Review of Nanoparticle-Based Drug Delivery Methods in Conjunction With Antisense Oligonucleotide for the Treatment of Spinal Muscular Atrophy

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1 - Overview of Nanotechnology

1.1 Introduction

Recent studies, experiments, and marketed treatments have brought about the vast benefits of nanotechnology and the hope it brings with it. With rare diseases increasing at an alarming rate, in both the number of diseases and their prevalence, now more than ever, effective treatments are needed to combat this unyielding battle. Nanotechnology provides practical solutions to lingering obstacles in the healthcare industry, enabling further progress in treating rare diseases.

Despite recent advancements in the field, nanotechnology is considered relatively new, with modern nanotechnology emerging in 1981, especially in medicine. A common consensus, nonetheless, is that despite sparse knowledge of the field, nanomedicine offers a surplus of benefits that have the potential to revolutionize the field of medicine. This work aims to review and summarize some of the most promising nanotechnology applications in the medical domain of rare diseases, specifically Spinal Muscular Atrophy (SMA).

1.2 Benefits in Medicine

Nanomedicine comprises devices and nanostructures for diagnosing, curing, mitigating, and treating diseases [1]. However, the most crucial aspect of nanomedicine is that nanoparticles can reach once-thought-inaccessible places of different biological systems, including the central nervous system (CNS), due to their smaller size.

As the name implies, nanomedicine works at a molecular level through various methods, the most effective being nanoparticle-based drug delivery systems. Nanoparticles (NPs) are chemically engineered organic or inorganic particles, also considered nanomaterials. They work by interacting, through the exploitation of their outer surfaces, with the molecular biological environments around them to perform nanoscale processes that accomplish the task at hand [1]. In the case of NP-based drug delivery systems, in most applications, NPs encapsulate the drug, are directed towards the targeted cells and deliver the drug to the targeted site with the controlled release [1]. These features alone make NPs a suitable medium for drug-delivery systems. Aside from drug-delivery systems, nanomedicine can accurately diagnose diseases, repair damaged tissues, conduct gene therapy, and provide immunity (e.g., vaccines) [1]. Nanomaterials have an advantage when it comes to diagnosis over current methods. Through non-viral administration, they can provide more accurate diagnoses through an environmental sensor. NPs modified with an environmental sensor relay the patient's inner body to the outside environment (e.g., MRIs), a task that invasively-administered methods of diagnosis cannot entirely achieve. Nanodevices can repair targeted tissues and cells without negatively impacting the surrounding biological environment (e.g., tissue nanotransfections). Along with tissue repair, nanomedicine also provides genetic treatments through chemically-modified nanoparticles. One such example is CRISPR, a chemically-modified protein whose original purpose by one's cells is to help form RNA transcripts and was repurposed for genetic editing. Nanoparticles have also been implemented into vaccines because of their advantages as a drug-delivery system. Their profound ability, in this case, is their protection of RNA from nuclease degradation, a focus of this review. Nanoparticles have been implemented within vaccines and administered on a global

scale; the Pfizer and Moderna SARS-CoV2 or Covid-19 vaccines are manufactured with lipid-nanoparticles (LNPs) for RNA protection from nuclease degradation and increased immunity [2, 3].

1.3 Types of Nanoparticles

Nanoparticles have an extensive database comprising two categories: organic and inorganic NPs. Organic NPs originate from within a biological system, such as protein-nanoparticles (PNPs), while inorganic NPs are particles originating from external elements such as gold nanoparticles (AuNPs). While the studies have shown the usage of inorganic NPs to report better overall efficacy over organic NPs, cytotoxicity due to metallic poisoning and low biocompatibility/biodegradability seems to be the primary factor limiting their usage. After reviewing multiple studies, it can be concluded that organic NPs are the better choice due to their low cytotoxicity, higher biocompatibility and biodegradability, and efficiency, despite their lower efficacy.

There are multiple types of organic NPs, including, but not limited to, polymer NPs, LNPs, PNPs, peptide NPs, liposomes, polymerases, exosomes, and dendrimers [4]. Each nanoparticle has its trade-offs and nuances for specific use cases, and multiple in-vitro/in-vivo studies have been conducted with these NPs. The most prominently studied organic NPs have been LNPs, peptide NPs, and PNPs. In terms of efficiency and efficacy, specifically in CNS-focused drug-delivery systems, polymer NPs, peptide NPs, and exosomes appear to be the best choices.

NP-based drug-delivery systems are created so that NPs would act as a carrier that makes up for the weaknesses of the drug, such as low solubility, poor cellular uptake, or intercellular trafficking, and increase its effectiveness. They are also advantageous for their protection from nuclease degradation, controlled drug release, site targeting specificity, and intercellular delivery. However, NPs can also overcome a significant deficiency of modern pharmaceuticals - accessing the CNS. Only 1% of modern pharmaceuticals can reach the CNS because of their inability to cross the blood-brain barrier (BBB) and brain-cerebrospinal fluid (BSCFB).

The BBB and BSCFB are biochemical membranes in the brain that are tightly packed endothelial cells with neuron pericytes and astrocytes [4]. These membranes allow nutrients and small lipid-soluble molecules to pass through while blocking almost everything else [5]. The difference, however, between these membranes and cell membranes is that these have tight junctions, specific transport and carrier proteins, and low rates of fluid-phase endocytosis [6]. NPs, such as the ones listed above, can break through these membranes and effectively deliver pharmaceuticals to the CNS without any chemical changes being done to the drug itself, which could affect its therapeutic effect.

As mentioned before, polymer NPs, peptide NPs, and exosomes are the best options for crossing the BBB and accessing the CNS due to their specific qualities.

Polymers are substances containing large sequences of macromolecules joined together, both natural and synthetic. Synthetic polymers originate from labs, including polyester, nylon, and Teflon, while natural polymers include nucleic acids, proteins, lipids, and carbohydrates. Polymer NPs are natural polymers that originate from living organisms and possess significant advantages in creating an effective drug-delivery system. One specific advantage is the versatility of their properties. They have varying natural compositions, as mentioned above, different releases and degradation abilities, and easy manipulation of their outer surface. They

offer long blood-circulation time, controlled release of pharmaceuticals, and good loading capacity [7, 8]. In addition, when modified with GLUT1-ligands (Glucose), they are very effective in permeating across the BBB due to the GLUT1 receptor proteins on its membrane [9]. Exosomes are derived from extracellular vesicles from cells used for cellular communication and the transportation of materials. Due to this, exosomes are incredibly effective in transporting pharmaceuticals between and in cells. Their specific advantages are their easily manageable surfaces, high biocompatibility and biodegradability, complete non-cytotoxicity, and originating solely from cells. As a carrier, exosomes show the most potential in carrying most pharmaceuticals, primarily due to their ability to resist nuclease degradation, penetrate the BBB, and deliver the drug with site-specific precision. Targeting moieties can be modified upon the surface of exosomes and have been shown to increase mRNA delivery into the BBB to suppress tumors [10].

Peptide NPs are amino acids that are bonded together by covalent peptide bonds. Peptides are molecules made up of amino acids joined through dehydration synthesis and helped to form ribosomes. They are an efficient delivery system due to their size, low cytotoxicity, high ability to reach the targeted sites, and transcapillary delivery of bio-cargoes [11]. Peptide NPs have shown high efficiency in crossing the BBB, and studies have shown tumor-suppressing functions in brain gliomas. Chemical modifications can be made upon the surface of peptide NPs with ligands to perform vital communication (through signals) with cellular membranes.

Nanoparticles serve as excellent potential candidates for NP-based drug delivery systems.

1.4 Strengths of organic NP-based drug delivery systems

One of the most significant advantages of NP-based drug delivery systems is that they are non-invasive. Most drugs designed for neurodegenerative diseases are administered through invasive methods, usually requiring intracranial or intrathecal injections, both of which can present severe limitations to patients and weaken them. Patients present numerous side effects, such as infection, edema, and neuronal damage [12]. Though these invasive methods are effective, non-invasive methods can offer equal or even better effectiveness without the excess burden on the patients and lessen the chances of adverse side effects.

Besides acting as a more effective vessel in medicinal delivery, NPs are incredibly advantageous in making up for the weaknesses the drug themselves lack. They have high malleability, which makes it easier to load the drug into NP, resulting in the ease of manufacturing. Chemical modifications increase the productivity and strength of the nanoparticle, and its small size acts as a natural carrier that escorts the drug to nearly inaccessible places to reach, such as the CNS [13]. Along with this, they have high biocompatibility with biological systems, high biodegradability, flow density, cellular penetration and uptake, and make up for solubility— all desirable properties in the context of drug delivery. Additionally, they outperform conventional drug delivery mechanisms with their ability to restore cytoarchitecture and connection patterns in CNS disorders. Nanocarriers exhibit the ability to absorb proteins, interacting with the BBB endothelial cell receptors, allowing them to cross the BBB without harming the barrier itself. Nanocarriers have significant potential concerning their efficiency as an NP-based drug delivery system, specifically towards their positive effects on pharmaceuticals [4, 10, 14].

1.5 Toxicology

While nanoparticles possess properties that are significant towards providing effective solutions to ongoing treatment problems, there are still questionable concerns that plague its progress.

Nanoparticles are abundant in every part of the human body. Nanoparticles are cells, vesicles, proteins, and nucleic material. On the other hand, Engineered nanoparticles align more with the definition of "nanotechnology." Despite nanoparticles' non-toxic properties within our living organisms, once engineered, they can exhibit toxic properties and be cytotoxic in living organisms.

As aforementioned, nanotechnology is a novel platform, and there is limited research and knowledge, resulting in several unknowns which need to be explored before utilizing it widely in medicine. The concerns of nanotechnology are the potential for toxicity, atypical immune responses, and the implications of the cost and manufacturing processes. Organic NPs engineered in a lab, usually through chemical modification, have raised toxicity concerns, despite originating from biological systems. The click-down within this realm of research suggests that cationic charges on NPs are toxic, while neutral and ionic NPs show little to no toxicity. Cationic NPs, especially LNPs (regardless of charges), have been shown to activate inflammatory responses and aggravate immune responses [1, 14]. LNPs and organic NPs can biodegrade, which means that side effects and toxicity might usually exist for a finite amount of time. Currently, inorganic NPs pose the most significant threat to biological systems due to the high toxicity presented in studies. With high cytotoxicity, inorganic NPs have little biodegradability, despite showing biocompatibility (usually due to chemical modifications), meaning that they will continue to exist within biological systems without degrading, which is an incredible danger to one's health.

2 - Antisense Oligonucleotides

2.1 - Applications

Antisense Oligonucleotides (ASOs) are a form of gene therapy that inhibit the viral reproduction of genes [15]. ASOs as potential gene therapies may be a relatively new drug class, but not the concept, as drugs like Fomivirsen were first released in 1998 [16]. ASOs work by penetrating cellular membranes, reaching the pre-RNA, transcribing specific genes to include exons to be transcribed into the RNA, and eventually into mRNA, which is then used to create proteins. Its innovation alone makes it an already highly sought gene therapy. However, when paired with its other strengths, its applications seem limitless in providing potential treatment options for genetic disorders. Some of ASOs' applications include (1) cryptic splicing mutations, which could prove effective in β-thalassemia, breast cancer, and cystic fibrosis [17]. Cryptic splicing refers to splice sites not used in pre-mRNA but spliced due to their mutations in genes. Through their ability to perform this function, ASOs provide a therapeutic effect by cleaving transcriptions consisting of genetic mutations that might otherwise be obscured or left undiscovered. (2) Therapeutic potential for inflammatory diseases, dystrophy, atrophy, anti-apoptosis, and cancers by switching between alternative splicing isoforms [17]. Alternative splicing is a cellular process in which exons in transcribed pre-mRNA are included or excluded to generate mature mRNA transcriptions. (3) Inducing exon inclusion for cancers, spinal muscular atrophy, and Duchenne muscular dystrophy [17]. The induction of exons is significant in acting as a suitable form of treatment for diseases, as they can increase the production of vital proteins lost due to mutations. (4) Correcting the reading frame to allow the production of

internally deleted partially functional proteins in Duchenne muscular dystrophy, spinal muscular atrophy, and dystrophic epidermolysis bullosa [17]. The internally deleted partially functional proteins refer to poorly synthesized vital proteins due to genes incapable of transcribing fully functioning proteins. (5) Induction of reading-frame disruptions to achieve partial protein knockdown in, for example, atherosclerosis and cancer [17]. Similar to correcting reading frames for synthesizing functioning proteins, this application of ASOs enables them to replace naturally degrading, partially-functioning proteins with fully functioning ones.

2.2 - Strengths & Weaknesses of ASOs

ASOs have tremendous potential as gene therapy; however, they are still considered a new drug with even newer technology. As such, they may have the potential to offer beneficial and effective genetic treatment options, but one needs to acknowledge their limitations which may result in low efficacy and effectiveness inside biological environments. Considering the cost of the released ASOs, which will be further discussed in the coming sections, ASOs' limitations could curb their potential as a prospective treatment for genetic disorders.

The strengths of ASOs include (1) High target specificity. ASOs can accurately reach a targeted site through specific conjugations of targeting ligands while exhibiting low toxicity and systemic exposure. Systemic exposure refers to the amount of time that a drug can exist within a biological system before being retained by the immune system. (2) Longer half-life dosing. Through higher circulation within the body, ASOs exhibit longevity in their gene-splicing processes resulting in effective therapeutic effects. Prolonged half-life dosing also results in a lower dose and dosage, which can be especially beneficial cost-wise. (3) Exhibit multiple methods of treatments for the specific downregulation of disease-relevant genes. Due to their several applications, ASOs can be used for many disorders and purposes, and through this, they have efficient gene knockdown and precise gene-splicing. (4) The most significant strength of ASOs is that their effects are reversible [12]. Reversible effects are an attribute not previously demonstrated in most other genetic therapies, and it sets ASOs apart from the rest. This attribute is vital because permanent effects upon the pre-mRNA can result in irreversible side effects and burden upon patients due to their permanent alterations in pre-mRNA.

Despite these strengths, the limitations of ASOs must be acknowledged and weighed against the benefit before considering them as a candidate for gene therapy. (1) Unconjugated ASOs exhibit poor cellular uptake and intracellular trafficking. Cellular uptake refers to molecules interacting with plasma membranes or, in other words, poor cellular permeability. Without this crucial property, ASOs' ability to cross into cells is not guaranteed, meaning they cannot splice pre-mRNA if it cannot reach the proper site. Intracellular trafficking refers to the ability of a drug to travel in the cytoplasm of cells. Without the property of intracellular trafficking, ASOs cannot reach the nucleus to splice pre-mRNA, posing the same access issue. (2) Poor BBB crossing. Most genetic disorders are neurodegenerative disorders, so it is essential that ASOs can reach the CNS, if needed, and access neuronal cells. Poor BBB crossing is not just due to the poor cellular uptake of ASOs but is associated with the strength and extreme selectivity that properties of the BBB have compared to regular specialized cells. (3) Poor endosomal escape. Endosomes are vesicles that transport proteins, lipids, and other materials from the plasma membrane to organelles in the cytoplasm and vice versa. Related to ASOs, they must use endosomes to reach the cytoplasm to reach the pre-mRNA in the nucleus; however, if they cannot influence chemical changes in the endosomes to escape, it results in the ASOs being secreted back outside of the cell. (4) Nuclease degradation, The biggest weakness

of ASOs is nuclease degradation. Nuclease degradation refers to the degradation of foreign genetic material through the immune system. Unlike the weaknesses mentioned above, the ASOs can still reach the targeted site. However, through nuclease degradation, ASOs might be degraded through white blood cells before exhibiting proper retention in blood circulation and reaching the targeted site [10, 12, 15].

In terms of efficacy and efficiency, ASOs exhibit subpar results due to their weaknesses that generally outweigh their strengths. At the same time, there is still the indubitable fact that the weaknesses of unconjugated ASOs act as a liability towards the overall performance of ASOs. However, their reversible effects and alternative splicing concept continue to set them apart from modern genetic therapies.

2.3 - ASOs in Duchenne Muscular Dystrophy and Spinal Muscular Atrophy

While listing out applications of ASOs, its therapeutic effects were most evidently shown upon muscular disorders such as Duchenne Muscular Dystrophy (DMD) and Spinal Muscular Atrophy (SMA). These two disorders are perhaps the most common rare genetic disorders exhibited in infants, with SMA being the leading cause of genetic mortality in infants. The fatality rate of these two disorders is incredibly high relative to the prevalence of the disorders, and the lifespan of the patients is limited to around two years in infants. Due to their status as rare diseases, finding an effective treatment for them can be incredibly difficult, but new treatment avenues have opened due to new technology leveraging ASOs.

Two treatments, Nusinersen and Eteplirsen, were released for SMA and DMD, respectively. While specific weaknesses do plague the overall efficacy of the unconjugated ASOs, they serve as suitable candidates for these muscular disorders. Despite having two different genetic targets, their treatment processes are nearly identical: antisense-mediated exon-skipping therapies. SMA and DMD patients experience genetic mutations that induce partial functioning proteins; SMA is the lack of survival motor neuron proteins that inhibits muscular growth. DMD is the lack of dystrophin proteins while strengthening muscle fibers and protecting them from injury through muscular contractions. Antisense-mediated exon-skipping therapies refer to the ability of ASOs to skip (exclusion) or induce exons in pre-mRNA before translation into mRNA, which is used in the Endoplasmic Reticulum (ER) to synthesize proteins. Exons refer to specific parts of a gene that can help the gene synthesize partially or fully functioning proteins through its inclusion or skipping. Nusinersen and Eteplirsen work in this manner. The gene that Nusinersen targets is the copy of the survival motor gene, SMN2, due to a mutation that causes the loss of the original survival motor gene, SMN1, which transcribes fully functioning SMN proteins. Research and studies have shown that including exon 7 within SMN2 increases the full functioning that it transcribes. As such, Nusinersen splice-splits the inclusion of exon 7 with the pre-mRNA transcript before its maturation. Unlike SMA, DMD's gene referred to as the DMD gene, inclusion of exon 51 into the mRNA transcript results in the partial functioning of dystrophin proteins, resulting in a progressive muscular loss. As such, Eteplirsen split-splices the exclusion or skips exon 51 of the pre-mRNA before its maturation resulting in a greater production of functioning dystrophin proteins [11, 12, 15, 17].

Studies have shown positive results in the production of fully functioning SMN or dystrophin proteins has increased; however, there are still side effects and burdens that need to be addressed. As aforementioned, their reversible effects lighten the concern of their side effects, but not patient burdens, as they assure patients and examiners that they will not last for long.

3 - Spinal Muscular Atrophy

3.1 - Rare Diseases

Despite their deceptive name, rare diseases affect over 300 million people worldwide, with approximately 7,000 diseases existing today, of which 80% percent is genetic. Compounded with the fact that they are difficult to quantify at scale, the severity of rare diseases is also typically underestimated. Rare diseases are challenging to treat for multiple reasons: they are difficult to diagnose, they cannot be classified objectively, and there is a sparse understanding of pathology and progression [18]. The biggest issue concerning rare diseases is their frequently changing and geographically-varying classification criteria. Each country has its guidelines that are relative to its population size. For example, the United States, with a population of 356 million people, has stated that any disease with a total prevalence of fewer than 200,000 cases is considered rare. However, the United Kingdom, with a population of approximately 37 million people, has set any disease with a total prevalence of fewer than 33,000 cases as a rare disease. This stark contrast in "prevalence limits" will subside numerous diseases considered rare in the United States as to their classification in the United Kingdom. As a result, it is difficult to find universal cures, studies, or research on the most prevalent rare diseases. In addition, finding a cure/treatment for rare diseases is extremely difficult due to: low prevalence, patient isolation, limited research literature, and lack of financial motivation for pharmaceutical companies [18]. Low prevalence is the most significant obstacle in finding effective treatments for rare diseases since it results in patient isolation, limited understanding, and low financial contributions toward them. Patient isolation refers to patients feeling isolated from their long diagnostic processes and lack of understanding/support from scientists and the public. The limited knowledge stems from low prevalence, lack of studies, and improper diagnostics of these diseases. Additionally, such a wide breadth of rare diseases makes it difficult for scientists to understand any one disease deeply.

Focus on rare diseases began to increase attention when President Reagan, in 1983, signed the Orphan Drug Act in response to a lack of medical research and attention to rare diseases. Orphan drugs are pharmaceuticals produced to treat rare medical conditions in which the lack of prevalence would cause the drug to be unprofitable. This act was a significant milestone in the rare disease treatment market as it incentivized the private sector to increase research in rare disease treatments by offering government assistance. Since its approval, 600 orphan drugs have been released over the past 40 years, including ASO drugs such as Nusinersen and Eteplirsen [18]. Moreover, the government established the National Organization for Rare Disease (NORD) as a platform for research and information into rare diseases. The US National Institute of Health (NIT) and Food and Drug Administration regulate the training and guidance to improve the quality and marketing of NIH-funded rare disease orphan drugs. Over the recent decade, the rare disease treatment market (RDTM) has been exponentially increasing. As of 2019, the RDTM has exceeded $144.3 billion and is expected to grow by at least 12% over the coming years, with the US spending the most on treatments. Despite no cure, these efforts have not gone to waste, especially with the rise of orally-administered treatments. With a market share of $80 billion in the RDTM in 2019, orally-administered treatments are becoming the preferred medium of administration for rare disease treatments [18]. Their significance compared to invasively administered treatments is becoming more recognized. Such benefits include, but are not limited to, increased safety, higher patient compliance, ease of ingestion, and significantly lowered chances of pain.

Scientists have already developed an effective orally-administered treatment for Fabry disease, a rare genetic disease, that has proven to exhibit both high efficiency and efficacy in treatment.

However, even with all these efforts, 70% of all rare disorders remain without treatment. Effective treatment is desperately needed, with most rare disorders classified as fatal and approximately 60% developed in childhood [18].

3.2 SMA Background

Spinal Muscular Atrophy (SMA) is a rare autosomal recessive genetic muscular disease. It is considered the leading cause of genetic infant mortality rates worldwide and the 7th most common rare disease in a survey of 100,000 people worldwide [19]. SMA is a highly severe disease with a moderately high prevalence, mostly in children. SMA has four types defined by motor milestones patients can achieve [20, 21]. Types I - III are the ones that will be discussed within this article, as they are the deadliest and affect the more significant population.

SMA Type I is the most common type of SMA and the most fatal. With a prevalence of 1 in 6,000 to 10,000 neonates, type I has the highest mortality rate and primarily affects infants at birth. Type I patients display overall muscular weakness and hypotonia, low limb movements, lack of tendon reflexes, fasciculations, swallowing and feeding difficulties, and impaired breathing around 6 months of age. They never gain the ability to sit upright by themselves, and for most of their life, they are situated with a respirator. Their expected lifespan is less than 2 years, though modern clinical care has improved this statistic. Some cases have shown SMA type I exceeding this age and reaching ages up to 5, but it is rare without treatment. Eventually, most infants with type I SMA die due to their rapid progression of muscular weakness, leading to respiratory failure [20].

SMA Type II still mainly develops within infants, with onset around 6-18 months after birth. While prevalence is not as high as type I, the expected lifespan remains short and has a high mortality rate. Patients will still experience respiratory difficulties and may require ventilation, but they can sit upright without support from others. Most patients have a lifespan into young adulthood/adolescence [20].

SMA Type III is developed in children after 18 months of birth, but patients usually retain the ability to walk independently. However, most other milestones cannot be achieved adequately without support, including running, standing up, or climbing, since leg muscles are affected first. Multiple complications often accompany this disease, including scoliosis, joint problems, muscular weaknesses, and respiratory infections. However, most patients can expect an average lifespan [20].

SMA patients all lack a common attribute: the survival motor neuron gene (SMN1). This gene provides transcriptions to synthesize survival motor neuron proteins (SMN), which are responsible for maintaining motor neurons. Without proper expression of the SMN1 gene, the body, mainly in the spinal cord, lacks functioning SMN proteins leading to the degeneration of motor neurons and poor motor movement. Though all SMA patients have an identical copy of SMN1, known as SMN2, its transcript results in poorly synthesized SMN proteins, with only about 10% fully functioning. As a result, SMN2 cannot synthesize enough proteins needed for normal motor neural movement resulting in SMA. Researchers and studies have shown that splicing pre-mRNA transcripts to include exon 7 within the SMN2 genes increases the output of fully functioning SMN proteins by approximately 50-60%, serving as the basis for treatments for SMA [11, 20, 21 22].

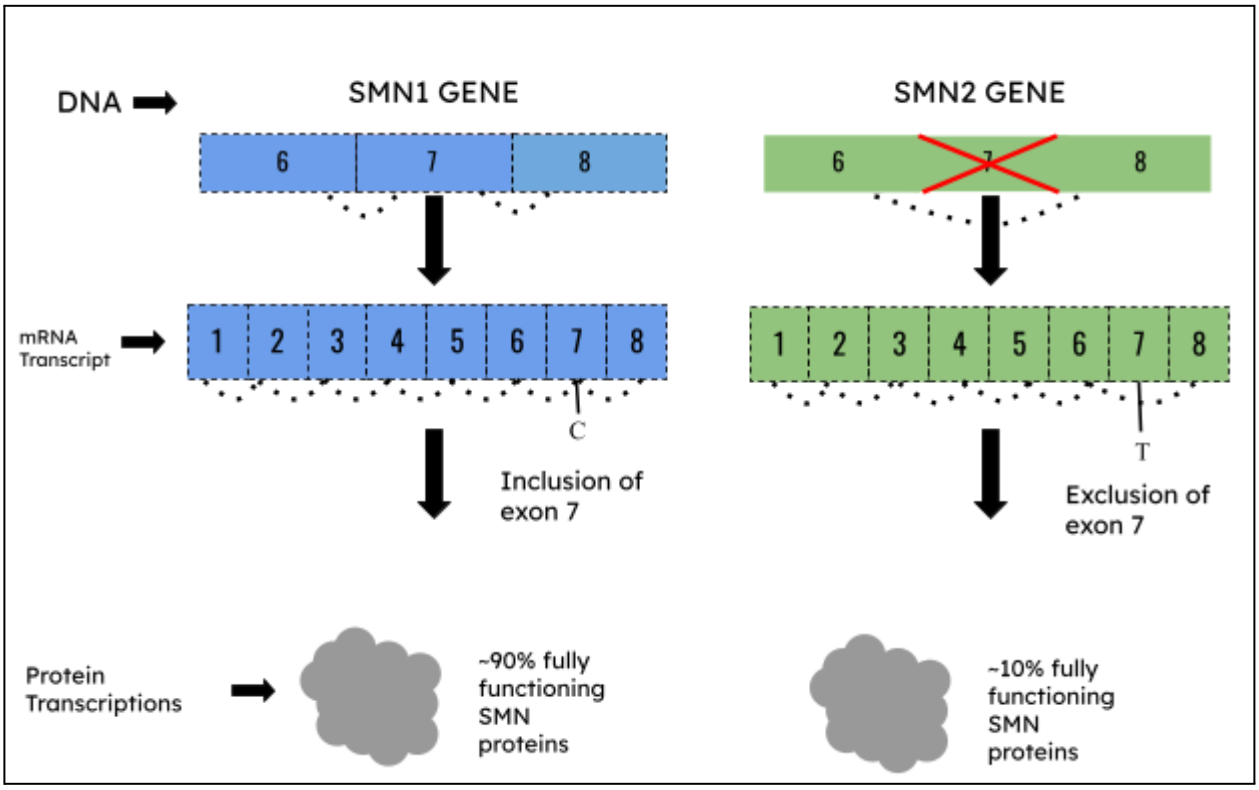

Fig. 1 - SMN Protein Synthesis Outputs From SMN1 And SMN2

3.3 SMA Diagnostic Methods

SMA is a rare disease, but despite its low prevalence, multiple methods exist to diagnose one with SMA, including the PCR-RFLP method, blood tests, genetic testing, nerve conduction testing, and muscle biopsies. PCR-RFLP stands for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method— it works by digesting the PCR based on the digestion of PCR amplicons with appropriate restriction enzymes to produce distinct polymorphic fragments used as markers for species identification [23]. Along with this method, blood tests are accurate enough to act as a marker for SMA. Blood tests pick up samples of proteins and enzymes and check for creatine kinase. Deteriorating muscles release this chemical, acting as a marker for muscular disorders. However, this chemical merely indicates muscular disorders, whereas other methods are more accurate in diagnosing one definitively with SMA. Lastly, genetic testing is a blood test specifically focused on nucleic acids. With its 95% overall efficiency, genetic testing can find mutations, deletions, or alterations with the SMN1 gene [20]. The nerve conduction test is an electromyogram that directly scans the nerves within muscles. The test measures neural activity, and, as such, lower neural activity within muscles indicates SMA. Muscular biopsies are periodic tests in which examiners remove a tiny piece of muscle and have a lab analyze the sample for any muscle loss or atrophy. In almost all cases, except for muscular biopsies, the cost is not a significant issue for these tests, and they are easily accessible [24]. In addition, genetic testing, with an average efficiency of 95%, confirms SMA diagnosis and, in some states, is part of the routine screening process for newborns.

3.4 Treatments

Besides Nusinersen, two other treatments have recently been released since Nusinersen's release in 2016. Besides the performance of the drug itself, the main problem is the costs. Ridisplasm, the first non-viral administered treatment of SMA, Zolgensma, a one-time dose invasively-administered Adeno-Associated Virus drug, and Nusinersen, a multiple-dose ASO invasively-administered drug. It is hard to differentiate the effectiveness of each drug due to limited studies of each one, which primarily stems from these drugs being set at an inaccessible price. In terms of material the treatments are made up of, ASOs seem to be the most promising.

Nusinersen seems to have a tremendous impact mainly on SMA types I and II, as they are the deadliest forms of SMA, but they can still be used to treat all forms of SMA. The FDA approved Nusinersen in 2016, however, it remains to be determined how cost-effective the treatment is. Due to the low prevalence of SMA and the cost of manufacturing, Nusinersen is priced extremely high and must be priced lower in order to have a more significant impact on the population of individuals who suffer from SMA, it must be priced lower. Currently, 4 doses are required for the first year, costing a total of $750,000. Afterward, a maintenance dose is required every 4-8 months till the patient's death, which is $375,000 per dose [12, 20, 21]. Zolgensma was released in 2019, and though significantly cheaper than Nusinersen (in terms of the overall cost) and intravenously administered, its high cost still needs to be justified. The one-time dose will cost the patient $2.1 million, with results of improvement not completely guaranteed [25]. Lastly, Risdiplam, the first at-home, orally-administered treatment of SMA, is cheaper and has demonstrated strong potential since its 2021 US FDA approval. Despite being incredibly expensive compared to most drugs, Risdiplam still maintains the lowest cost of all current SMA drugs on the market, at an annual price of approximately $100,000 and a maximum annual price of $340,000 (cost varies upon weight) [26]. However, progress is being made. Aetna, a private healthcare insurance company, has recently launched a new program with full coverage for these three genetic therapies, significantly lowering the burden of these high prices. Without effective healthcare, people affected by SMA may not be able to access any of these therapies, leaving them to rely solely on clinical care.

Despite the cost-effectiveness of these drugs being justified, from a company standpoint, their impact will be limited to specific populations without lower prices. Through speculation, nanotechnology cannot only increase the efficacy of these drugs but increase their cost-effectiveness. By offering non-invasive methods of administration (Nusinersen and Zolgensma), intrathecal and intracranial injections would no longer be necessary and would offer hope to patients who cannot afford high-cost treatments. In addition, nanotechnology provides reassurance for increased effectiveness of the drugs with fewer and milder side effects and greater access to biological systems (CNS) that are crucial for treating SMA.

4 - NPs in Conjunction with ASOs for SMA Treatment

4.1 Strengths

Organic nanocarriers are excellent drug delivery systems for ASOs, especially for treating SMA. NPs can protect ASOs from harsh biological environments and nuclear degradation while improving biocompatibility, cellular permeability, and uptake [10]. The purpose of ASOs is to splice pre-mRNA to transcribe higher levels of fully functional proteins. In turn, ideal NPs should provide safer transport to the cells and make up for the weaknesses that ASOs exhibit. The most vital aspect of NPs is their ability to better ASOs in terms of efficiency. Their attribution of

targeted delivery and controlled release allows for greater distribution of ASOs with increased accuracy of the drug. Due to their small size, nanocarriers can also act as effective transports within cells, increasing their ability to reach the nucleus.

Another significant advantage is that NP-based drug delivery systems can reach the CNS, allowing for non-invasive methods of delivery for ASOs into the patient's body. The need for current treatments to employ invasive administration methods is to access the CNS, primarily blocked by the BBB. Nusinersen, which employs invasive administration through intrathecal bolus injections through a lumbar puncture needle, has reported severe side effects, primarily due to the method of administration [12]. These adverse side effects include headaches, vomiting, pyrexia, respiratory infection, and upper respiratory tract infection, but they vary depending on the type of SMA the patient is diagnosed with [12]. Converting the method of administration to non-invasive would be incredibly beneficial towards the efficacy of the ASO itself and eliminate a significant burden upon patients.

As aforementioned, nanoparticles that show these strengths include exosomes, peptides, and polymers. All three have a common factor: their ability to penetrate through the BBB and access the CNS [7, 10, 11]. Exosomes have shown great potential due to their intercellular trafficking ability, cellular permeability, and effectiveness in crossing the BBB. In addition, they demonstrate high biocompatibility and biodegradability, with targeted delivery and controlled release, which could help to prove exosomes as the most effective organic NPs for drug-delivery systems, not just for ASOs.

Aside from exosomes, peptide NPs exhibit similar potential and, unlike exosomes, have shown efficacy as NP-based drug delivery systems for ASOs. Br-ApoE (K→A) is a form of peptide NPs, considered conjugated P-PMOs, which have been identified as a candidate for NP-based drug delivery systems for Nusinersen. Despite the in-vivo study being conducted on adult mice with SMA, they serve as an effective marker of the significant potential of nanoparticles to benefit, in terms of efficacy, Nusinersen [11]. Evaluation of Br-ApoE (K→A)-PMO resulted in a significant increase in the median survival of the SMA mouse pups from 78 days to two mice surviving to 282 and 290 days, respectively [11]. In addition, Br-ApoE (K→A)-PMO-treated mice exhibited numerous markers of increased health, including increased weight gain and higher muscle strength [11].

Polymer NPs are highly advantageous in chemical modification due to their varying natural compositions, different releases and degradation abilities, and easy manipulation of their outer surface. They have high blood circulation retention, controlled drug release, good loading capacity, and high BBB crossing. Despite all of these strengths, NP-based drug delivery systems, as a whole, exhibit considerable weaknesses.

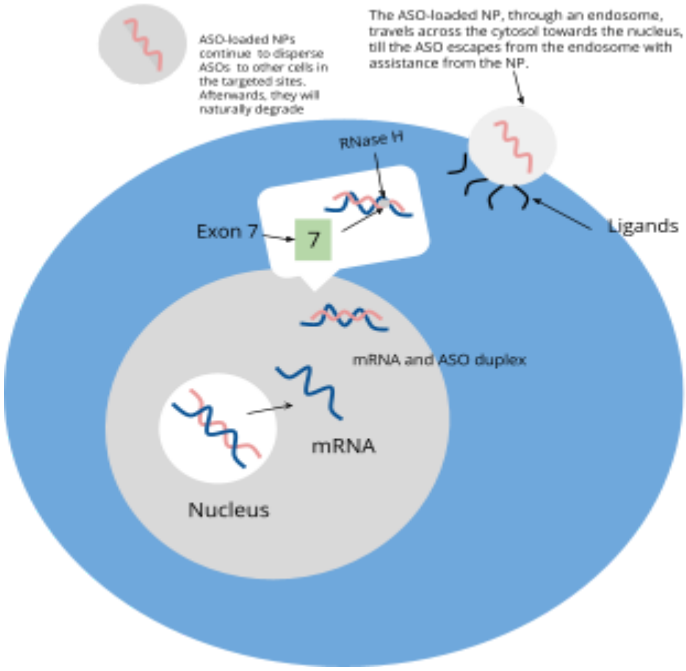

Fig 2. Demonstration of an NP-based drug delivery system for ASO

4.2 - Limitations

While the overall advantages of NP-based drug delivery systems outweigh the current and other systems, their weaknesses should still be assessed. The primary concerns are cytotoxicity and provoked immune responses. It should also be noted that there needs to be more knowledge, mainly due to the lack of in-vivo studies on humans concerning their weaknesses [27]. The full scope of these weaknesses is yet to be understood entirely.

According to current research, organic NPs exhibit cytotoxicity, such as cationic NPs, as aforementioned. However, the effect on one's immune system is yet to be learned. Scientists understand that, especially when carrying foreign nucleic material, including mRNA, it is more than likely that without proper "stealth," an immune response is inevitable. Stealth relates to the ability to remain undetected by white blood cells (WBCs) within an organism through cellular membranes. This property can also be seen within studies conducted for nanoparticles, regardless of whether they are organic or inorganic. Particles that exhibit high retention (not due to low biodegradability) within the blood circulation also have higher properties of stealth [4]. Without stealth properties, nanoparticles will attract an inflammatory and aggravate immune response for the NPs to be eliminated from one's body through the kidneys.

The real issue arises from the side effects of such aggravated immune responses, especially in patients exhibiting SMA, and the burden, if any, on the patients. Despite the low prevalence of this property exhibited within organic NPs, the possibility continues to exist. Another weakness is poor endosomal escape. Though not exhibited within all nanoparticles, it is highly plausible that without proper endosomal escape, similar to that of ASOs, NPs cannot escape vesicles transporting them to reach the cytosol and reach the nucleus to release ASOs. LNPs and exosomes have been shown to combat this weakness heavily, improve endosomal escape within ASOs and increase intracellular trafficking.

4.3 - In-Vivo/In-Vitro in Animal Models

Due to nanomedicine being a new technology, in-vivo studies have been sparse or inconclusive, and there has been a greater prevalence of in-vitro studies. While in-vivo studies consisting of nanoparticles have been conducted with more than promising results, they have mainly been tested on mice or other rodents. Such actions imply that these results may be inaccurate for humans as well. Such as any organism, humans' anatomical structure varies significantly from that of rodents. With such vast anatomical differences, it is to be expected that the results of in-vivo studies on rodents will vary for humans, meaning that nanoparticles that have shown effective BBB crossing in rodents, may not exhibit that same property in humans [27]. The significance of this could be misleading of nanoparticle efficacy and efficiency in humans. To validate the beneficial nature of nanoparticles for treating diseases, elements, such as the one mentioned, must first be studied in detail [27].

That being said, LNPs have recently undergone mass production in vaccinations, becoming the first instance of an mRNA-LNP vaccine. Moderna and Pfizer's SARS-Cov19 (COVID-19) mRNA vaccinations are constructed with LNPs to protect the Covid-19 mRNA from nuclease degradation and increase the vaccine's overall effectiveness against the infectious disease [2, 3].

LNPs were chosen as a drug-delivery system for the mRNA due to their ability to protect the mRNA from nuclease degradation, assist with intracellular trafficking and endosomal escape, and aid in prolonged retention within the bloodstream. LNPs within the vaccines were constructed with a mixture of phospholipids, cholesterol, PEGylated lipids, and cationic or ionizable lipids [3]. These materials act to stabilize and provide structure to the nanoparticle. In addition, LNPs exhibit fusogenic compatible properties, allowing them to fuse with cellular lipid membranes. LNPs can fuse with target cells and effectively release their cargo into the cell cytosol [28].

While this may be the first in-vivo LNP study, liposomes have been used to deliver mRNA cargoes to mice in vivo since as early as the 1990s. Lipidic-delivering agents, such as Lipofectamine, Stemfect, and TransIT-mRNA, were released to assist in in-vitro cell transfection of nucleic materials [15].

The LNPs have proven incredibly effective as natural protectants for mRNA in vaccines; however, side effects have been presented. Though some were mild and others more severe, the exact cause of these side effects is yet to be determined. No studies have been conducted to determine the cause of these local and systemic side effects. Some insist that immunogenic material within the vaccine induces an inflammatory response within the respiratory system, resulting in side effects. However, others claim that introducing LNPs aggravates the immune response, provoking a robust inflammatory response and causing the observed side effects [2].

4.4 - Cost-Effectiveness

Regarding negative implications, NP-based drug delivery may offer a speculative solution to a critical problem with Nusinersen: cost. Despite justified cost-effectiveness for patients, high costs still heavily plague the overall impact of Nusinersen. At approximately $750,000 for the first year of dosage, following annual maintenance doses at $375,000, Biogen claims that this pricing is reasonable due to its classification as an "ultra-orphan drug" and the other due to the rarity of the disease and its sought-out effectiveness. Many orphan drugs have been priced around $500,000, but most insurance companies will not provide coverage due to their high

prices (except for Aetna HealthCare), rendering the treatment inaccessible to specific populations [29]. However, there is some hope for lowering their cost.

NP-based drug delivery systems, when conjugated with ASOs, can lower the frequency of doses and the dosage amount while providing a form of non-invasive administration. Through speculation, this could prove evidence of the ability of NP-based drug delivery systems to lower the cost of Nusinersen, though how much is unknown. Effectively, lowering the frequency of doses could potentially result in overall annual deductions of the drug without altering the cost of a single dose. In addition, lowering the amount of dosage required would lower the cost of manufacturing Nusinersen for a single dose, hence decreasing the cost of the drug. Lastly, preliminary evidence could provide a link between non-invasive administration and lower costs. Despite being a different type of drug, Risdiplam employs oral administration through a liquid form and is priced at a significantly lower cost than all current SMA therapies at an annual cost of ~$100,000-400,000 [26]. Extrapolating this trend further, if Nusinersen were to employ oral or non-invasive administration methods, it could result in a significantly lower cost, essentially eliminating the currently operated methods of administration which are both expensive and risky. As such, the probability that NPs will be conjugated within drugs for mass production can be reasonably assumed to be within the near future, in which, afterward, cost-effectiveness can be measured.

5 - Conclusion

Nanotechnology is an up-and-coming technology with seemingly limitless applications in medicine. The most prominent of its applications lie within nanoparticle-based drug delivery systems for traditional pharmaceuticals, especially for neurological and rare diseases. Spinal Muscular Atrophy, and diseases similar to it, can all benefit from its usage within existing treatments. NP-based drug delivery systems have the potential to provide, in simple terms, protection, increased efficiency and efficacy, access to the CNS, increased dispersion of the drug, and specific targeting. NP-based drug delivery systems have many applications but potentially serve most effectively as mediums for genetic therapies, such as ASOs. A commercial joint venture between ASOs and NPs could potentially prove incredibly beneficial in treating SMA, not just in terms of efficiency but lowering costs and making it safer for the consumers. Nanoparticles, however, face obstacles in achieving mass industrialization of joint ventures with pharmaceuticals: the limited knowledge about them and their potential effects on humans. Regardless, some nanoparticles have already been released to the public for use as drug-delivery systems, as seen in the recent SARS-CoV-2 vaccine released by Moderna and Pfizer. Nanoparticles can apply for their benefits in various methods that could revolutionize current medical treatments and technology. In addition, they potentially serve to effectively provide or innovate upon current treatment for illnesses such as cancer, ALS, Alzheimer's, cardiac diseases, and much more. Nanotechnology can offer plenty of medicine, but until medical researchers fully understand its full extent, it may be some time till they are commercially introduced to the medical market.

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