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Review

Non-viral delivery of nucleic acid for treatment of rare diseases of the muscle

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Rare muscular disorders (RMDs) are disorders that affect a small percentage of the population. The disorders which are attributed to genetic mutations often manifest in the form of progressive weakness and atrophy of skeletal and heart muscles. RMDs includes disorders such as Duchenne muscular dystrophy (DMD), GNE myopathy, spinal muscular atrophy (SMA), limb girdle muscular dystrophy, and so on. Due to the infrequent occurrence of these disorders, development of therapeutic approaches elicits less attention compared with other more prevalent diseases. However, in recent times, improved understanding of pathogenesis has led to greater advances in developing therapeutic options to treat such diseases. Exon skipping, gene augmentation, and gene editing have taken the spotlight in drug development for rare neuromuscular disorders. The recent innovation in targeting and repairing mutations with the advent of CRISPR technology has in fact opened new possibilities in the development of gene therapy approaches for these disorders. Although these treatments show satisfactory therapeutic effects, the susceptibility to degradation, instability, and toxicity limits their application. So, an appropriate delivery vector is required for the delivery of these cargoes. Viral vectors are considered potential delivery systems for gene therapy; however, the associated concurrent immunogenic response and other limitations have paved the way for the applications of other non-viral systems like lipids, polymers, cell-penetrating peptides (CPPs), and other organic and inorganic materials. This review will focus on non-viral vectors for the delivery of therapeutic cargoes in order to treat muscular dystrophies.

Keywords. Gene therapy; non-viral vectors; rare muscular diseases

1. Introduction

Rare muscular disorders (RMDs) are the group of muscular dystrophies that cause progressive weakness of skeletal, respiratory, and cardiac muscles and lead to the development of a dystrophic pathological phenotype. Duchenne muscular dystrophy (DMD), GNE myopathy, spinal muscular atrophy (SMA), and limb girdle muscular dystrophy are some rare muscular dystrophies with an estimated prevalence of approximately 1 in 3,500–1 in 9,300 male births worldwide for DMD (Ryder *et al.* 2017), 1–9 per 1000,000 individuals for GNE myopathy (Orphanet.com) (Carrillo *et al.* 2018), 1 in 10,000 live births for spinal muscular atrophy (Lunn and Wang 2008; Verhaart *et al.* 2017) and 1 in 123,000 for limb girdle muscular dystrophy (Wicklund and Kissel 2014). RMDs are mostly associated with mutations in single genes such as DMD and GNE myopathy which are caused due to mutations in the dystrophin gene (Blake *et al.* 2002) and GNE gene (Eisenberg *et al.* 2001), respectively. The mutations, however, can vary in number and position across different populations with incidences of founder mutations observed in some regional clusters (Argov and Mitrani Rosenbaum 2015). All these RMDs consist of diverse collections of disorders with different pathological characteristics including weakness in skeletal,

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cardiac, and respiratory muscles. Along with the involvement of muscles, some other internal organs are also involved like the brain, ear, eye, skin, etc. The prognosis, comorbidities, onset, and severity of the condition vary substantially depending on the type of the disease (Emery 2002). Clinical guidelines are being implemented based on understanding the problems associated with specific disorders (Bushby *et al.* 2010). Moreover, increased research on the underlying disease processes is leading to the development of novel therapeutic strategies, some of which are currently undergoing clinical trials (Eagle *et al.* 2002; Emery 2002).

Gene therapy is one of the promising approaches towards treatment for monogenic disorders and even for some complex disorders like malignancies (Kirschner and Cathomen 2020). Research into potential gene therapy strategies for the management of various fast progressing RMDs has been ongoing for decades (Morgan 1994; Matsuo 1996; Hartigan-O'Connor and Chamberlain 2000). In general, there have been several setbacks in this approach, leading to slow progress in the development of gene therapy for RMDs (Ferrer *et al.* 2000). However, some of the recent successes for SMA and DMD treatments has triggered renewed interest in the area (Mendell *et al.* 2017, 2023; Kirschner and Cathomen 2020; Ogbonmide *et al.* 2023). Gene therapy offers added advantages in treating rare disorders with limited known information regarding their inherent backgrounds and genotype–phenotype connections since complete gene replacement approaches are mutation independent. Various approaches towards gene therapy are being developed for RMDs currently, which include gene augmentation (Nóbrega *et al.* 2020; Elangkovan and Dickson 2021; Mendell *et al.* 2023; Ogbonmide *et al.* 2023), exon-skipping (Miyatake *et al.* 2018; Filonova and Aartsma-Rus 2023; van Deutekom *et al.* 2023), and gene editing (Zhu *et al.* 2017; Min *et al.* 2019; Bhokisham *et al.* 2023; Eslahi *et al.* 2023). Gene augmentation therapy involves delivery of the normal wild-type version of the gene for the monogenic recessive disorder in which the mutation is non-functional. The introduction of a functional copy of the gene is anticipated to reverse the disease phenotype by restoring the production of the mutated or deficient protein (Nóbrega *et al.* 2020). Exon-skipping relies on the usage of tiny fragments of nucleotides known as antisense oligonucleotides (ASOs), which target and bind particular mRNA sequences (Miyatake *et al.* 2018; Lejman *et al.* 2023) and eliminate the targeted mutated exons from a mature mRNA. Genome editing, as opposed to exon skipping, results in the permanent correction of gene mutation and is turning out to be a game changer in the field of genetic methods of disease correction. The aim is to improve the quality of life of patients by reducing the frequency of administration, which would promote compliance with the treatment (Fischer *et al.* 2010; Naldini 2015), and delivering individualized therapy to repair genetic defects (Dunbar *et al.* 2018). In the early days of genome editing, the defective gene's splicing sequences used to be permanently removed using engineered zinc finger nucleases (ZFNs) (Ousterout *et al.* 2015) and transcription activator-like effector nucleases (TALENs) (Ousterout *et al.* 2013), enabling the expression of the wild-type protein. The restoration of the functional gene through genetic correction is currently being explored using the clustered regularly interspaced short palindromic repeats CRISPR/CRISPR-associated protein 9 (Cas9) system which is a class of RNA-guided endonucleases found in bacterial and archaeal immune systems (Ousterout *et al.* 2013; Doudna and Charpentier 2014; Min *et al.* 2019; Eslahi *et al.* 2023).

Although one of the most effective strategies for treatment of RMDs is gene therapy, delivery of the genetic payload or the gene editing components remains challenging due to the inherent instability of such biomacromolecules leading to degradation and quick clearance before the action is completed. Therefore, in order to deliver these cargoes, a suitable delivery vector is needed. Compared with other methods, viral vectors have been studied for a long time in both preclinical and clinical studies in a wide range of diseases including RMDs (Bish *et al.* 2012; Mitrani-Rosenbaum *et al.* 2012; Vulin *et al.* 2012; Tal-Goldberg *et al.* 2014). However, viral vectors are associated with some limitations like strong undesired immunogenic response (Nayak and Herzog 2010; Mingozzi and High 2017; Park *et al.* 2019), very high production cost as well as low packaging capacity (Kofron and Laurencin 2006; Park *et al.* 2019), and sometimes these are also associated with insertional mutagenesis (Davé *et al.* 2004; Park *et al.* 2019). To address these issues, novel non-viral vectors have been developed in order to treat RMDs (Andreana *et al.* 2021; Cui *et al.* 2022). Non-viral vectors that include cationic lipid, polymers, cell penetrating peptides, inorganic nanoparticles, etc. show several advantages like relatively lower manufacturing cost, low immunogenicity, and high packaging capacity (Magin-Lachmann *et al.* 2004; Bondì and Craparo 2010; Park *et al.* 2019). The aim of the review is to focus on summarizing the various non-viral gene therapy approaches used for RMDs as well as the challenges

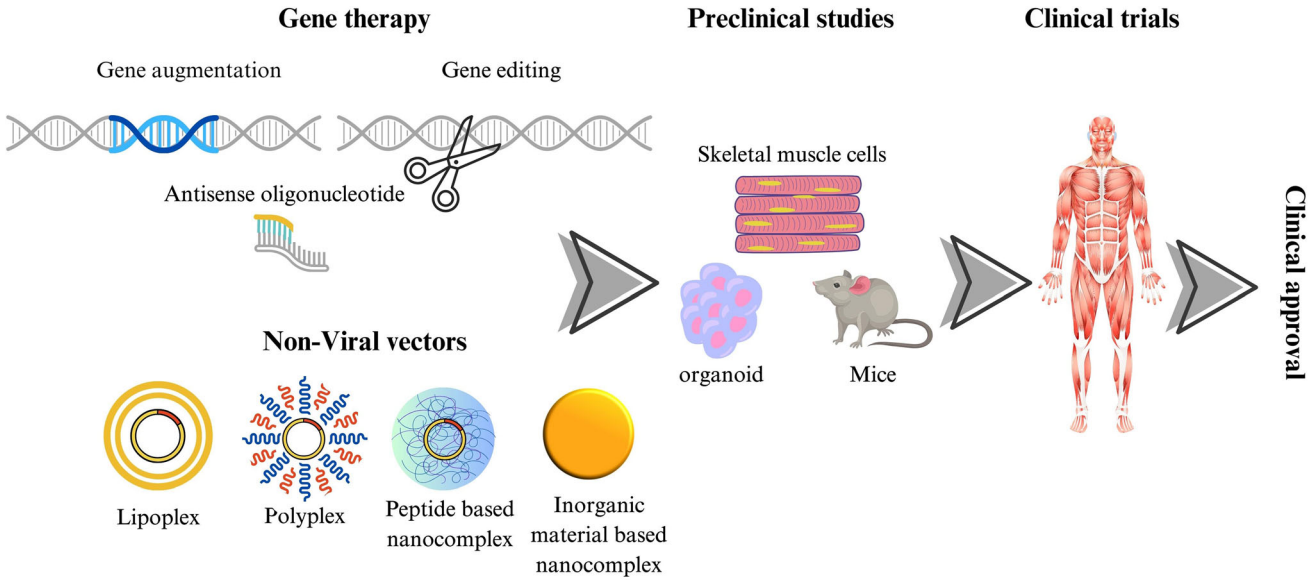

Figure 1. Overview of the clinical translational pipeline for developing gene therapeutics based on non-viral vectors.

associated with delivery to the skeletal muscle using such methods. The overview of the clinical translational pipeline for these non-viral vectors is depicted in figure 1.

2. Current status of clinically approved/under clinical trial gene therapy approaches for RMDs

There are various small molecules that are being used for the treatment of RMDs; some of them have FDA approval while others are still under preclinical research and development. Due to the rarity of these disorders, not much is known about the precise biochemical or biological processes that underlie the progression of these diseases, and these small molecules treat the symptoms partially instead of providing a permanent cure (Papaioannou *et al.* 2023). As mentioned earlier, one of the methods of gene therapy involves replacement of the functional copy of the gene. Successful gene replacement therapies, Zolgensma (AVXS-101, Onasemnogene Abeparvovec) for SMA treatment, and SRP9001 (Sarpeta Therapeutics) for DMD treatment (Mullard 2023), which are FDA approved in 2019 and 2023, respectively have increased the interest in this area (Crossrates 2019; Shahryari *et al.* 2019). Now companies such as Genthon, Pfizer, etc., are conducting clinical trials using microdystrophin or minidystrophin constructs for DMD treatment (Elangkovan and Dickson 2021; Philippidis 2022a).

Since delivery of large genes is quite challenging, other approaches like exon skipping are also important in the case of RMDs. There are several products based on exon skipping which employ ASOs to target specific portions of the mRNA, resulting in the correction of the reading frame by excluding the mutated exon. This leads to the generation of a functional but shortened protein (Miyatake *et al.* 2018; Lejman *et al.* 2023). The FDA and the European Medicines Agency (EMA) authorized nusinersen as one of the first ASO-based medications for the treatment of SMA in December 2016 and June 2017, respectively (Chiriboga 2017). FDA also granted rapid approval to Eteplirsen, a phosphorodiamidatemorpholino antisense oligonucleotide (PMO) that controls splicing for the treatment of DMD patients, in late 2016 (Aartsma-Rus and Krieg 2017). Exon 53-skipping PMO golodirsen was the second ASO approved by the FDA in 2019 for DMD (Heo 2020). In August 2020, Viltolarsen (NS Pharma, Inc.) was also been approved by the FDA for the treatment of DMD patients using exon 53 skipping (Dhillon 2020a). Table 1 gives a list of different types of therapies, based on gene augmentation/exon skipping, etc., as well as other treatment options that are either FDA-approved or in different stages of clinical trial for these diseases.

Table 1. Therapeutic interventions for treatment of RMDs in clinical trials

Type	Disease	Cargo/drug name	Clinical status	Trial registration number	Reference
Small molecules	SMA	Risdiplam	FDA approved		Dhillon (2020b); O'Keefe (2020)
		Branaplam	Phase 1/2 clinical trial	NCT02268552	Shorrock *et al.* (2018); Keller *et al.* (2022)
	DMD	Ataluren	EMA approved		Andreana *et al.* (2021); Mercuri *et al.* (2023)
	GNE	Intravenous immunoglobulins (IVIG)	Phase 2 clinical trial	NTR6160	Lim *et al.* (2021)
		Aceneuramic acid	Phase 3 clinical trial	NCT04671472 UMIN000020683	Mori-Yoshimura *et al.* (2023); Suzuki *et al.* (2023)
		ManNAc	Phase 2 clinical trial	NCT04231266	Carrillo *et al.* (2021)
Gene augmentation	SMA	SMN1 gene (Onasemnogene - Zolgensma)	FDA approved		Crossrates (2019)
	DMD	Microdystrophin (SRP-9001)	FDA approved		Mullard (2023)
		Minidystrophin (PF06939926 pfizer)	Phase 3 clinical trial	NCT04281485	Philippidis (2022a)
		Microdystrophin (GNT 0004)	Clinical trial ongoing	EudraCT #2020-002093-27	Elangkovan and Dickson (2021); Cernisova *et al.* (2023)
		Microdystrophin (SGT-001)	Phase 1 and 2 clinical trials	NCT03368742	Rao *et al.* (2021)
	GNE	GNE gene lipolex	Single patient response		Nemunaitis *et al.* (2010, 2011)
Exon skipping	SMA	Nusinersen	FDA approved		Chiriboga (2017)
	DMD	Eteplirsen	FDA approved		Aartsma-Rus and Krieg (2017)
		Golodirsen	FDA approved		Heo (2020)
		Viltolarsen	FDA approved		Dhillon (2020a)
		Drisapersen	Phase 3 clinical trials	NCT01803412	Goemans *et al.* (2018)

3. Delivery vectors

Adeno-associated viruses (AAVs) that do not replicate are often used for gene therapy by encapsulating the plasmid DNA into its capsid. Various AAV capsids display specific tissue tropisms which help them increase gene delivery efficiency towards specific organs. It has been reported that the class of capsids having the RGD motif, when injected systemically into animal models including mice and non-human primates, transduce muscles with greater efficiency and selectivity. These AAVs are referred to myoAAVs as they show high muscle tropism (Tabebordbar *et al.* 2021). Luxterna, an AAV vector-based gene therapy for biallelic RPE65-mutation-associated retinal dystrophy, is the first AAV-based product that was approved clinically in 2017 (Russell *et al.* 2017; Smalley 2017). The FDA-approved Zolgensma to treat children with SMA is an AAV vector-based gene therapy (Crossrates 2019; Shahryari *et al.* 2019) which has received a lot of attention as the 'world's most expensive drug' (Nuijten 2022). SRP9001 (delandistrogene moxeparvovec-rokl), an FDA-approved drug developed by Sarpeta Therapeutics, is also based on an AAV based viral vector for the delivery of microdystrophin in 4- to 5-year-old DMD patients (Mullard 2023). AAV-based vectors were used in a single clinical trial for delivery of ASOs which show minimal toxicity (Gushchina *et al.* 2021; Takeda *et al.* 2021). It has been demonstrated that using AAV vectors, CRISPR components can be expressed *in vivo* and the edited gene transcripts can lead to some therapeutic benefits (Choi and Koo 2022). AAV8-based vectors have been used for the delivery of wild-type human GNE cDNA in GNE myopathy

patient-derived cells as well as in healthy mice (Mitrani-Rosenbaum *et al.* 2012). Furthermore, a human-directed trans-splicing (TS) construct carried by an AAV8-based viral vector produced wild-type GNE transcripts from the primary muscle cells derived from a patient with GNE myopathy who carried the homozygous M712T mutation (Tal-Goldberg *et al.* 2014). However, AAV-based viral vectors have a number of clinical limitations. Several studies have demonstrated the possible toxicity associated with high systemic doses of AAV in large animals, despite the fact that it is safer in small animal models (Duan 2018a). AAV-based viral vectors are susceptible to losing their potency due to the effect of the neutralizing antibodies generated (Nayak and Herzog 2010). Furthermore, they have low packaging capacity, poor target specificity, and inadequate targeting of muscle cells. Therefore, in order to transfer genes into skeletal muscles, it is necessary to explore alternative methods, ideally employing a non-viral gene delivery system. Many initiatives have been attempted in this direction, but they are still far from clinical applications (Nance *et al.* 2018). Non-viral vectors have various advantages, including minimal immunogenicity and high packaging capacity (Magin-Lachmann *et al.* 2004; Bondì and Craparo 2010; Park *et al.* 2019), as well as being highly biocompatible and can be easily modifiable to target specific tissues (Wang *et al.* 2022). In the following sections we have highlighted different categories of non-viral vectors including inorganic NP and organic (peptide, lipid, and polymer-based) non-viral vectors that are used for RMDs.

3.1 *Peptide-based non-viral vectors*

One of the approaches used for non-viral gene delivery to muscles towards developing solutions for RMD, is to use muscle-targeting peptides. Laminin is one of the main components of the basement membrane. It interacts with receptors anchored in the plasma membranes of cells next to basement membranes. Two laminin-derived peptides A2G78 (GLLFYMARINHA) and A2G80 (VQLRNGFPYFSY) have been screened and shown to have ability to bind to the alpha-dystroglycan (α-DG) receptor (Suzuki *et al.* 2010; Negishi and Nomizu 2019). Therefore, an A2G80-modified oligoarginine (R9) and oligohistidine (H8) (A2G80-R9-H8) peptide was designed in order to create an efficient gene delivery system for skeletal muscle tissue. The oligoarginine residues, because of their positive charge, can interact with negatively charged DNA and can form nanocomplexes, whereas the addition of oligohistidine residues improves the endosomal escape capability of nanocomplexes. In C2C12 myoblast cells, A2G80-R9-H8 showed α-DG-dependent cellular uptake and significantly increased gene transfection efficiency. Furthermore, in Duchenne muscular dystrophy mouse models, the delivery system promoted efficient and sustained gene expression. The A2G80-R9-H8 peptide can be used as a potential carrier for gene therapy in RMDs (Nirasawa *et al.* 2021). For DMD, CPP-mediated administration of phosphorodiamidatemorpholino oligomers (PMOs) through local or systemic administration has shown significant success as an exon-skipping strategy. The previously developed muscle-targeting B-MSP-PMO delivery platform was improved for DMD treatment. B-MSP-PMO is composed of muscle-targeting heptapeptide (MSP) and arginine-rich CPP (B-peptide) which is conjugated with PMO. It was shown that B-MSP-PMO resulted in restoration of dystrophin protein expression in various muscle areas at extremely low systemic dosages (6 mg/kg), resulting in functional improvement and correction of the mdx dystrophic phenotype (Yin *et al.* 2009, 2010). Pip(PNA (peptide nucleic acid) internalization peptides)5e, an arginine-rich CPP with sequence RXRRBRRXR ILFQY RXRBRXRB, conjugated with PMO, has been used in *mdx* mice and resulted in effective exon skipping and dystrophin protein restoration (Yin *et al.* 2011; Betts *et al.* 2012). Later, a Pip5e-PMO derivative was developed, called Pip6a-PMO, which is the conjugated product of Pip6a (RXRRBRRXR YQFLI RXRBRXRB) and PMO, and was shown to be very effective for DMD mice models, although the detailed mechanism underlying its enhanced activity is yet to be investigated. The intracellular localization and biological activity of Pip6a-PMO has been assessed in primary cardiomyocytes and skeletal myocytes. According to the findings, endocytosis of Pip6a-PMO in skeletal muscle cells occurs through caveolae-mediated endocytosis, whereas in cardiomyocytes clathrin-mediated endocytosis seems more prevalent. These differences in cellular trafficking explained the differences in exon skipping and dystrophin protein restoration in both cardiomyocyte and skeletal myocytes in DMD mice models (Betts *et al.* 2012; Lehto *et al.* 2014). Cell-penetrating peptide DG9 (YARVRRRGPRGYARVRRRGPRR)-conjugated PMO delivery in DMD mice models improves dystrophin protein expression by exon-skipping strategy. Local delivery of a DG9-PMO combination that skips exons 45 to 55 restored dystrophin synthesis. This work offers a proof-of-concept for therapy for DMD

Table 2. Non-viral vectors tested *in vivo* for application in RMDs

Class of non-viral vectors	Nano carrier composition	Disease	Cargo	Animal model	Administration route	References
Peptide-based non-viral vectors	A2G80-R9-H8	DMD	pDNA	mdx mice	Intramuscular	Nirasawa *et al.* (2021)
	B-MSP-PMO	DMD	PMO	mdx mice	Intravenous	Yin *et al.*(2009, 2010)
	Pip5e-PMO	DMD	PMO	mdx mice	Intravenous	Yin *et al.* (2011)
	Pip6a-PMO	DMD	PMO	mdx mice	Intravenous	Betts *et al.* (2012); Lehto *et al.* (2014)
	DG9-PMO	DMD	PMO	mdx mice	Intramuscular	Lim *et al.* (2022)
	Pip6a	SMA	PMO	Smn1tm1Hung/ WT; SMN2tg/tg	Intravenous	Hammond *et al.* (2016)
Lipid-based non-viral vectors	LNP-CRISPR	DMD	CRISPR-Cas9/ sgRNA	hEx45KI-mdx mice	Limb perfusion	Kenjo *et al.* (2021)
	A2G80-LSP-Lip	DMD	-	mdx mice	Intravenous	Sasaki *et al.* (2021)
	Bubble liposomes	DMD	PMO	mdx mice	Intramuscular	Negishi *et al.* (2014)
	Nanolipodendrosome	DMD	myoD and Myogenin	SW-1	Intramuscular	Afzal *et al.* (2013)
	Nanoliposomes	DMD	MPS	mdx mice	Intravenous	Turjeman *et al.* (2019)
	GM-HL	DMD	Gentamicin	mdx mice	Intraperitoneal	Yukihara *et al.* (2011)
	Lipopetide based LNP	DMD	CRISPR/ CAS9	Ai14 mice	Intramuscular	Zhu *et al.* (2023)
Polymer-based non-viral vectors	PLGA-PEG-M12	DMD	PTEN inhibitor	mdx mice	Intravenous	Huang *et al.* (2020)
	PEI-PEG/PLGA	DMD	2'O-methyl AO	mdx mice	Intramuscular	Sirsi *et al.* (2009)
	ZM2	DMD	ASO	mdx mice	Intraperitoneal	Ferlini *et al.* (2010)
	PEI	DMD	pMDysE	-	-	Campeau *et al.* (2001)
	G5-PAMAM	DMD	pμDys	mdx mice	Intramuscular	Hersh *et al.* (2021)
Inorganic non-viral vectors	perfluorocarbon	DMD	rapamycin	mdx mice	Intravenous	Bibee *et al.* (2014)
	Gold	DMD	CRISPR/ Cas9	mdx mice	Intramuscular	Lee *et al.* (2017)

using exon 45 to 55 skipping that is more practical and affordable (Lim *et al.* 2022). In a mouse model of SMA, phosphorodiamidate oligomer (PMO)-internalizing peptide (Pip) peptides successfully deliver splice-switching oligonucleotide (SSO) throughout the body at doses lower (2.5, 5, and 10 μg/g) than those needed by naked SSOs (10 μg/g) (Hammond *et al.* 2016). For Pip6-PMO, all three different doses used have shown significant improvement in muscle strength, whereas for only PMO, a 10 μg/g dose was used and there was no significant improvement over and above the untreated group.

Various CPP-conjugated PMOs have been formed and used for exon-skipping therapy for DMD and other neuromuscular disorders (Tsoumpra *et al.* 2019). The majority of cell-penetrating peptides used for the treatment of muscular dystrophies are mentioned in Table 2.

3.2 *Lipid-based non-viral vectors*

Cationic lipids and liposomes are the most commonly employed systems in non-viral gene delivery, and shown to improve *in vitro* gene transport in a variety of cell types. Due to the positive charge of these lipids, they can easily form complexes with negatively charge DNA, resulting in the formation of lipoplexes which

help in protection of DNA from nucleases. Furthermore, lipoplexes are efficient in intracellular delivery of DNA mediated by endocytosis and also show good endosomal release assisted by the phase switching of lipid layers and a proton sponge effect due to the buffering capacity of amine groups present in the cationic/ionisable lipids (Stamatatos *et al.* 1988). Liposomes modified with polyethylene glycol (PEG) can also alleviate the problem of wide particle size distribution of liposomes and bring about effective drug release as well as improved *in vivo* circulation (Gabizon and Martin 1997; Lila and Ishida 2017).

Trivedi and Dickson (1995) have investigated the potential application of a variety of polycationic liposomes as a gene delivery vector for skeletal muscle. They have used four different kind of liposomes, LipofectAMINE, Lipofectin, LipofectACE, and DOTAP, to form the nanocomplex with the dystrophin-encoding plasmid. LipofectAMINE showed the best transfection efficiency followed by DOTAP in C2C12 cells. A new platform, stearoyl-CH2K3, a self-cross-linked lipopeptide has been developed for DNA delivery. The LP-DNA nanocomplex is coated with PEG which improves the distribution and gene expression by neutralizing its charge (Ho *et al.* 2018).

A2G80-LSP-Lip is a A2G80-modified long- and short-chain PEG (polyethylene glycol)-coated liposome. The long and short chain refer to 2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2K-OMe: PEG2K) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-750] (DSPE-PEG750-OMe: PEG750), respectively. To prepare A2G80-LSP-Lip, first, the A2G80 peptide is conjugated with the long chain, i.e., PEG2K, and then the short and long chain of PEG are coated over the already formed liposome. A2G80-LSP-Lip accumulated more efficiently in muscle tissue of DMD mice models compared with unmodified liposomes. These findings imply that systemic administration of A2G80-LSP-Lip can serve as a muscle-targeting liposome for DMD and may be a useful for its treatment (Sasaki *et al.* 2021). Bubble liposomes are PEG-modified liposomes which consists of DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and DSPE-PEG2000-OMe (1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-polyethyleneglycol). The bubble liposome along with ultrasonic exposure increases the uptake of PMO and hence increases PMO-mediated exon skipping. This results in significantly higher dystrophin expression. This suggests that the combination of bubble liposomes with ultrasonic exposure can be used for PMO delivery which helps in treatment of DMD and other muscle dystrophies (Koebis *et al.* 2013; Negishi *et al.* 2014).

Previous research found that in addition to muscular dystrophy, immunological inflammation is caused due to inflammatory cell invasion, such as T-lymphocyte markers (CD^{8+}/CD^{4+}). Inflammatory mechanisms play a significant role in muscle fibrosis in people with muscular dystrophy. In this context as well, non-viral carriers have been used to enhance the delivery.

For example, as compared with the nandrolone control drug, the loaded nanolipodendrimer (a class of nanocarriers where the dendrimer is connected with a liposome consisting of cholesterol, phosphotidylcholine, and dimethyl dioctadecyl ammonium bromide) with the candidate drug induced a considerable gain in muscle mass, decrease in CD^{4+}/CD^{8+} inflammatory indicators, and no notable toxicity. This suggests that this nanolipodendrimer can be an effective technique for treating muscle degeneration and muscle dystrophy (Afzal *et al.* 2013). Methylprednisolone hemisuccinate (MPS) is a glucocorticoid-based compound which has anti-inflammatory and immunosuppressive properties and is used to treat various pathological conditions including autoimmune disease, allergic reactions, etc. The nanoliposomes loaded with MPS in short- (4-weeks) and long-term (58-weeks) therapy in the DMD mouse model improves muscle strength and mobility (Turjeman *et al.* 2019; Al-Hakkani 2023). Gentamicin (GM) belongs to a class of aminoglycosides which has the potential to enable the ribosome to read through the nonsense mutation and restore normal translation of the dystrophin protein by avoiding premature termination of protein synthesis. Hybrid liposomes (HL) consisting of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(23) lauryl ether ($C_{12}(EO)_{23}$) together with GM show higher accumulation in muscular dystrophy (*mdx*) mice compared with normal mice. GM-HL results in an increase of dystrophin-positive fibers along with a reduction of cytotoxicity (Yukihara *et al.* 2011). In the DMD animal model, the administration of Cas9 RNP and donor DNA through lipopeptide-based NPs resulted in dystrophin protein restoration and increase in muscle strength (Zhu *et al.* 2023). Lipid nanoparticles (LNPs) have been used to deliver Cas9 mRNA and sgRNA in

DMD patient myoblasts and can induce exon skipping (Kenjo *et al.* 2021). In 2010 and 2011, a phase 1 clinical trial was done for GNE gene therapy using both intramuscular and intravenous injections of GNE lipoplex (consisting of DOTAP, cholestrol and GNE gene) (Nemunaitis *et al.* 2010, 2011).

3.3 *Polymer-based non-viral vectors*

Carrier systems comprising cationic polymers have the extra benefit of forming smaller homogeneous particle sizes, resulting in higher transfection efficiency. Negatively charged nucleic acids tend to compress and pack in cationic polymers (Boussif *et al.* 1995). The first cationic polymer studied for DNA transfection was poly-L-lysine (PLL) (Wu and Wu 1987). Later, linear poly-ethylenimine (PEI) as well as different branched and modified variants with different levels of protonation have been used extensively as well. PEI has better transfection efficiency due to the buffering capability of numerous amino groups (Boussif *et al.* 1995; Wahane *et al.* 2020). Multiple biodegradable polymers have been designed and synthesized for a large number of applications. Some polymers used for gene delivery in RMDs are highlighted in table 2. Hyper-branched poly(ester amine)s (PEAs) were used to deliver plasmids into mouse muscle cells (C2C12) and dystrophic muscles (Wang *et al.* 2012) with minimal toxicity. Furthermore, research has shown that nanopolymers PEG-PEI and PLGA-encapsulated PEG-PEI facilitate ASO delivery in *mdx* mice and mediate exon skipping (Williams *et al.* 2008; Sirsi *et al.* 2009; Colapicchioni *et al.* 2022). In addition, polymethyl methacrylate PMMA/N-isopropyl acrylamide (ZM2) NPs were employed by Ferlini *et al.* 2010 as ASOs delivery vectors into *mdx* mice skeletal muscle (Colapicchioni *et al.* 2022). *Ex vivo* gene therapy is a promising treatment option for DMD because myoblast transplantation in primates can be an effective strategy. The low delivery efficacy of large DNA constructs in human primary myoblasts is one impediment to this therapy. However, hybrid viral–non-viral systems are being used to overcome this limitation. For PEI adenofection, the DNA is first complexed with PEI, and then these complexes are linked to an adenovirus. This helps in the delivery of large-size plasmids. DNA attachment to adenovirus with the use of polyethylenimine (PEI) helps in the delivery of 12 kb pMDysE large plasmids into human primary myoblasts (Campeau *et al.* 2001).

In some cases, muscle targeting is achieved by conjugating the polymer with other moieties. Using phage display screening it was found that the M12 (RRQPPRSISSHP) peptide predominantly binds to muscle cells compared with other organs (Gao *et al.* 2014). As a result, it has now been employed to modify polymers so that they can specifically bind to the cell surface receptors of muscle cells. Phosphatase and tensin homolog (PTEN) inhibitors were delivered into dystrophic *mdx* mice using poly(lactide-*co*-glycolide)-*b*-poly(ethylene glycol) nanoparticles (PLGA-PEG NPs) conjugated with the muscle-targeting peptide M12 (Huang *et al.* 2020; Colapicchioni *et al.* 2022). Other new systems based on the modified G5 polyamidoamine (G5 PAMAM) dendrimer–DNA complex have been developed which can enable cell-specific targeting to skeletal muscle cells and transport of DNA via different components of the intracellular machinery. The skeletal muscle-targeting peptide (SMTP) with peptide sequence ASSLNIA is known for its specificity towards muscle cells. G5 PAMAM conjugated with SMTP (G5-SMTP) along with a dynein light chain 8 protein (DLC8)-binding peptide (DBP) for intracellular transport can enhance targeted gene delivery efficiency (Jativa *et al.* 2019). DBP (CHHHKKKKETQTKKKHHHC) along with a nuclear localization sequence (PKKKRKVEDPYC) and microdystrophin plasmid DNA (Dys) can form a polyplex with G5-SMTP, and its administration has been viewed as a therapy option for patients with the disabling DMD condition (Hersh *et al.* 2021).

3.4 *Inorganic non-viral vectors*

The 'BALL' porous silica nanoparticle, which enhanced serum stability and bioavailability, allows safe and direct intracellular Cas9 RNP delivery. Furthermore, a localized injection of the Cas9 RNP-BALL complex into the muscle, which targets the myostatin (*MSTN*) gene known to prevent muscle growth, achieved successful knockout of the *MSTN* gene, leading to an increase in muscle mass and improved motor functions (Chae *et al.* 2022). Rapamycin-containing perfluorocarbon (PFC) inorganic NPs were recently employed to control autophagy in the DMD mouse model. Intravenous treatment of rapamycin-loaded PFC NPs enhanced

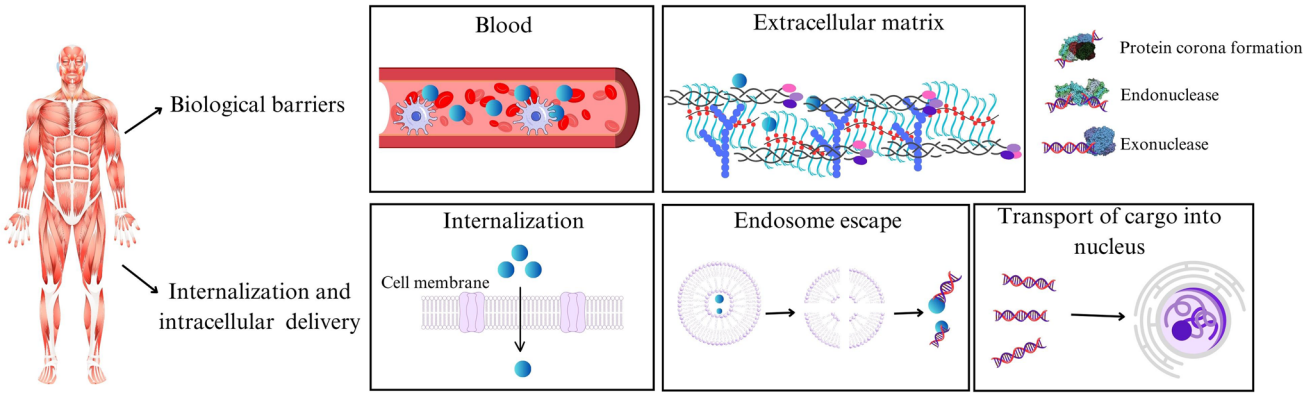

Figure 2. Challenges for gene delivery to muscles.

muscular function and regulated autophagy response more than oral preparations (Bibee *et al.* 2014). Intramuscular injection of gold NPs containing a Cas9/sgRNA RNP and donor DNA results in the acquisition of fully functional dystrophin protein using HDR mechanisms (Lee *et al.* 2017).

4. Future perspectives and challenges

Although gene therapy has considerable potential for the treatment of RMDs, one key hurdle is the transport of these components to skeletal muscle tissues.

As discussed above, the success of gene therapy strategies for RMDs depends to a large extent on the nature of the delivery system. Several viral-based systems have not only been used in the literature for gene delivery relevant to RMDs but have also been translated into clinical trials and some have even arrived in the market. However, prohibitvely high cost, problems associated with possible immune reactions, as well as limited packaging capability are causes for concern. Therefore, there is a strong need to develop appropriate non-viral systems for this purpose. Several biological and pharmacological hurdles exist for non-viral vector distribution to skeletal muscles. Biological barriers consist of the intricate architecture of the skeletal muscle, which includes the skeletal muscle parenchyma, connective tissue, blood vessels, and nerves. One of the most significant barriers to delivery to skeletal muscles is the presence of a thick extracellular matrix (ECM), which accounts for 1 to 10% of muscle mass (Gillies and Lieber 2011; Yhee *et al.* 2017; Sleboda *et al.* 2020). This impedes NP penetration by holding them in the ECM via electrostatic and mechanical interactions using different fibrous-forming proteins (collagens, glycoproteins, proteoglycans, and glycosaminoglycans) (Stylianopoulos *et al.* 2010; Engin *et al.* 2017). A simplified diagram outlining the biological barrier components is available in figure 2.

Since non-viral vectors may be retained at the ECM, particularly in advanced stages of the disease, the challenge of accessing injured muscle fibers needs to be carefully addressed in preclinical research (Yhee *et al.* 2017; Sleboda *et al.* 2020). Surface-engineered nanocarriers can be used to enhance interaction of nanocarriers with muscle cells (Ebner *et al.* 2015). Gene therapy for RMDs uses a variety of non-viral vectors, each of which has benefits and drawbacks of its own. Surface modification can easily be done in case of liposomes and polymers; for example, modification of liposomes and polymers with muscle-targeting peptides increases their specificity towards muscle (Hersh *et al.* 2021; Sasaki *et al.* 2021). Some properties of the peptide-based vector can be enhanced by adding oligopeptides; for example, A2G80-R9-H8 was created by combining the A2G80-R9 peptide with a functional octamer histidine to increase endosome escape capacity (Nirasawa *et al.* 2021). In addition, pharmaceutical barriers include formulation and related challenges of scaling up nanomedicine products. Formulation strategies based on scalable processes have recently been developed to enable the application of nanomedicine in an industrial setting (Đorđević *et al.* 2022; Xu *et al.* 2022). In addition to these obstacles, NPs must be biocompatible in order to avoid further muscle degeneration in severely damaged skeletal muscles. For example, in DMD patients, the sarcolemma membrane is badly damaged, making any treatment more likely to cause injury (Ebner *et al.* 2015; Andreana *et al.* 2021). Along with extracellular barriers there are some intracellular barriers for the delivery of nucleic acids including nanocarrier internalization, endosomal escape capability, etc. (Karlsson *et al.* 2020). In order to enhance the delivery efficacy of the non-viral vectors towards muscle, they can

be chemically modified. A PEI modification with xylitol increases its endosome buffering ability, which in turn enhances its transfection efficiency in muscle tissue compared with only PEI (Lee *et al.* 2014).

While different approaches of gene therapy are being followed in these diseases including gene augmentation and exon skipping, the ideal situation of gene correction through genome editing is being pursued to a large extent.

Initially, ZFNs (Ousterout *et al.* 2015) and TALENs (Ousterout *et al.* 2013) were employed to delete splicing regions from mutant genes and functional gene production. Currently, CRISPR has been studied in conjunction with a specialised DNA endonuclease protein called Cas9, targeting DNA sequencing, to restore genetic mutation. Cas9 nuclease requires a single-guide RNA (sgRNA) to form a complex with DNA by recognising a specific 20-bp DNA sequence known as the protospacer. The protospacer sequence is immediately followed by a short sequence known as the protospacer-adjacent motif (PAM), for which Cas9 need to begin DNA cleavage and genome editing (Ousterout *et al.* 2013; Doudna and Charpentier 2014; Min *et al.* 2019; Eslahi *et al.* 2023). The gene editing strategy has several drawbacks, including poor efficiency in deleting numerous exons, inefficiency in delivering the CRISPR-Cas9 system *in vivo*, the possibility of repeated doses, and the off-target effects of Cas9 activity (Ramos and Chamberlain 2015), and immunity against Cas9 (Crudele and Chamberlain 2018; Kwon *et al.* 2020). However, CRISPR-based therapy has been successfully used to modify muscle stem cells (Nance *et al.* 2019). A single-patient clinical trial was done using CRD-TMH-001, CRISPR-based exon skipping for the treatment of DMD. However, the sole patient enrolled in the trial passed away during the trial; the cause of death was stated as severe ARDS (acute-respiratory distress syndrome) with diffuse alveolar damage, a possible toxicity effect due to the high-dose recombinant-AAV gene therapy (Philippidis 2022b; Bhokisham *et al.* 2023; Lek *et al.* 2023). Further development is needed in order to treat rare muscle disorders using the genome editing method.

5. Conclusion

Non-viral vectors, as outlined in this review, show great potential for the development of effective and safe RMDs treatments. Exon skipping, gene augmentation, and gene editing have emerged as major approaches for the development of RMD therapies (Mitrani Rosenbaum *et al.* 2012; Zhu *et al.* 2017; Duan 2018b; Min *et al.* 2019). Currently, mostly viral vectors are available for therapeutic purposes in order to treat RMDs. Clinical translation of non-viral vectors is a protracted and difficult process as it needs to address a number of challenges, including biocompatibility, their interaction with the biological environment (serum proteins and ECM), effectiveness, scalability, cost, regulatory requirements, and intellectual property (Zor *et al.* 2019). Additional advances and modifications are required for nanocarrier development and their clinical approval so that they can offer unprecedented opportunities to RMDs.

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