaterials

Another focus of recent research has been inorganic nanobiomaterials, since, compared with organic materials, these show high thermal/chemical stability and resistance to corrosion under physiological conditions. Moreover, they are characterized by good biocompatibility and low degradation rates. Silica is one of the biocompatible materials most used; it is an endogenous substance present mainly in bones and it has been used commercially as excipient in several drugs.

Additionally, silica has been used to synthesize one of the recently developed organic-inorganic hybrid nanomaterials, which combine the advantages of both organic and inorganic materials. Thanks to their high porosity and biocompatibility, mesoporous silica nanoparticle (MSNs) systems, synthesized by co-assembly of organic structural directing agents and inorganic silicate species, provide efficient drug-delivery of siRNA (Na et al., 2012). MSNs with 23-nm pores have demonstrated highly efficiency and specificity, without significant cytotoxicity, both *in vitro* and *in vivo* studies. However, especially for therapeutic purposes, the degradation behavior of these NPs still requires clarification and refinement (Chen et al., 2013).

Silver has also been used in the synthesis of new types of nanoparticles. These 60- to 80-nm silver nanoparticles are decorated with thiol-terminated photolabile DNA oligonucleotides and showed enhanced stability and resistance to enzymatic degradation, greater cellular uptake, and, after photoactivation, a controlled restoration of therapeutic activity of antisense delivery, as compared with naked nucleic acids (Brown et al., 2013).

Oligodeoxynucleotides have also been efficiently delivered into cells by a calcium phosphate (CP)-based carrier, developed by Hou and coworkers. The spherical porous ODN-CP nanoparticles with a diameter of 50–70 nm used in this case were prepared by a facile microemulsion approach (Hou et al., 2013). Fabrication of micro- and nanoparticles

with defined features, such as shape, size, surface charge, stiffness, and chemical composition, can also be performed by a high-resolution molding technology known as particle replication in non-wetting templates. This technique enables the synthesis of particles from various biocompatible and biodegradable materials (PEG, proteins, and PLGA), and is widely used to create hydrogel and nanogel nanoparticles (with a diameter of 80 nm and lengths ranging from 180 to 5000 nm), which have been studied as a delivery system for cellular transfection with siRNA (Dunn et al., 2012; Kersey et al., 2012).

All nanomaterials reported in the two sections above are briefly described in Table 1.

Exon Skipping Therapies for Duchenne Muscular Dystrophy

Duchenne muscular dystrophy, one of the most common inherited neuromuscular disorders, is caused by mutations in the *DMD* gene, which encodes the protein dystrophin and lies at chromosomal locus Xp21 (Emery, 2002). The dystrophin mutations usually seen in DMD patients cause the loss of the C-terminal domain of dystrophin and result in a nonfunctional protein.

However, mutations involving a part of the central rod domain may still allow production of a functional dystrophin protein, as seen in the milder condition Becker muscular dystrophy (BMD) (Kingston et al., 1983). This less severe phenotypic form of the disease provides the basis for DMD treatment by splice modulation (Douglas and Wood, 2013). The deletion of some *DMD* gene exons can cause a shift of the open reading frame in the spliced transcript. These frameshifts lead to the incorporation of a premature STOP codon in the downstream region of the transcript, and produce a protein that is nonfunctional and possibly unstable. Moreover, a premature termination codon in the middle of a transcript could lead to degradation of the transcript via activation of the nonsense-mediated mRNA-decay cellular pathway (Buvoli et al., 2007). In contrast, maintenance of the normal open reading frame leads to a functional dystrophin in the BMD phenotype (Monaco et al., 1988).

Conversion of an out-of-frame transcript into an in-frame transcript that codes for a functional protein is the goal of the antisense-mediated exon skipping strategy for DMD (Aartsma-Rus and van Ommen, 2007). Two clinical trials to this end are ongoing: the European companies Prosensa Therapeutics and GlaxoSmithKline (GSK) are exploring 2'OMePS chemistry (PRO051/GSK2402968, Drisapersen), and AVI-Biopharma (now Sarepta) is investigating the chemistry of PMO (AVI-4658, Eteplirsen). In 2007, Prosensa published initial results from their clinical trial, conducted in The Netherlands: four DMD boys received a single dose of 0.8 mg of PRO051 injected into the tibialis anterior muscle, and a specific skipping of exon 51 and a percentage range of 64%–97% of dystrophin-positive myofibers was detected in all (van Deutekom et al., 2007).

The PMO (AVI-4658) chemistry was used in a similar study in 2008 in the United Kingdom, in which seven DMD boys received either 0.09 or 0.9 mg in one of the two extensor digitorum brevis muscles. Exon skipping was observed in muscles treated with both doses; however, dystrophin restoration was only detected in those receiving the higher dose, specifi-

cally 44%–79% of fibers. Encouragingly, no significant side effects were noted in either trial (Cirak et al., 2011), and both AON chemistries produced dystrophin rescue in patients, albeit with various degrees of intensity and skipping percentage.

Actually the two AON chemistries are subject of systemic clinical trials (www.clinicaltrials.gov/ct2/results?term=NCT01396239; <http://www.clinicaltrials.gov/ct2/results?term=DMD&pg=2>).

Animal Models

Before the clinical trial phase, AONs need to be tested *in vivo* on animal models of the disease. Such studies have provided much pre-clinical evidence for the potential of exon skipping-based DMD therapy. The most widely studied and used animal DMD model to date is the *mdx* (X-chromosome-linked muscular dystrophy) mouse, which carries a spontaneous mutation identified in the C57BL/10-ScSn mouse line (Bulfield et al., 1984). This mutation is a single nucleotide substitution in exon 23 that results in a premature stop codon in the dystrophin gene. This causes an absence of the protein in the muscle, even though some rare revertant myofibers may be present (Sicinski et al., 1989; Hoffman et al., 1990). While in humans DMD leads to an early loss of muscle functionality, dystrophin-null mice show very mild symptoms until old age (Chamberlain et al., 2007), probably due to the presence of utrophin, an autosomal homologue of dystrophin in these animals (Nakagaki and Camilli, 2012). Hence, to obtain a phenotypic model closer to human disease, utrophin/dystrophin double-knockout mice have been created, which show a more severe phenotype and many signs of human dystrophy (i.e., severe muscle weakness, reduced lifespan, cardiomyopathy, and growth retardation) (Durbecq and Campbell, 2002; Vainzof et al., 2008).

Several dog breeds have also been proposed as dystrophy models, although very few have been characterized. In current studies, the most-used dog colonies are the beagle, corgi, and golden retriever muscular dystrophy models (GRMD) (Duan, 2011). The beagle and GRMD models share the same mutation, a splice site mutation in intron 6, although the latter has been more widely studied (Smith et al., 2007). This mutation induces exon 7 skipping, the formation of a premature stop codon in exon 8, and the resulting absence of dystrophin, accompanied by a severe phenotype. Several mutations in other canine DMD models have also been identified (Duan, 2011; Kornegay et al., 2012), but these remain costly and require double exon skipping, making the *mdx* mouse the preferred model.

The first exon skipping approach tested on *mdx* mice used 2'-O-methylated phosphorothioated (2'-OMePS) AON. This resulted in an efficient local restoration of dystrophin (Lu et al., 2003), and its efficiency has been boosted in studies using PMOs, peptide-tagged PMO, and non-peptide polymer-tagged PMO (Wu et al., 2009; Goyenvallé et al., 2010). The results of these studies have shown that muscular dystrophy can be ameliorated in mouse models through repeated intravascular or intraperitoneal AON injections. Moreover, an enduring dystrophin expression can be achieved through a combination of AON-mediated exon skipping and adeno-associated virus (AAV) gene transfer (Goyenvallé et al., 2004).

Other AON chemistries used to improve dystrophin restoration in *mdx* mice are 2'-O-methoxyethyl phosphorothioate

TABLE 1. OVERVIEW OF DIFFERENT NANOMATERIALS

<i>Nanomaterials</i>	<i>Properties</i>	<i>Advantages/limitations</i>	<i>Application</i>	<i>Reference</i>
PLA (Poly (d,l-lactide))	Aliphatic polyester, hydrophobic	Good biocompatibility and biodegradation rate Low stiffness	Medical implants, vaccines, periodontal drug-delivery systems	Sah et al., 2013
PLGA (Poly (d,l-lactide-co-glycolide))	Aliphatic polyester, hydrophobic, negative charged	High biodegradation rate poor loading High burst release, aggregation, vulnerable to opsonization	Vaccination, cancer therapy, treatment of cerebral disorders, gene silencing	Danhier et al., 2012; Woodrow et al., 2009
PEG (poly(ethylene glycol))	Non-ionic, hydrophilic	Biocompatible, non-toxic, high stability, biologically inert	Cancer therapy, gene silencing, exon skipping therapies	Raemdonck, 2013; Liang et al., 2012; Williams et al., 2008
PEI (poly(ethylene imine))	Amine-rich cationic polymer, pH responsive	Low stiffness and mechanical properties Easily dissolvable in aqueous buffers	siRNA transport, gene silencing, exon skipping therapies	Patil et al., 2010; Liang et al., 2012; Williams et al., 2008
Polymersomes	Polymetric colloidal vesicular systems, with an aqueous inner core (made of PEG-PLGA copolymers)	Long blood circulation time, good loading capacity and controlled release of encapsulated therapeutics	siRNA and antisense oligonucleotides delivery, cancer therapy	Kim et al., 2009
Lipid nanoparticles	Ionic amino-lipids	Good biocompatibility, long circulation time, low degradation rate Difficulty of large-scale production	Drug delivery, gene delivery, imaging	Jayaraman et al., 2012; Tao et al., 2010
Lipidoids	Lipid-like molecules, made of alkyl-acrylate or alkyl-acrylamide tails added to primary or secondary amine-containing cores	Easy synthesis, immunostimulatory effect	Delivery of siRNA to stimulate innate immune responses	Nguyen et al., 2012
Chitosan/pluronic F68-coated poly(isobutylcyanoacrylate) (PIBCA) nanoparticles	Hydrophilic chains of chitosan and pluronic F68 on the surface, positively charged	Activation of the complement system, high stability, siRNA protection Pluronic reduce siRNA accessibility to chitosan positive charges	Delivery of siRNA to tumors	Zandanel and Vauthier, 2012; de Martimprey et al., 2010
Cell penetrating peptides (CPPs)	Short cationic peptides	Traverse the plasma membrane, good stability and protection especially of RNA molecules Limited tissue targeting, very high dosage of therapeutics to obtain activity, increased off-target effects	Delivery of siRNA and PMO	Jarver et al., 2011; Dupont et al., 2011; Betts et al., 2012; Ezzat et al., 2012
Mesoporous silica nanoparticles	Organic agents and inorganic silicate species	Encapsulate drugs with different physicochemical properties, good loading capacity Degradation behavior not yet clarified, drug release too rapid	Delivery of siRNA	Na et al., 2012; Chen et al., 2013
Silver nanoparticles	Synthesis via ligand exchange of citrate with 30 thiol-modified DNA oligonucleotides	Large surface-area to-volume ratios, enhanced stability of surface-bound nucleic acids, unique photophysical properties, resistance to enzymatic degradation Difficulty of synthesis and functionalization, silver toxicity	Delivery of antisense-oligonucleotides	Brown et al., 2013
Calcium phosphate (CP)-based carriers	Spherical and porous nanoparticles, 50–70 nm of diameter, anionic molecules	High loading capacity, efficient intracellular delivery, good biocompatibility and biodegradability, low toxicity	Delivery of antisense-oligodeoxynucleotides, cancer therapy	Hou et al., 2013

isRNA, immunostimulatory RNA; PEG-PLGA, poly(ethylene glycol-poly(d,l-lactide-co-glycolide)); PMO, morpholino phosphorodiamidate oligomers, siRNA, small interfering RNA.

RNA (MOePS) AONs (Geary et al., 2001). Indeed, Yang and coworkers have demonstrated that MOe AONs can induce efficacious exon skipping, both *in vitro* and in *mdx* mice through local intramuscular injections. Interestingly, the significantly higher levels of dystrophin expression seen in mice treated in this way indicate that this could be a promising efficient exon skipping strategy for DMD (Yang et al., 2013). Unsurprisingly, therefore, many other animal model studies are now being focused on the identification of new delivery systems for AON molecules, in order to improve the efficiency and safety of this strategy.

Nanoparticle-Antisense Oligoribonucleotide Complexes for DMD Therapy

In recent years, many AONs have been synthesized, but only few molecules have been employed in AON-mediated molecular therapy. In order to be used in drug applications, the AON design must allow the molecule to enter in cell nucleus, specifically hybridize against the target RNA, and biodistribute to the target organs (Jarver et al., 2013). AON intake is also dose-dependent, raising concerns that increasing doses to a therapeutic range could have major side effects, especially in terms of renal overload (Henry et al., 2012). Furthermore, naked AONs are internalized poorly by cells, tending to localize in endosomes/lysosomes, where they are ineffective.

Nonetheless, AON uptake occurs through active transport, which depends on adsorptive endocytosis and fluid phase pinocytosis, meaning that AON internalization might be achieved through interaction with membrane-bound receptors (de Diesbach et al., 2002). Various techniques of improving cellular uptake have been tested, and the most

successful to date in terms of efficacy is the coupling of AON with basic peptides with membrane translocation properties. In particular, systemic delivery of morpholino oligomers conjugated with cell-penetrating peptides (Pip) containing a stretch of arginines have yielded excellent results, bringing about considerable dystrophin rescue, even in the heart, which is normally poorly targeted by AON (Yin et al., 2011). Although AON-conjugated transducing peptides are very promising, concerns about Pip toxicity persist, due to its ability to activate many signaling pathways (Juliano et al., 2012).

In order to enhance *in vivo* AON applications, by increasing stability and enabling the use of lower concentrations, several vectors or chaperones have been developed. Among these, chimeric small nuclear RNA (snRNA) molecules and U7 snRNAs (using AAV or retrovirus) have both been shown to efficiently induce exon skipping (Mendell et al., 2012). A number of other transporters, such as liposomes, cationic polymers, and molecular sponge mechanisms, have been tested with various results (Dias and Stein, 2002). In particular, two new classes of nonviral biocompatible nanoparticles, called T1 and ZM2, have recently been shown to bind and deliver 2'OMePS M23D AON in *mdx* mice treated by intraperitoneal (i.p.) administration (Rimessi et al., 2009; Ferlini et al., 2010).

T1 nanoparticles

T1 nanoparticles, made up of a polymethyl methacrylate (PMMA) core surrounded by a shell whose cationic carrying groups are ideal for AON binding, have been shown to mitigate desorption and instability issues (Fig. 5a). This follows

FIG. 5. T1 nanoparticle characteristics. (a) Representation of the interactions between AON molecules and quaternary ammonium groups on the surface of nanoparticles; (b) Scanning electron microscope image of T1 nanoparticles showing their size. Scale bar = 500 nm.

TABLE 2. INJECTION SCHEDULE

Group of mice	Treatment	No. of injections (i.p.)	First sacrifice	Second sacrifice
Naked AON	M23D-AON 45µg/injection	3 (1/week)	1 week after last injection	6 weeks after last injection
T1-AON	T1 2.5 mg M23D-AON 45µg/injection	3 (1/week)	1 week after last injection	6 weeks after last injection
T1	T1 2.5 mg/injection	3 (1/week)	1 week after last injection	6 weeks after last injection
Untreated	—	—	1 week after last injection	6 weeks after last injection

AON, antisense oligonucleotide; i.p., intraperitoneal.

previous reports that anionic and cationic PMMA-based nanoparticles are feasible delivery systems for protein and DNA vaccines (Caputo et al., 2004). T1 nanoparticles are able to convey the 2'OMePS M23D oligoribonucleotide with a good efficiency at a loading value of 18 µg AON/mg NPs. M23D (5'-GGCCAAACCUCGGCUUACCUGAAAU-3') AON is a 2'-O-methyl modified RNA that contains a full-length phosphorothioate backbone, and has been designed against the boundary sequences of exon and intron 23 of the mouse dystrophin gene (Gebbski et al., 2003). The quaternary ammonium groups on the nanoparticle surface and matrix facilitate strong lipophilic interaction with the hydrophobic 2'OMePS oligoribonucleotide (Rimessi et al., 2009).

Rimessi and coworkers showed that administration of M23D AON conjugated with T1 was able to restore dystro-

phin protein expression in skeletal muscles by increasing the dystrophin transcript and protein expression. Their biodistribution studies also demonstrated that T1 nanoparticles abound in various tissues and organs of *mdx* mice treated, via i.p. injections, with fluorescent AON-free T1 NPs. Under an electron microscope, T1 nanoparticles, which appear as electron-translucent round structures with an expected size of 500 nm (Fig. 5b), were found inside the cytoplasm of macrophages in lymphatic vessels and endothelial cells in blood vessels. Table 2 summarizes the schedule of i.p. injection tested in this approach. Thanks to NPs' body-wide distribution and protein synthesis restoration, this approach could be considered an effective means of delivering AON in DMD therapies. However, these nanoparticles biodegrade slowly, potentially leading to accumulation and potential adverse

FIG. 6. ZM2 nanoparticle characteristics. (a) Representation of the interactions between AON molecules and quaternary ammonium groups + N-isopropyl-acrylamide (NIPAM) on the surface of nanoparticles; (b) Scanning electron microscope image ZM2 nanoparticle showing its size (diameter 137nm). Scale bar = 500 nm.

effects. Moreover, T1 NPs can form small aggregates, and would therefore not be suitable for intravenous administration (Rimessi et al., 2009).

ZM2 nanoparticles

Another class of polymeric cationic core-shell NPs, the ZM2 NPs, are composed of a PMMA core and a random copolymer shell formed by units derived from N-isopropylacrylamide (NIPAM) and reactive methacrylate-bearing cationic groups (Fig. 6a). By electron microscope analysis, they have an expected diameter of 137 nm (Fig. 6b). ZM2 nanoparticles are nontoxic *in vitro*, and have demonstrated great efficiency in adsorbing M23D AON, with a loading value of 90 μ g of AON per mg of NPs.

Figure 7 shows the IP injections of ZM2-AON or naked AON given to *mdx* mice by Ferlini and coworkers, who showed, by immunohistochemical quantification analysis, that ZM2-AON complex induces dystrophin restoration in both skeletal muscles and heart. This dystrophin restoration was achieved using only 52.5 mg of AON/kg of animal, suggesting that lower doses of 2'OMePS AON administered for longer period might be sufficient to induce detectable dystrophin restoration (Ferlini et al., 2010).

In a further study, Bassi and collaborators tested the protective effect of ZM2 nanoparticles on AON molecules at 3 months from the end of the same low dosage treatment, and showed that the effect of ZM2-AON on dystrophin expres-

sion persists long after the suspension of treatment. They demonstrated that restored dystrophin is correctly localized on the sarcolemmal membrane of muscle fibers, and their Western Blotting analysis revealed a protein with the exact molecular weight in ZM2-AON treated mice, while this was undetectable in naked-AON treated mice (Bassi et al., 2012).

Taken as a whole, these findings clearly indicate the potential of ZM2 nanoparticles as nontoxic delivery vectors for 2'OMePS AON, and their protective effect on AON molecules is a particularly appealing characteristic. Indeed, such NPs could reduce the dose of AONs required, thereby reducing both the possible side effects and the duration of the administration schedule.

However, it is clear that there is the need to synthesize and test novel NPs with different binding capacity (based on covalent or electrostatic charge with higher stability) with AONs in order to increase the efficacy of effects in terms of dystrophin rescue.

Conclusions and Future Prospects

Drug delivery is certainly a key factor in the optimization of treatment efficacy and efficiency, and may enable us to reduce undesirable off-target effects, side effects, and the dose requirement.

Antisense oligoribonucleotides (AONs) have shown great potential in the treatment of various diseases, as for Duchenne muscular dystrophy. Nevertheless, the selective

FIG. 7. Injection schedule (*upper panel*); immunohistochemical analysis of *mdx* skeletal muscles of first (*lower left panel*) and second (*lower right*) sacrifice. Muscle sections (20 μ m) were labeled with a polyclonal anti-dystrophin antibody and revealed with anti-rabbit Cy3-conjugated secondary antibody (green signal). Inj., injection; i.p., intraperitoneal.

delivery to a target tissue (striated muscle) has proved challenging. Striated muscles, comprising both skeletal and cardiac muscles, are proven difficult to target, both for the high level of organization of the sarcolemma membrane, and for their representation, since the percentage of body weight that is muscle is between 38% and 54% for the men and 28% and 39% for the women. AONs should therefore improve both for the targeting, that should be auspiciously being in muscle only, avoiding overload on other organs, especially kidney, and for dose regimen, since the current trials use quite high doses of these molecules.

Significant progress has been made during the recent years in nanoscale drugs and delivery systems employing diverse chemical formulations to facilitate the rate of drug delivery and to improve its pharmacokinetics. Biocompatible nanomaterials have been mainly used as drug-delivery systems. The nature of the elements used for producing advanced biomaterials is of great importance and medicinal chemistry is required to face several challenges related to nanoparticle synthesis and conjugation. NP surface modification is another key issue to optimize the binding capacity on AON by introduction of various reactive functional groups on the surface of nanomaterials.

Nonetheless, concerns emerge about toxicity and pharmacokinetics of biocompatible nanomaterials, indicating the need for not only rigorous *in vivo* experiments in animal models but also novel nanomaterials for practical applications in the clinical settings. Indeed, we need to define the pharmacokinetics of NP-drugs after systemic administration, but especially the biophase bioavailability and pharmacokinetics. Studying the biodistribution and bioavailability of NP is still limited by the low labeling systems, and in this field also we do need extensive research. Elimination is another crucial issue that must be finely explored on order to propose NP in clinical trials.

In conclusion, nanomaterials are certainly very appealing as drug vehicles and show great promise in this regard.

Future studies aimed at addressing all the above issues as well as identifying novel biocompatible or even better biodegradable compounds will doubtless improve the clinical applicability of these delivery vehicles. Such novel delivery systems are also expected to open up enticing new avenues in terms of administration route, with a move toward noninvasive skin and oral administration. Progress in these areas made to date indicates that a real revolution in RNA therapy, with particular reference to DMD, is just around the corner.

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