Advances in Duchenne Muscular Dystrophy gene therapy: Test of BSA nanocapsules for nucleic acid delivery

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“We used to think that our fate was in our stars. Now we know, in large measure, our fate is in our genes.”

— James Watson, 1989

Resumo

A Distrofia Muscular de Duchenne (DMD) é uma doença recessiva associada ao cromossoma X causada principalmente por mutações *nonsense* e de *frame-shift* no gene que codifica a distrofina, uma proteína essencial para a manutenção da integridade da membrana das células musculares. Os doentes de DMD apresentam uma completa ausência de distrofina nos seus músculos. O *skipping* de exões *antisense-mediated* consegue recuperar o quadro de leitura do gene *distrofina* permitindo assim a síntese de distrofina funcional em cultura de células de doentes DMD. Neste momento, o *skipping* de exões mediado por oligonucleótidos *antisense* (AONs) é uma abordagem molecular terapêutica que se encontra em desenvolvimento para a Distrofia Muscular de Duchenne uma vez que é espectável que os doentes de DMD melhorem consideravelmente se os seus músculos forem capazes de produzir alguma proteína distrofina funcional ainda que em baixas quantidades e parcialmente truncada.

A terapia de modelação do *splicing* com AONs direcionados para induzir o *skipping* de exões para corrigir a produção de distrofina encontra-se em ensaios clínicos mas os resultados são limitados. O uso de AONs para media terapia humana ainda apresentam inúmeras barreiras que necessitam de ser ultrapassadas: algumas relacionadas com a farmacocinética, eliminação/acumulação no organismo, entrega-alvo, e a baixa capacidade de atravessar a membrana plasmática.

As nanopartículas tem sido exploradas como veículos de entrega de várias entidades terapêuticas, apresentando vantagens como a possibilidade de dirigir para o tecido-alvo, protecção contra a degradação, melhoramento na dose eficaz e diminuição de efeitos secundários. Estudos recentes mostram um melhoramento significativo na entrega de ácidos nucleicos quando associados a nanopartículas após administração IV em animais experimentais.

Neste projecto, testamos uma nova química de AONs modificados para tratar um modelo de ratinhos DMD através de terapia de modelação do *splicing* para induzir o *skipping* do exão 51, restaurando assim o quadro de leitura e a produção de distrofina nos músculos. Tendo em conta os bons resultados obtidos, colocamos a hipótese que moléculas terapêuticas de DNA terão um *uptake* melhorado nos tecidos-alvo e consequente correcção do fenótipo, se os ácidos nucleicos terapêuticos foram encapsulados em nanopartículas antes de serem administrados.

De forma a testar o efeito do encapsulamento de ácidos nucleicos *in vivo*, um plasmídeo repórter, pEGFP-NLS foi encapsulado em nanocapsulas de BSA para posterior injecção num modelo animal. O objectivo deste projecto consiste em avaliar a eficiência da entrega do gene após administração sistémica das nanopartículas num modelo animal. O nível de expressão da proteína EGFP-NLS foi avaliado em órgãos-alvo dos animais injectados através de análises de imunohistoquímica e através de análise de citometria das células dispersar do fígado e pulmão. O estudo foi complementado com avaliação *in vitro* e caracterização das nanopartículas. São necessários estudos futuros de forma a melhorar o desenvolvimento de nanopartículas desta classe como veículos de entrega controlada de ácidos nucleicos.

Key-words: Distrofia Muscular de Duchenne; terapia genética; *skipping* de exões; *mdx52*, LNA-AON; sistemas de entrega controlada; nanocapsules de BSA; imunohistoquímica; microscopia de fluorescência; citometria de fluxo.

Abstract

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disease caused mainly by frame-shifting or nonsense mutations in the gene of dystrophin, a protein essential for maintaining muscle cell membrane integrity. It was shown in DMD patients a complete absence of dystrophin in their muscles. Antisense-mediated exon skipping can restore the open reading frame and allow synthesis of a partially functional dystrophin in cultured DMD patient derived cells. Currently, antisense oligonucleotides (AONs) mediated exon skipping is a molecular therapeutic approach under development for DMD because it is expected that DMD patients would improve considerably if their muscle cells were able to produce some dystrophin even if it is at low level and even besides it is partially truncated, but functional.

Splicing modulation therapy, with AONs directed to induce exon skipping, to correct the production of dystrophin is under clinical trials but with limited results. AONs mediated human therapy still presents several barriers needed to be overcome: some related to pharmacokinetics, elimination/accumulation in the body, targeted delivery, and the low capacity to cross the plasma membrane unaided.

Nanoparticles have been explored as carriers to several therapeutic entities, presenting advantages such as tissue directed delivery, protection from degradation, improvement of dose efficiency and decrease of harmful side effects. Recent studies show a significantly improvement in nucleic acids delivery when associated to nanoparticles after IV administration in experimental animals.

Here we tested a new chemistry of modified AONs to treat a DMD mouse model, MDX52, by splice modulation gene therapy directed to induce skipping of exon 51 and therefore restore the reading frame and the production of the protein dystrophin in muscles. Regarding the suboptimal results, we hypothesized that therapeutic DNA molecules would have an increased uptake on target tissues, and subsequent correction of the phenotype, if the therapeutic nucleic acid was encapsulated on nanoparticles before being administered.

In order to first test the effect of encapsulation on nucleic acid in in vivo delivery we encapsulated a reporter plasmid pEGFP-NLS in BSA nanoparticles for IV administration on an animal model. The goal of this project was to evaluate the efficiency of gene delivery after systemic administration of the nanoparticles on experimental animals. The level of EGFP-NLS protein expression was accessed on target organs of injected animals by immunohistochemical analysis and by flow cytometry of dispersed cells from liver and lung and the study was complemented with an in vitro evaluation and characterization of the nanoparticles. Further studies will be needed to improve the development of this class of nanoparticles as carriers of nucleic acids for controlled delivery.

Key-words: Duchenne Muscular Dystrophy; gene therapy; exon skipping; mdx52; LNA-AON; controlled delivery systems; BSA nanoparticles; immunohistochemical analysis, fluorescent microscopy, flow cytometry.

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cytometry analysis in approximately 10 000 detected events for each experiment. *****: with transfection agent

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List of Acronyms

DMD	Duchenne Muscular Dystrophy
cDMD	Canine model of Duchenne Muscular Dystrophy
dsDNA	double-stranded DNA
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
AONs	Antisense Oligonucleotides
SFDA	China's State Food and Drug Administration
EMA	European Medicines Agency
AAVv	Adeno-associated viral vector
LPLD	Lipoprotein lipase deficiency
DNA	Deoxyribonucleic Acid
BSA	Bovine Serum Albumin
LNA	Locked Nucleic Acid
DAP	Dystrophin-associated Protein Complex
2OMP	2´-O-methyl-phosphorothioate
PMO	Morpholino Oligomer
NP	Nanoparticle
PLGA	Poly(lactic-co-glycolic acid)
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
PEG	Polyethylene Glycol
PVA	Polyvinylalcohol Polymer
MPS	Mononuclear Phagocytes System
siRNA	Small Interfering Ribonucleic Acid
PMMA	Polymethylmethacrylate
IP	Intraperitoneal
NIPAM	N-Isopropylacrylamide
PEI	Polyethylenimine
FA	Folic Acid
DGL	Dendrigraft poly-L-lysine
PGA	Polyglycolide
DOPS	1,2-dioleoyl-*sn*-glycero-3-phospho-L-serin
IV	Intravenous
LNA-AON	Lock Nucleic Acid Antisense Oligonucleotides
pEGFP-NLS	Plasmid Enhanced Green Fluorescent Protein with Nuclear Localization Signal
Kan	Kanamycin
NLS	Nuclear Localization Signal
FBS	Fetal Bovine Serum
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
DAPI	4', 6-diamidino-2-phenylindole
bp	base pairs
FSC	Forward-scattered light
SSC	Side-scattered light
FL1-H	Green fluorescence channel

Motivation

Duchenne Muscular Dystrophy (DMD), a recessive X-linked disease caused by mutations in the dystrophin gene that lead to deficiency of dystrophin protein in all body muscles.

The majority of DMD cases arise from partial dystrophin gene deletions or duplications or from mutations that affect the normal splicing of the dystrophin RNA transcript into mRNA. Each of these types of gene mutations is a problem when it disrupts the normal dystrophin mRNA open reading frame such that a functional dystrophin protein cannot be produced in muscle cells. An approach to restore the normal mRNA reading frame involves exon skipping. This can be induced by short, synthetic fragments of nucleic acids known as ‘‘antisense oligonucleotides’’ (AONs), which are designed to bind (anneal) with RNA sequences that regulate how a gene’s initial RNA transcript is spliced into a functional mRNA. Antisense oligonucleotides (AONs) have been reported to modulate pre-mRNA splicing in several studies.

Controlled delivery systems have several advantages as compared to the traditional methods of systemic delivery once they protect the drug from rapid degradation or clearance and enhances drug concentration in target tissues, therefore lower doses of drug are required. Cell-specific targeting can be achieved by attaching drugs to individually designed carriers. Recent developments in nanotechnology have shown that nanoparticles (structures smaller than 100nm) have a great potential as drug carriers. Due to their small size, the nanostructures exhibit unique physicochemical and biological properties that make them a favourable material for biomedical applications.

Transfection of foreign plasmid DNA or RNA into host cell nucleus to modify, change, or silence expression of a gene is a challenging task. The cellular nuclease enzymes constitute the major obstacle in this task as they can degrade naked plasmids in the cytoplasm before entry into the nucleus leading to low transfection efficiency. Recent reviews have focused on different types of gene-delivery systems, barriers encountered during nonviral gene delivery and the techniques to overcome them.

Considering these, there is a need for development of efficient and specific delivery vehicles either natural or synthetic. Proteins, such as albumin can very well satisfy all such requirements to act as a gene-delivery vector.

1. Introduction

So far there is no cure for Duchenne Muscular Dystrophy (DMD). An ultimate solution to Duchenne Muscular Dystrophy requires production of a functional protein and to achieve this goal distinct molecular therapeutic approaches have already been introduced into the clinic. Recent studies provide realistic hope that molecular therapies may help DMD patients (Aartsma-Rus et al, 2004; Kole et al, 2015; Spitali et al, 2012). However, novel methods are still needed for improved systemic delivery of therapeutic nucleic acids and more effective correction of the mutated protein in all affected muscles.

In order to be effective, the therapeutic agent, such nucleic acids, must reach the target cell. Some natural and synthetic vehicles have been studied and tested to improve delivery of the nucleic acid into the cell without degradation (Ibraheem et al, 2014; Lou et al, 2000). Nanoparticles made of albumin have been described as potential carriers (Kothamasu et al, 2012; Musyanovych, Landfester, 2014; Shimanovich et al, 2014).

1.1. Duchenne Muscular Dystrophy

1.1.1. Historical Perspective and Clinical Presentation

Clinical descriptions of Duchenne Muscular Dystrophy have occurred since the mid-1800s. The first clinical description of this disorder was made by Meryon and Little in 1852. However, DMD is named after the French neurologist Guillaume Benjamin Amand Duchenne, who, in 1861 described his first case of the dystrophy, under the title "*Paraplegie hypertrophique de l'enfance de cause cerebrale*" (Emery et al, 2015; Emery et al, 2011). Duchenne's scientific persuasion was complemented by an interest in photography and he was even one of the first people to use photographs instead of drawings to document pathological conditions for medical reference. Thus in 1862 he presented photos of his patient in his "*Album de photographies pathologiques*" (Duchenne, 1862).

Duchenne Muscular Dystrophy is the most common muscular dystrophy: it has an incident of 4700 and 3917 male births in Canada and southeast Norway, respectively (Dooley et al, 2010; Tangsrud & Halvorsen, 1989). In most cases, the disease is diagnosed on the basis of gait abnormalities at 4–5 years of age including difficulties in standing from a seated position, climbing stairs, and keeping up with their peers during play. In 1886, Gowers described a number of boys with DMD and observed the classical sign named after him (Gowers's sign). He drew sketches depicting the wat in which patients "walk up their legs" by using their hand to push up from the floor, then push on their knees to arise to a sating position (Figure 1) (Chamberlain and Rando, 2006). By 8–10 years of age, deterioration of the patient's condition necessitates wheelchair use. At their early teens, patients are wheelchair dependent and, in some cases, neurological and cardiological symptoms are apparent. Progression of muscle degeneration takes place and replacement with fibrous/fatty tissue occurs, which ultimately results in muscle contraction and ultimately death by respiratory or cardiac failure by late teens to mid-twenties. The introduction of positive-pressure ventilation has since increased life expectancy into late-

twenties and early thirties. Severe cognitive impairment such as reduced verbal skills and delayed reading learning are seen in approximately 30% of Duchenne Muscular Dystrophy cases (Emery, Walton, 1967; Mah et al, 2014; MDA Inc., 2015).

Figure 1 – Boys with Duchenne Muscular Dystrophy have a distinctive way of rising from the floor, called a *Gower's maneuver* (MDA Inc., 2015).

The disease is a fatal X-linked recessive disorder. Females will typically be carriers for the disease once their muscles are multinucleate mosaics of affected and unaffected cells due to random X-inactivation in muscles precursor cells, where compensatory dystrophin expression is sufficient to render a normal phenotype. Males, on the other hand, will be affected. Typically, a female carrier will be unaware they carry a mutation until they have an affected son. The son of a carrier mother has a 50% chance of inheriting the defective gene from his mother. The daughter of a carrier mother has a 50% chance of being a carrier. In all cases, the father will either pass a normal Y to his son or a normal X to his daughter (Figure 2).

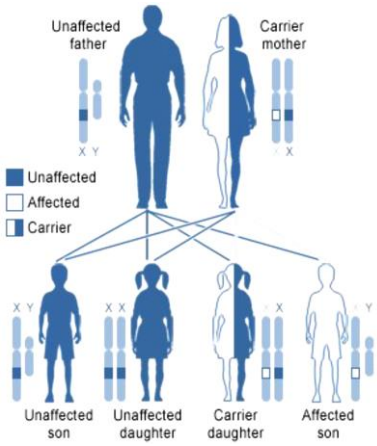

Figure 2 - X-linked recessive inheritance (https://en.wikipedia.org/wiki/X-linked_recessive_inheritance).

1.1.2. Molecular Pathology of DMD

In most of the cases, DMD occurs due to frame-shifting deletions or nonsense mutations in the *DMD* gene encoding the dystrophin protein. Another related disorder that occurs because of mutations in the *DMD* gene is Becker Muscular Dystrophy (BMD) with an incidence of 18450 live male births in northern England (Bushby et al, 1991). In DMD the gene mutations lead to complete absence of functional protein while in BMD a partially functional dystrophin protein is typically produced, leading to an attenuated clinical course and attenuated muscle pathologic abnormality (Wein et al, 2015).

The difference between an absent or a partially functional dystrophin relies in the concept of the "reading frame rule". Mutations that ablate the open reading frame ("out-of-frame" mutations) leads to

translation termination and Duchenne Muscular Dystrophy; in other hand, those that maintain the open reading frame ("in-frame" mutations) leads to Becker Muscular Dystrophy, where a smaller, but functional protein is produced, or no clinical symptoms at all (Wein et al, 2015) (Figure 3). The dystrophin is one of the largest known genes. Its more common transcript contains 79 exons, 13956 bp, and codes for a 427 kDa protein composed of 3685 amino acid residues.

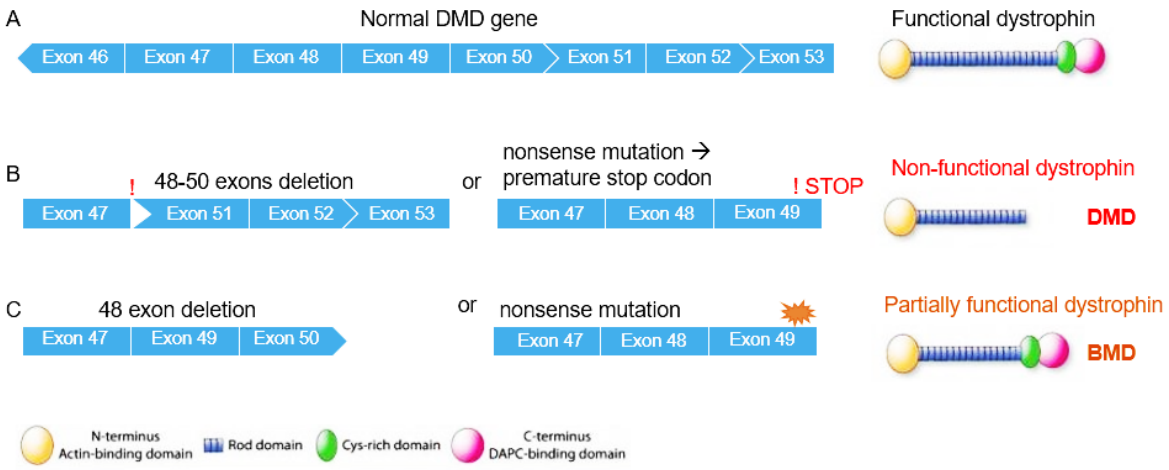

Figure 3 – Differences between (A) Normal DMD gene, leading to production on functional dystrophin; (B) mutations in *DMD* gene caused by deletion or premature stop codon leading to out-of-frame that results in non-functional dystrophin, observed in Duchenne Muscular Dystrophy patients and (C) deletion in *DMD* gene that does not cause out-of-frame but in-frame mutations, and so partially but functional dystrophin is produced resulting in the least severe DMD, called Becker Muscular Dystrophy.

The protein localizes to the sarcolemma and has a scaffolding function in muscle fibers where it connects the actin cytoskeleton to the extracellular matrix. The amino-terminus of dystrophin binds to filaments of actin (globular multi-functional protein that forms microfilaments) and the carboxyl-terminus to the dystrophin-associated protein complex (DAPC). This complex is a combination of membrane and transmembrane proteins like dystroglycan, sarcoglycans, integrin and caveolin-3 (Nowak, Davies, 2004).

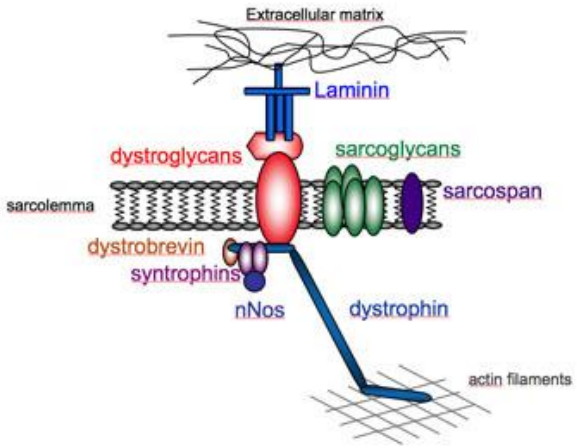

Figure 4 – Organization of the dystrophin-associated protein complex (DAPC) at the sarcolemma of skeletal muscle (http://www.umd.be/DMD/W_DMD/protein.html).

The sarcolemmal dystrophin-associated protein complex provides a crucial structural link between the extracellular matrix and the intracellular actin cytoskeleton. The lack of dystrophin leads to destabilization of the DAPC which results in diminished levels of the membrane proteins and, ultimately progressive fiber damage and membrane leakage is observed (Fairclough et al, 2013; Nowak et al, 2004).

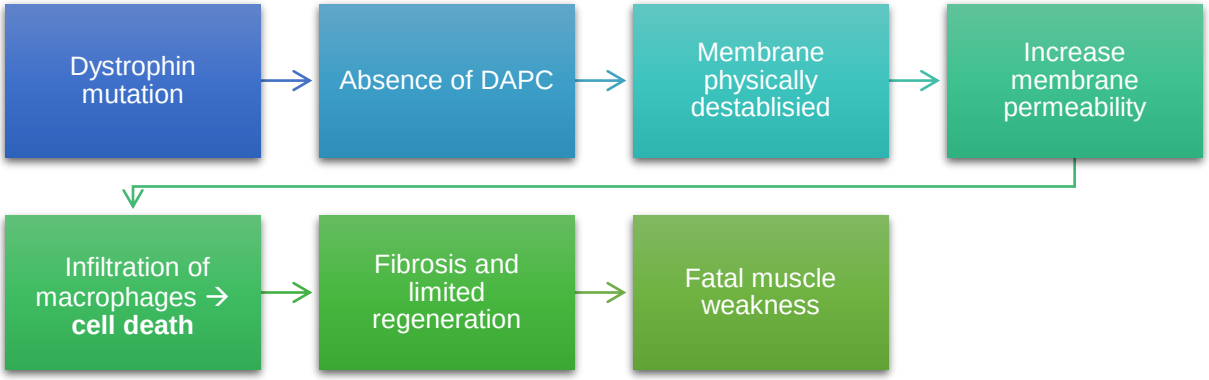

Figure 5 - Muscle fibers become vulnerable to mechanical stress through the loss of mechanical stability and/or loss of some so far unknown signaling capability. Increased membrane permeability, and impaired calcium homeostasis and activation of proteases contribute to increased levels of cell death. In the long term, insufficient regeneration and progressive fibrosis lead to an ultimately fatal muscle weakness (Nowak et al, 2004; Kharrax et al, 2014).

Being one of the largest gene in the human genome, *DMD* gene have many thousand mutations recorded. Deletions of one or more exons account for approximately of 60-70 % of mutations in individuals with Duchenne Muscular Dystrophy and Becker Dystrophy. Duplications account for the disease-causing mutations in about 5-10 % of males with DMD and BMD, while point mutations (small deletions, insertions, splicing-mutations) account for approximately 25-35 % of mutations on males with DMD and about 10-20 % of males with BMD. There are no particular common point mutations or point mutations hotspot, and each affected family may carry a unique mutations in this enormous gene (private mutations). However, it is fortunate that many of the large gene deletions within the *DMD* gene can be detected in specific “hotspot areas”. These “hotspots” are clustered in two main regions: (1) exons 1, 3, 4, 5, 8, 13, 19 and (2) exons 42-45, 47, 48, 50-53, 60 (Al-Hadithi et al, 2014).

1.1.3. Animal models of DMD

Both naturally occurring and laboratory-generated animal models are available to study the pathobiology of Duchenne Muscular Dystrophy and to develop innovate therapies for treating the disease. Currently there are nearly 60 different animal models for Duchenne Muscular Dystrophy (McGreevy et al, 2015). The most commonly used laboratory model of DMD is the *mdx* mouse. Whilst the *mdx* is a genetic and biochemical homologue of the disease, it has a somewhat milder phenotype. Muscle pathology is comparatively moderate and mechanical function is less seriously compromised (Collins and Morgan, 2003). However, canine models might bridge this gap. Confirmed dystrophin

deficiency has been reported in approximately 20 different dog breeds. Generally, the clinical phenotype of canine Duchenne Muscular Dystrophy (cDMD) is considered more severe than that of *mdx* mouse model and, as such, cDMD is regarded as a better model of human DMD (McGreevy et al, 2015).

1.1.3.1. *mdx* and *mdx52* mouse model

A naturally occurring dystrophin-deficient mouse mutant was first described in 1984 in a colony of C57BL/10 and has since been referred to as the "*mdx* mouse". This mouse, now called C57BL/10ScSn-Dmd^{mdx}/J is readily available from commercial breeders. It carries a point mutation in exon 23 of the *DMD* gene introducing a premature stop codon, which leads to the absence of full-length dystrophin. This kind of mutation resembles what is found in one third of DMD patients (McGreevy et al, 2015; Nakamura, Takeda, 2011). In the development of exon skipping therapy, the *mdx* mouse model is available for exon 23 skipping to convert an out-of-frame into an in-frame mutation.

To create a mouse model with large deletions in the DMD gene like those found in two thirds of human patients, exon 52 was disrupted on a C57BL/6J background. A new mouse model for DMD, known as *mdx52* was then generated by Katsuki and colleagues. In this model, exon 52 of the DMD gene was deleted. Like *mdx* mouse, *mdx52* lack dystrophin and presents dystrophic changes with muscle hypertrophy. It has been noted that exons 45 – 55 cover the main mutation "hot spot" of the DMD gene, so *mdx52* is a good mouse model to study potential therapeutics for DMD. Also, the targeting of exon 51 for exon skipping is theoretically applicable to the highest percentage (13%) of DMD patients (Nakamura and Takeda, 2011; Aoki et al, 2012).

Both mouse models have been used in exon skipping therapy. Systemic administration of 2´-O-methyl-phosphorothioate (2'OMP) antisense oligonucleotides to *mdx* mice revealed that dystrophin is expressed in the whole-body skeletal muscle (Nakamura and Takeda, 2011). Aoki and colleagues demonstrated that systemic administration phosphorodiamidate morpholino oligomers (PMO) in *mdx52* mouse model induced dystrophin expression at the sarcolemmal in skeletal muscles throughout the body (Aoki et al, 2012). Recently, Nakamura and Takeda conducted exon 51 skipping using PMO in *mdx52* mouse model to convert an out-of-frame mutation into an in-frame mutation resulting in whole-body skeletal muscles with amelioration of the dystrophic pathology and improved muscle function (Nakamura and Takeda, 2011).

1.1.4. Therapeutic Approaches

No effective treatment exists for DMD. Although there are pharmacological strategies, such as the injection of corticosteroids that treats inflammation and are able to delay symptoms by tackling the secondary effects of the disease, many are only partially effective because they treat just one aspect of the pathogenesis and they also may be toxic in longer term (Fairclough et al, 2013).

Pharmacological therapies and newer approaches involving the use of therapeutic oligonucleotides are described next.

1.1.4.1. Pharmacological therapies

The great advantage of a pharmacological approach is that nearly all drugs can be delivered systemically (orally, intravenously, subcutaneously) and thus will reach and potentially treat all muscles which is critical for clinical success in DMD (Pichavant et al, 2011).

While the search for effective therapies remains ongoing, the currently available pharmacological intervention broadly aim to manage/improve the phenotype for the patient. The corticosteroids prednisone and deflazacort are the only medications that have been shown to affect the clinical course of Duchenne Muscular Dystrophy and, although the precise mechanism of their effect is unknown, their use is considered standard of care. The classical randomized placebo-controlled trial of prednisone demonstrated that treatment of 0.75mg/kg/day resulted in improved muscle strength by 6 months. Deflazacort at an equivalent dose has also frequently been used. Nevertheless, the side-effects of both drugs are marked. Weight gain that often results in obesity, exacerbation of osteoporosis due to muscle weakness, hypertension and cataract formation are some of the reported side effects (Malik et al, 2013; Wein et al, 2015).

About 10-15% of DMD patients have a nonsense mutation that converts an amino acid into a premature nonsense codon, while the rest of the mRNA in unaffected. Some drugs are being developed to enable stop codon read-through by introducing an amino acid at the premature stop codon to continue the mRNA translation. This phenomenon called "*stop codon read-through*" is being intensively investigated (Wilton et al, 2011; Pichavant et al, 2011, Malik et al, 2012). Two pharmacologic tactics have shown pre-clinical efficacy (Malik et al, 2013).

In *mdx* mouse model, mutation suppression was shown with the aminoglycoside antibiotic, gentamicin. In a follow up clinical study, treatment of four Duchenne Muscular Dystrophy/Becker Muscular Dystrophy subjects failed to show a benefit (Barton-Davis et al, 1999). A subsequent clinical trial suggests that read-through had occurred, demonstrating full-length dystrophin as the product of gentamicin treatment (Malik et al, 2013).

Ataluren, formerly referred to as PTC124 (Translarna™) fulfilled the requirement of an orally administered pharmacological read-through product for stop codon mutations. Pre-clinical studies in the *mdx* mouse model demonstrated dystrophin expression in skeletal, cardiac and diaphragm muscle. It is an investigational new drug, in phase III clinical trials (Wein et al, 2015).

Although gentamicin treatment of Duchenne Muscular Dystrophy is possible and results in some increased dystrophin expression, it is not practical because of the requirement for intravenous injection on muscles. Thus, Ataluren, being an oral medication has advantages (Wein et al, 2015; Fairclough et al, 2013). Ataluren and gentamicin are therapeutic approaches specific for Duchenne Muscular Dystrophy patients with point nonsense mutations that converts an amino acid into a premature nonsense codon.

1.1.4.2. Gene therapy

Gene therapy is a promising therapeutic strategy based on using nucleic acids as a medicine to cure a wide range inherited diseases. Conceptually, gene therapy is a straightforward therapeutic method depending on either replacing a distorted gene by a healthy one, or adding a missing gene in order to express the required protein. Nevertheless, in practice this is the most complex operation due to several obstacles that must be overcome by the transgene in order to reach the targeted human cell-nucleus, where it should be expressed correctly and has not been achieved yet (Ibraheem et al, 2013; Misra, 2013).

The most common form of gene therapy research relies on using a DNA molecule that encodes a functional, therapeutic gene to replace a mutated gene. In 1990, doctor Anderson performed one of the first successful gene therapy study on a 4 years old girl, Ashanti DeSilva, who was born with ADA-SCD, a type of Severe Combined immune Deficiency (SCD) due to mutations the gene of adenosine deaminase (ADA). The lack of production of this enzyme had made her immune system weak so she had become susceptible to many severe diseases. Anderson and his colleagues extracted her white blood cells (WBCs – leucocytes), implanted genes producing ADA using a retroviral vector containing correct ADA gene and then transferred the cells back to her body (*ex vivo* gene therapy). Those corrected cells strengthened Ashanti's immune system and made it possible for her to survive (Ibraheem et al, 2013; Misra, 2013; Nienhuis, 2013; Boudes, 2013).

Most recently, the type II bacterial CRISPR/Cas9 system has been demonstrated as an efficient gene-targeting technology with the potential for directed genome editing. CRISPR is actually a naturally-occurring ancient defense mechanism found in a wide range of bacteria. Bacteria uses CRISPR (clustered regulatory interspaced short palindromic repeat) and Cas9 (CRISPR-associated) proteins to detect and destroy invading viruses and plasmids. Cas9 is an enzyme that snips DNA and CRISPR is a collection of DNA sequences that tells Cas9 exactly where to cut. Theoretically, if the right sequence is given to Cas9 (guide RNA), it is possible to cut and paste bits of DNA sequence into the genome wherever necessary (Wang et al, 2013).

Several research groups are currently investigating not only the therapeutic application of CRISPR/Cas9 to Duchenne Muscular Dystrophy (Li et al, 2015; Ousterout et al, 2015; Long et al, 2015) and also the possibility to generate in a more efficient way animals models to study this disease (Nakamura et al, 2014).

As genetic therapy correction of patient-derived induced pluripotent stem cells (iPSCs) by transcription activator-like effector nuclease (TALEN) or CRISPR-Cas9 holds promise for DMD gene therapy, Li and coworkers performed three correction methods (exon skipping, frameshifting and exon knockin) *in vitro*, in DMD-patient-derived iPSCs. The most promising approach was exon knockin. Corrected iPSCs were then differentiated in skeletal muscle cells and full-length dystrophin protein was successfully detected (Li et al, 2015).

The viability of CRISPR-Cas9 approach to restore the expression of dystrophin was also accessed by a group of researchers from Duke University, North Carolina, USA by using CRISPR-Cas9 system to restore the expression of the dystrophin gene in cells carrying dystrophin mutations

that causes DMD. They designed single guide RNA molecules to restore the dystrophin reading frame by targeting the mutational hotspot exons 45-55 and introducing shifts within exons or deleting one or more exons. Following gene editing in DMD patient myoblasts, dystrophin expression is restored *in vitro*. Human dystrophin is also detected *in vivo* after transplantation of genetically corrected patient cells into immunodeficient mice (Ousterout et al, 2015).

CRISPR/Cas9-mediated genome editing approach was applied to correct the genetic defect in the *DMD* gene of *mdx* mouse model *in vivo*. The degree of muscle phenotypic rescue in mosaic mice exceed the efficiency of gene correction, likely reflection an advantage of the corrected cells and their contribution to regenerating muscle (Long et al, 2015).

1.1.4.3. Therapeutic oligonucleotides

Gene therapy could also be used for therapeutic gene expression modulation which consists on altering the expression of a gene. It differs from basic gene therapy in that gene modulation seeks to alter the expression of a gene whereas gene therapy concerns the introduction of a gene. Modulation of gene expression can be mediated post-transcriptionally through RNA interference (RNAi), microRNAs and antisense oligonucleotides (AONs) (McClorey et al, 2015).

AONs that are small acid nucleic synthetic sequences that can include modified RNAs or DNAs, have been tested to: (1) down-regulate gene expression, (2) targeting micro RNAs (miRNAs) and (3) modulate splicing.

(1) Down-regulate gene expression: RNAi is a biological process in which RNA molecules inhibit gene expression. RNAi effector molecules, known as small interfering RNA (siRNA), are typically 21-mer AONs that are recruited into the RNA-induced silencing complex (RISC) to effect efficient cleavage of the target mRNA. Currently, ALN-TTR02 and ALN-TTRsc (Alnylam Pharmaceuticals), RNAi therapeutics intravenously administrated, have phase III clinical trials ongoing for treatment of familial amyloid polyneuropathy and familial amyloidotic cardiomyopathy respectively (McClorey et al, 2015). Both conditions are variant diseases from Transthyretin-related hereditary amyloidosis, an autosomal dominant neurodegenerative disease where mutant transthyretin (protein of the blood, TTR) is expressed causing abnormal accumulations of protein. In a phase I clinical trial, the efficiency of ALN-TTR02 was demonstrated with the mean reduction in TTR levels of 86.8% (McClorey et al, 2015).

(2) microRNAs: microRNAs are very small RNA functional molecules that can regulate post-transcriptionally gene expression, they are an attractive target for therapeutic gene expression modulation. One approach to modulate miRNA expressions is the use of single-stranded AONs that directly bind target miRNAs to inhibit their function (anti-miRs), and thus derepress their target genes. Roche Innovation Center Copenhagen (formerly Santaris Pharma) is currently undertaking phase II clinical trial (NCT02452814 clinicaltrials.gov) with Miravirsen, an AON with by 15-mer that binds to miR-122 to inhibit its binding to the Hepatitis C virus (HCV). miR-122 has been demonstrated to be important for HVC host replication (McClorey et al, 2015).

Within oncology, the first miRNA-based therapy approach, MRX34, has entered clinical testing in 2013 to inhibit prostate cancer stem cells. Cancer stem cells or tumor-initiating cells are involved in tumor progression and metastasis (Baumann V. and Winkler J, 2014; Liu et al, 2011).

(3) Splicing modulation: AONs could also be used to modulate gene splicing and an example of this approach is DMD, where antisense-mediated exon skipping is currently in clinical trials indicating that this could be a promising approach to restore the reading frame of *DMD* gene. Currently two AONs chemistries are in clinical trials, drisapersen (Prosensa Holding) and eteplirsen (Sarepta Therapeutics) (McClorey et al, 2015; Goyenvalle et al, 2015).

1.1.5. Splicing modulation therapy for DMD

DMD is characterized by a lack of dystrophin, which is frequently caused by mutations that shift the reading frame, most commonly from deletion or duplication of one or more exons resulting in no production of functional protein.

AONs induced restoration of the DMD reading frame is based on inducing the skipping of specific exons. The approach has been successfully applied in Duchenne Muscular Dystrophy patient-derived myotubes and in parallel in *mdx* mouse model (intramuscular injection). Treated mice showed dystrophin restoration in many muscles (Aartsma-Rus et al, 2003; Lu et al, 2005).

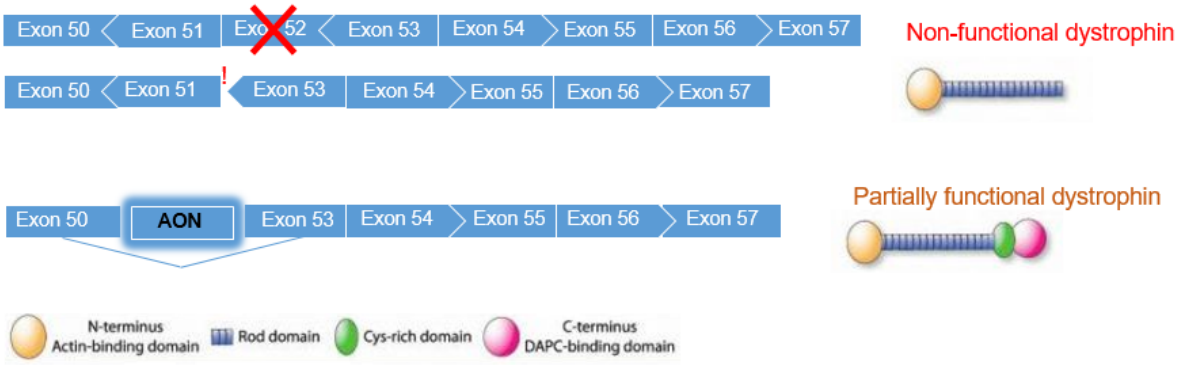

Figure 6 – Antisense-mediated exon skipping in *mdx52* mouse model. Absence of exon 52 in the dystrophy gene leading to an out-of-frame mRNA once exon 51 cannot join exon 53, so dystrophin synthesis during translation is aborted; Using AONs targeting exon 51 which is skipped during splicing restoring of the open reading frame of the transcript is achieved and it allows the synthesis of an internally deleted dystrophin.

To enhance the *in vivo* activity of AONs these nucleic acid molecules must overcome main obstacles in the whole body, such as nuclease degradation. Many artificial nucleic acids have been synthetized to improve some features like nuclease resistance and binding properties. Two drugs candidates are currently being evaluated for exon 51 skipping in advanced clinical trials. Both of them target and induce skipping of *DMD* exon 51 differing in their chemical modifications.

1.1.5.1. Drisapersen

Drisapersen (Prosensa Holding), is a 20 nucleotides AON with a sequence that is specific for induce skipping of exon 51 in splicing modulation of the *DMD* transcripts. It is modified in order to avoid nuclease degradation by a backbone substitution of an oxygen, called 2´-O-methyl-phosphorothioate (2'OMP). AONs with his chemical configuration acquire more resistance to nucleases once these enzymes do not recognize the modified phosphate group and, in that way, cleavage does not occur. After some promising results in Duchenne Muscular Dystrophy patient cells and in *mdx* mouse model, clinical trials on 4 DMD patients with drisapersen were done. The muscle injected with this therapeutic AON showed 64-97 % dystrophin-positive fibers with a level of dystrophin expression between 17 and 35 %. A phase III clinical trial has started with this AON on Duchenne Muscular Dystrophy patients (Pichavant et al, 2011; Aartsma-Rus et al, 2004; Aartsma-Rus et al, 2003; Lu et al, 2005).

1.1.5.2. Eteplirsen

Eteplirsen (Sarepta Therapeutics) is a AON also specific to induce skipping of exon 51 in *DMD* gene transcripts with the chemistry of an morpholino oligomer.This modification allow morpholinos to be resistant to nucleases once their structure is different from DNA and thereby nucleases do not recognize them. *mdx* mouse model was the first target of morpholinos. Restoration of dystrophin was observed in the treated mice muscle when morpholinos were intramuscularly injected and in man y muscles when intravenously administrated (Gebski et al, 2003; Alter et al, 2006). In humans, an initial phase I/II dose-escalation trial resulted in increased dystrophin level in 7 of 19 patients but to significant difference in 6MWD test between treatment and placebo groups (Aartsma-Rus et al, 2004; Pichavant et al, 2011; Bauman et al, 2009; Falzarano et al, 2014).

1.1.5.3. AONs alternative chemistries

Very recently, a new class of AONs incorporating a tricycle-DNA (tcDNA) has been tested to modulate splicing and revert phenotype in DMD gene with encouraging results in cell culture and in *mdx* mouse model (Goyenvalle et al, 2015). Also LNA modified AONs were tested in Duchenne Muscular Dystrophy patients cells with good results (Aartsma-Rus et al, 2003).

Table 1 – Clinical trials currently active or recruiting for select siRNA, splicing-switching and anti-miR therapeutics of the examples presented in text (McCLorey et al, 2015).

Drug	Target	Condition	Phase	Status	Clinical Trial ID
RNAi					
ALN-TTRSC	Transthyretin	TTR-mediated familial amyloidotic cardiomyopathy	I	Active	NCT01814839
			II	Active	NCT02292186
			III	Recruiting	NCT02319005
ALN-TTR02	Transthyretin	TTR-mediated amyloidosis	II	Active	NCT01961921
			III	Recruiting	NCT01960348

Splicing-switching					
Drisapersen	Dystrophin (exon 51)	DMD	I/II	Active	NCT01910649
			III	Recruiting	NCT01803412
PRO045	Dystrophin (exon 45)	DMD	I/II	Active	NCT01826474
Eteplirsen	Dystrophin (exon 51)	DMD	II	Active	NCT02286947
			II	Recruiting	NCT02420379
			II	Active	NCT01540409
			III	Recruiting	NCT02255552
Anti-miR					
Miravirsen	miR-122	Hepatitis C	II	Active	NCT01872936

1.1.6. LNA modified AONs

LNA modified AONs have been tested for the treatment of genetic and infectious diseases. Modulation of splicing using AONs is an attractive strategy for the treatment of DMD (Wojtkowiak-Szlachcic et al, 2015)

Locked nucleic acid (LNA) is an artificial nucleic acid derivative that was described by Wengel and coworkers in 1999 (Wengel et al, 1999). LNA contains a methylene bridge connecting the 2' oxygen to 4' carbon position in the furanose ring, which improves duplex stability to complementary DNA or RNA. LNAs are nuclease-resistant, nontoxic and have the highest affinity for complementary DNA and RNA yet reported for any DNA analog. Given these features, LNA-AONs can be used for various gene expression modulation techniques, such as antisense, short interfering RNA and blocking miRNA (Shimo et al, 2014; Aartsma-Rus et al, 2004).

Gupta et colleagues showed a reduction of proprotein convertase subtilisin/kexin type 9 (PCSK9), an important factor in the etiology of familial hypercholesterolemia, in cell lines and mouse liver through LNA AONs silencing PCSK9 mRNA (Gupta et al, 2010). Most recently, a short LNA AONs was tested in cell lines to correct splicing abnormalities in myotonic dystrophy (Wojtkowiak-Szlachcic et al, 2015).

A recent paper from a research group from Japan describes the use of LNA AONs complementary to the human dystrophin exon 58 sequence. Their ability to induce skipping of endogenous human dystrophin in primary human skeletal muscle cells showed very promising results (Shimo et al, 2014).

In our group an LNA-AON was also used to induce skipping of exon 51 in Duchenne Muscular Dystrophy patient's derived muscular cell lines with good results (results not published).

1.2. Gene delivery systems

The success of gene therapy essentially depends on ensuring that the therapeutic gene enters the targeted cell and the cell nucleus before DNA must be associated with the delivery system or vector that carriers the therapeutic gene into the targeted cell, protecting it from degradation by nucleases. DNA must be associated with the delivery system or vector that carriers the therapeutic gene into the targeted cell, protecting it from degradation by nucleases (Lou et al, 2000).

1.2.1. Viral vectors

Viruses were the first carriers to be used to deliver and protect the therapeutic gene, since they can penetrate into the cell nucleus of the host and exploit the cellular machinery to express its own genetic material and replicate it. In order to use a virus as a vector to transfer a gene, it have to be modified by genetic engineering, removing the pathogenic part of it and replace it by the therapeutic gene. To date, viral vectors are the vectors most often used to transfer genes, due their high transfection efficiency *in vivo*, despite their disadvantages (Munier et al, 2005; Ibraheem et al, 2014; Nienhuis, 2013; Misra, 2013; Touchefeu et al, 2010).

The first viruses to be used as vectors in gene therapy experiments were retroviruses (Ashanti DeSilva at 1990). Once inside the host cell cytoplasm, the retrovirus use their own reverse transcriptase enzyme to produce DNA from its RNA genome, the reverse of the usual pattern. This DNA is then incorporated in to the host cell genome by a viral integrase enzyme. That way, the host cell genome gains a new gene and if such modified host cells divide later, their descendants will maintain the new genes. (Gaspar, 2005; Ibraheem et al, 2014; Nienhuis, 2013; Boudes, 2013; Touchefeu et al, 2010).

Adenoviruses belong to a class of viruses that have their genetic material in the form of a double-stranded DNA. When infecting a host cell, these virus introduce their DNA molecule into the host but the genome of the adenovirus is not incorporated into the genetic material of the host cell. Adenoviruses are more likely to be attacked by the patient's immune systems, and high levels of viruses are required for treatment (Nienhuis, 2013; Boudes, 2013; Touchefeu et al, 2010). In 2003, China became the first country to approve a gene therapy based product for clinical use. Gendicine™ (SiBiono Gene Tech Co.) is an adenoviral vector that was approved by China's State Food and Drug Administration (SFDA) for the treatment of head- and neck cell carcinoma (Kumar et al, 2014; Wirth et al, 2013).

In 2012, the European Medicines Agency (EMA) recommended for the first time a gene therapy product (Glybera) for approval in the European Union. Glybera (UniQure) is an adeno-associated viral vector (AAVv) engineered to express lipoprotein in the muscle tissue for the treatment of adult patients diagnosed with familiar lipoprotein lipase deficiency (LPLD) (Wirth et al, 2013).

1.2.2. Non-viral vectors

The drawbacks of viral vectors, especially immune response, have led to research for safer alternatives. Consequently, non-viral vectors have been designed for transferring DNA. Non-viral vectors are relatively safe since they generally cause low immune response. They can be divided into two groups:

1- Physical approaches: the principle of these approaches is to use mechanical, ultrasonic, electric, hydrodynamic or laser-based energy (electroporation – creation of electric field induce pores in plasma membrane; sonoporation – ultrasonic frequencies to disrupt cells membrane; gene gun – shoots DNA coated gold particles into cells by using high pressure; hydrodynamic injection) in order to create temporary weak point in the membrane of the target cell, by causing transient injuries or defects in it allowing the DNA to inter the cell by diffusion (Ibraheem et al, 2013; Misra, 2013; Touchefeu et al, 2010; Chou et al, 2011).

2- Chemical vectors: proposed as promising alternatives to viral ones to overcome the drawbacks of the latter. These vectors have three main goals to improve gene transfer into the cell nucleus: (a) mask DNA-negative charges; (b) compress the DNA molecule to make it smaller and, (c) protect it from degradation by intracellular nucleases. These objectives can be achieved through encapsulating the nucleic acid molecules it in biodegradable polymers, or by adsorbing it. Among the several nonviral approaches, the use of DNA conjugated with liposomes has been the most widely used to undergo cellular uptake through endocytosis, with subsequent gene expression in cell culture, but has got serious limitations in order to be used *in vivo* (Ibraheem et al, 2013; Misra, 2013; Touchefeu et al, 2010).

The research of non-viral vectors led to the field of nanotechnology with the development of potential nonviral delivery system. These vehicles could deliver a variety of drugs and macromolecules, such as oligonucleotides, via various administration routes, including intravenous, oral, intramuscular, pulmonary, intranasal, ocular, rectal and intraperitoneal. Nanobiotechnology aims the delivery through nanoformulation and provide desired release kinetic for prolonged periods of time (Devulapally and Paulmurugan, 2014).

1.3. Nanotechnology

Generally, nanotechnology can be understood as a technology, which allows in the controllable way not only to create nanomaterials but also to operate them, i.e. to influence them or to use them according to their intended purpose. Accordingly, nanomaterial can be understood as the materials, which are characterized at least in one of three measurements by nanometer scale concerning both the sample of a material as a whole and its structural elements (Logothetidis, 2012). The nanometer is often used to express dimensions on the atomic scales: the diameter of a helium atom, for example, is about 0,1nm and that of a ribosome is about 20nm.

Nanotechnology is considered an emerging technology due to the possibility to advance well-established products and to create new products with totally new characteristics and functions with enormous potential in a wide range of applications. In addition to various industrial uses, great innovations are anticipated in information and communication technology, in biology and biotechnology, in medicine and medical technology, in metrology, etc. Significant applications of nanosciences and nanoengineering lie in the fields of pharmaceutics, cosmetics, processed food, chemical engineering, high-performance materials, and environmental sciences (Logothetidis, 2012).

The development of a wide spectrum of nanoscale technologies is beginning to change the foundations of disease diagnosis, treatment and prevention. These technological innovations, referred to as nanomedicines by the National Instituted of Health (Bethesda MD, USA), have the potential to turn molecular discoveries arising from genomics and proteomics into wide spread benefit for patients. Nanomedicine is a large subject area that included nanoparticles, nanofibers and polymeric nanoconstructs as biomaterials and nanoscale microfabrication-based devices. Furthermore, there is a vast array of nanoscale technologies capable of targeting different cells and extracellular elements in the body to deliver drugs, genetic materials, and diagnostic agents specifically to these locations. Indeed, research into the rational delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents via intravenous and interstitial routes of administration with nanosized particles is at the forefront of projects in nanomedicine (Moghimi et al, 2005).

1.3.1. Nanoparticles as biological carriers

In general, the size of a nanoparticle (NP) spans the range between 1 and 100nm. Metallic nanoparticles have different physical and chemical properties from bulk metals, properties that might prove attractive in various industrial application. However, how a NP is viewed and is defined depends very much on the specific application (Heiligtag and Niederberger, 2013).

In this size range, a wide variety of materials, including metals, metal oxides and semiconductors, exhibit unique optical, electrical and magnetic properties that can be tuned based on their size and shape. Other types of material, such as small molecules, lipids, polymers and other organic molecules, can be assembled into carriers for contrast agents and drugs to enhance payload and solubility. Collectively these materials can be synthesized, assembled into desirable geometries and configurations, and coated with targeting agents, and provide novel material properties for application in molecular and cellular labeling, tracking, detection, drug delivery and medical imaging with high sensitivity and functionality. So, such modularity offers an infinite matrix of nanoparticles (NPs) with different properties, making nanoparticle-based contrast agents and therapeutics more versatile than either small molecules (Heiligtag and Niederberger, 2013; Horikoshi and Serpone, 2013).

NPs research has become a key area in drug delivery systems for treatment of cancer and several other metabolic disorders. The drug is dissolved, entrapped, encapsulated or attached to a NP matrix. Depending upon the method of preparation, NPs, nanospheres or nanocapsules can be obtained (Musyanovych and Landfester, 2014).

The major goals in designing NPs as a delivery system are to control particle size, surface properties and release of pharmacologically active agents in order to achieve the site-specific action of the drug ate the therapeutically optimal rate and dose regimen (Mohanraj, W. Chen, 2006).

Extensive libraries of nanoparticles, composed of an assortment of different sizes, shapes and materials have already been constructed, for example: Fullerenes (molecule composed entirely of carbon), solid lipid nanoparticles (solid phase at room temperature), liposomes (vesicular structures with an aqueous core), *quantum dots* (semiconductor nanocrystal) dendrimers (micellar nanostructures) (Mudshinge et al, 2011).

Table 2 - Examples of various types of nanoparticles being developed for intracellular applications (Chou et al, 2011).

Type of NP	Size range (nm)	Application	References
Fullerene (C_{60})	~ 1	HIV proteases	Bakry et al, 2007
Solid lipid NPs (Oleic Acid)	50 – 1000	Inflammation	Panga et al, 2009
Liposomes (Hepatic targeted liposomes)	>= 15	Diabetes mellitus	Spangler, 1990
***Quantum dots* (encapsulated in phospholipid micelles)**	2 – 10	Cell tracking and color imaging of live cells	Dubertret et al, 2002; Jaiswal et al, 2003
Dendrimers (PAMAM – polyamidoamine)	~ 20	Diagnose certain disorders of the heart, brain and blood vessels.	Wiener et. al, 1994

To ensure the arrival of a transgene into a cell nucleus without degradation it is necessary to use gene delivery systems that are capable of protect the transgene from degradation and pass through the plasma membrane to the nucleus (Lou et al, 2000; Gao et al, 2007). A god delivering system (carrier) must fulfil the following criteria: (a) it must not interact with vascular endothelial cells and blood components, (b) it must be capable of avoiding uptake by the reticuloendothelial system, (c) it must be small enough to pass through the cell-membrane and reach the nucleus (Ibraheem et al, 2014; Lou et al, 2000; Gao et al, 2007; Labhasetwar, 2005).

In 2009, Rimessi et al, report that using cationic polymethylmethacrylate (PMMA) nanoparticles loaded with a low dose of a certain AON delivered by weekly intraperitoneal (IP) injection in *mdx* mouse model, could restore dystrophin production in body-wide striated muscles. Also, they tested injection of an identical dose of naked AON but this test did not resulted in detectable dystrophin expression (Rimessi et al, 2009).

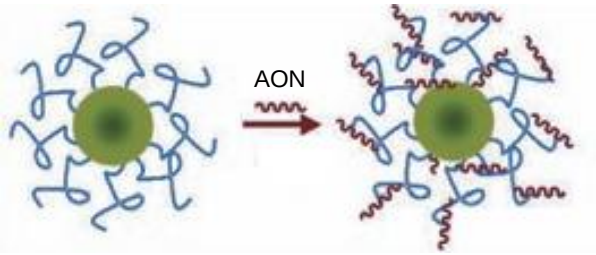

Figure 7 – Schematic representation of the lipophilic interaction between AON molecules and the surface onto nanoparticles (Rimessi et al, 2009).

The same group of investigators, a year later, made some modifications concerning T1 nanoparticles, designed and prepared a novel type of cationic core-shell nanoparticle made up of a predominantly PMMA core and a random copolymer shell consisting of units derived from N-isopropyl-acrylamide+ (NIPAM). These new PMMA nanoparticles bind and convey AONs very efficiently and systemic injections restored dystrophin protein synthesis in both skeletal and cardiac muscles of *mdx* mouse, allowing protein localization in up to 40% of muscle fibers (Ferlini et al, 2010).

In 2011, Kurosaki et al, take a step forward and developed a novel vector, electrostatically coated poly(ethylenimine) (PEI)/pDNA) (plasmid DNA) complexes with folic acid (FA) since the addition of FA markedly decrease the cytotoxicity of the cationic PEI/pDNA complexes to the melanoma cell line, B16-F10 cells, which regularly expressed FA-specific receptor (FR). Thus, we are now thinking not only in the safe and effective delivery of AONs but also on target them to specific locations. After the intravenous injection of Fa60/PEI/pDNA complexes into mice, a higher transgene efficiency than PEI/pDNA complexes were observed in the liver, kidney, and lung with FR (Kurosaki et. al., 2011).

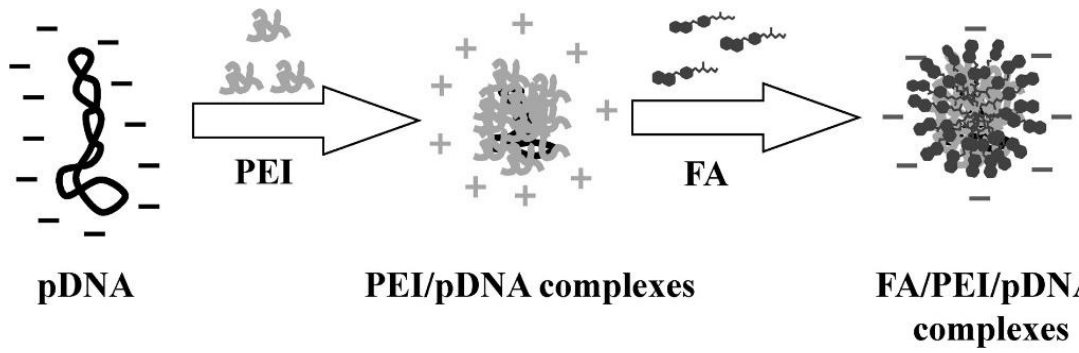

Figure 8 - Schematic representation of FA/PEI/pDNA complexes production (T. Kurosaki et. al., 2011).

The cytotoxicity is also an important feature to consider in nanoparticles synthesis. In 2014, Kodama et al, tested a novel gene vector composed of dendrigraft poly-L-lysine (DGL). The transgene expression efficiently of the pDNA/DGL complexes was markedly higher than that of the control pDNA/poly-L-lysine complex. However, the DGL complexes caused cytotoxicity and erythrocyte agglutination at high doses. To overcome that issue, the investigators added a biodegradable anionic polymer and the resultant ternary complexes (DGL/γ-PGA) were shown to be stable nanoparticles. The transgene expression efficiency of the DGL/γ-PGA complexes was similar to that of DGL

complexes; however they exhibited lower cytotoxicity and did not induce erythrocyte agglutination at high doses (Kodama et al, 2014).

For Kurosaki et al, the cytotoxicity of their PEI/pDNA complexes studied in 2011 was also a problem. In 2015, the group continued their way on targeting and selected DOPS (1, 2-dioleoyl-*sn*-glycero-3-phospho-L-serin) as an analogue for phosphatidylserine for splenic gene delivery of pDNA. The addition of DOPS changed the zeta potential of PEI/pDNA complexes to negative and, surprisingly, the PEI/pDNA/DOPS complex showed relatively high transgene efficacy *in vitro*. Also, the injection of the PEI/pDNA complex killed most mice within 24 hours at high doses, but all the mice in the PEI/pDNA/DOPS complex group survived (Kurosaki et al, 2015).

So, many studies had reported the efficacy of using nanoparticles as a vehicle to the delivery of AONs but the use of nanocapsules for the same purpose remains poorly documented. Table 3 summarizes some of the studies on this field.

Table 3 – Literature review of studies using nanocapsules delivery systems

Mouse model	Injected compound	Dose	Injection (/mouse)	Incubation time	Organs evaluated	Reference
Male *mdx*	T1/M23D	0.9mg/kg (AON)	250µL IP injection	24 hours	Liver	Rimessi et al, 2009
Male ddY	PEI/pDNA	2mg/kg (pDNA)	300µL I.V. injection	6 hours	Liver Lung	Tomoaki K. et al, 2011
Male ddY	DGL/γ-PGA	2mg/kg (pDNA)	250µL I.V. injection	6 hours	Liver Lung	Kodama Y., et al, 2014
Male ddY	PEI7pDNA/ DOPS	2mg/kg (pDNA)	300µL I.V. injection	6 hours	Liver Lung	Tomoaki K. et al, 2015
7-week-old BALB/c	pDNA-EGFP	1mg/kg (pDNA)	200µL I.V. injection	48 hours	(tumor)	Junjie et al, 2015

1.3.1.1. Albumin as a carrier

Albumin is the most abundant protein in blood plasma and in addition probably the most extensively researched plasma protein to date. In 1985, Theodore Peters Jr. published his seminal review article "*Serum albumin*" and in 1996, the same author, published the first comprehensive book. Medical applications were restricted to intravenous administration of human serum albumin (HSA) as a blood substitute for treating patients with severe burns or cachexia, however, in mid 1990s a few research groups began to investigate the potential of albumin as a carrier protein (Kratz, 2014).

HSA is one of the smallest proteins present in blood plasma. Both size and abundance explain the fact that so many metabolic compounds and therapeutic drugs are transported by this protein. The Danish pharmaceutical company Novo Nordisk was the first to exploit the long half-life of HSA of

approximately 19 days in order to improve the pharmacokinetic profile and compliance of insulin for treating diabetes (Kratz, 2014).

Albumin is non-toxic and degradable *in vivo*, so the nanoparticles generated by using it, which can be easily functionalized chemically, are easily adaptable to the human body (Elzoghby et al, 2011; Jun et al, 2010). Functionalized nanoparticles can be used for drug delivery, improving cellular uptake and ameliorating undesired toxic side effects associated with conventional chemotherapeutic agents (Loureiro et al, 2015).

Recently, a group of researchers from India tested incorporation of plasmid DNA isolated from *E. coli* strain into BSA nanoparticles formulated by the coacervation method. These BSA-NPs were employed as gene-delivery vehicles to load pDNA such as pUC18 and this protein-conjugated pDNA was successfully and integrally transfected into *E. coli* DHα5 bacterial cells. This study demonstrated the efficacy of BSA-NPs as delivery vehicle for pDNA transfections (Wagh et al, 2014).

Levemir® (insulin detemir from Novo Nordisk) was approved in 2004 in Europe by European Medicines Agency to control high blood sugar in adults and children with *diabetes mellitus* type 1 and 2. More recently, in 2010, Victoza® (Novo Nordisk) was approved by US Food and Drug Administration (FDA) to improve glycemic control in adults with type 2 *diabetes mellitus*. Abraxane® (paclitaxel), produced by American Bioscience was the first albumin-based drug delivery systems to be approved by FDA in oncology. It was approved at the beginning of 2005 for the treatment of metastatic breast cancer in USA and meanwhile also in Europe, China, Russia and several other countries. Trials in non-small lung cancer, metastatic pancreatic cancer and metastatic melanoma as well as expanded applications for breast cancer were undergone registration. Is currently being investigated in a phase II trials, the treatment of bladder and ovarian cancer (Elsadek et al, 2011).

Recently, Qian et al suggested a novel nanocapsules consisting of protein and lipid produced by double emulsion technique, is composed of three layers, forming a compact capsule that offers separated microenvironment for cell imaging and drug delivery. Bovine Serum Albumin (BSA) in the outer aqueous phase could be labeled by fluorescence agents for imaging, the separated oil phase can dissolve anticancer lipophilic molecules (paclitaxel or doxorubicin) for therapeutic application, and the interested proteins are encapsulated to the inner aqueous phase to provide a potential way for protein delivery (Qian et al, 2015).

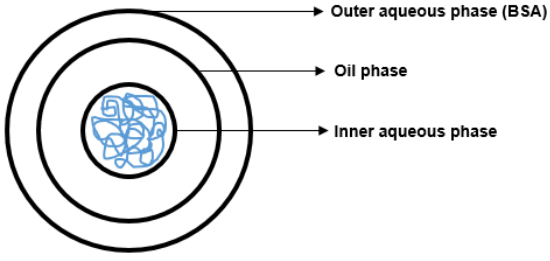

Figure 9 – Schematic representation of the novel hybrid protein-lipid polymer nanocapsules tested by Qian et al as an effective and nontoxic delivery and imaging carrier.

To identify the cell transduction ability of the delivery system, TRITC-BSA and NLS-GFP was assembled as shell and core of capsules, respectively, to observe the distribution of fluorescence.

After 1 hour of incubation with the nanoparticles, the cytosol of Hela cells showed obvious red fluorescence, whereas strong green fluorescence appeared in the cell nuclei, indicating successful intercellular delivery of NLS-GFP into the nuclei (Qian et al, 2015).

1.3.2. Nanocapsules synthesis

Polymeric nanocapsules can be made from a variety of synthetic and natural monomers/polymers. Different preparation methods have been published, which describe the formulation of NPs from the polymers, such as single and double emulsion technique, phase separation coacervation technique and ultrasonic emulsification technique. The selection of the method mainly depends on the physical-chemical properties of the used polymer and entrapped biological material, however the size and size distribution of the obtained nanocapsules is usually difficult to control (Kothamasu et al, 2012; Musyanovych, K. Landfester, 2014; Shimanovich et al, 2014). To synthesize micro- or nano-containers from biopolymers by using single emulsions, the precursor molecules, commonly proteins, are dissolved or dispersed in the aqueous medium followed by emulsification in a non-aqueous medium such as oil (Kothamasu et al, 2012; Shimanovich et al, 2014; Musyanovych, Landfester, 2012; Elzoghby et al, 2011).

The double emulsion technique allows the production of more complex structures. Like in single emulsion technique, the aqueous protein solution is dispersed in a lipophilic organic continuous phase. The primary single water-in-oil emulsion obtained in this manner is then added to an aqueous solution of a second biopolymer that will then from the outer shell of the capsule (Kothamasu et al, 2012; Shimanovich et al, 2014; Musyanovych, Landfester, 2012; Elzoghby et al, 2011; Qian et al, 2015).

Through the choice of monomers/polymers and chemical reactions used to form nanocapsules with a hydrophilic or hydrophobic core, the possibilities are unlimited. Moreover, the surface of nanocapsules can also be easily functionalized with biomolecules for specific targeting or biosensing. Thus, this technique gives the opportunity to produce capsules with the desire properties for a broad range of bioapplications (Kothamasu et al, 2012).

A class of materials that has emerged as being particularly promising in that of biocompatible polymers, protein capsules and hybrid polymer-protein capsules. These structures consist of a polymeric shell with dimensions on the micro- or nanoscale encapsulating active species in their interior. Multiple synthetic pathways are available to generate these materials, and a crucial challenge is the engineering of the precursor components and the assembly pathway to generate capsules with the desired properties (Devulapally and Paulmurugan, 2014, Shimanovich et al, 2014)

1.3.3. Properties of Nanocapsules

1.3.3.1. Particle size

The physical and chemical properties of nanomaterials depend not only on the composition but also on the particle size. This last and size distribution are the most important characteristics of nanoparticle systems. They determine *in vivo* distribution, biological fate, toxicity and the targeting

ability oh nanoparticle systems. Also, they can influence the drug loading, drug release and stability of nanoparticles (Khanbabaie and Jahanshahi, 2012; Shimanovich et al, 2012)

Generally nanoparticles have relatively higher intracellular uptake compared to microparticles and available to a wider range of biological targets due to their small size and relative mobility. Desai et al have demonstrated that 100nm size nanoparticles showed 2.5 fold greater uptake compared to a 1μm microparticles and 6 higher uptake compared to 10μm microparticle in Caco-2 cell line (M. Desai et al, 1997). Thus, particle size significantly affects cellular and tissue uptake and in some cell lines, only submicron nanoparticles can be taken up efficiently but not the larger size particles. Smaller particles have larger surface area, therefore, most of the drug associated would be at or near the particle surface, leading to fast drug release. On other hand, larger particles have large cores which allow more dug to be encapsulated and slowly diffuse out. Smaller particles also have greater risk of aggregation of particles during storage and transportation on nanoparticle dispersion. It is always a challenge to formulate nanoparticles with the smallest size possible but maximum stability (Khanbabaie and Jahanshahi, 2012; Shimanovich et al, 2012; Kothamasu et al, 2012).

Particle size can also affect the polymer degradation as the rate of poly (PLGA) polymer degradation revealed an enhancement with an increase in particle size *in vitro*. Panyam et al prepared PLGA particles with different size ranges and found that the polymer degradation rates *in vitro* were not substantially different for different size particles (Panyam et al, 2003).

Currently, the fastest and most routine method of determining particle size is by photon-correlation spectroscopy or dynamic light scattering. The first method requires the viscosity of the medium to be known and determines the diameter of the particle by Brownian motion and light scattering properties. The results obtained by photon-correlation spectroscopy are usually verified by scanning electron microscopy (SEM or TEM) (Singh and Lillard, 2009).

In the field of nanocapsules, when they are used to carry a drug like small molecules, the chemical and physical nature of the encapsulated drug can affect not only the capsules' size but also the hydrophobicity of the internal part of the capsule. For example, the size dependence of Bovine Serum Albumin (BSA) protein capsules has been shown to be affected by the length of encapsulated RNA molecules. The results showed that the size of the RNA loaded capsules increases with an increase of the number of nucleotides in the RNA chain, *i.e.*, with an increase in the size of the encapsulated molecule (Shimanovich et al, 2011). Regarding to albumin nanocapsules, Loureiro et al suggested, recently, a novel method that enables the fabrication of highly stable albumin emulsions in the nano-size range, greatly desirable for controlled drug delivery (Loureiro et al, 2015).

1.3.3.2. Stability

In order to be effective, nanocapsules must have high stability. That characteristic is dependent on the protein sequence and size, and may be enhanced by the presence of surfactant molecules that are added to the precursor mixture.

The stability of mixed proteins capsules can also be enhanced through the addition of a biocompatible synthetic polymer to the mixture of proteins. For example, the silk fibroin capsules, prepared by the phase separation method, were showed to be stabilized by polyvinylalcohol polymer (PVA). This stabilization can in part originate from the change in the secondary structure observed for silk fibroin following the addition of PVA. A further agent hat has been shown to stabilize protein capsules is polyethylene glycol (PEG). In addition to the enhanced stability, the interactions of the capsules with biological systems are modified by pegylation (Shimanovich et al, 2014).

As potential delivery systems, an important parameter of interest of protein capsules is their ability to interact with biological systems and in particular their biocompatibility. So, the principal goal is to develop chemistry, which is compatible both with the structural requirements as well as minimizing the toxicity of the capsules. The use of potentially toxic covalent cross-linkers will lead to capsule stabilization but at the same time can induce an increase in toxicity of the resulting capsule.

1.3.3.3. Surface Properties

When nanoparticles are administered intravenously, they are easily recognized by the body immune systems and are then cleared by phagocytes from the circulation. Apart from their size, the surface hydrophobicity of nanoparticles determines the amount of adsorbed blood components, mainly proteins. This in turn influences the *in vivo* fate of nanoparticles. Binding of these proteins into the surface of nanoparticles called opsonization acts as a bridge between nanoparticles and phagocytes. The association of a drug to conventional carriers leads to modification of the drug biodistribution profile, as it is mainly delivered to the mononuclear phagocytes system (MPS) such as liver, spleen, lungs and bone marrow. Actually, once in the blood stream, surface non-modified nanoparticles (conventional nanoparticles) are rapidly opsonized and massively cleared by the macrophages of MPS rich organs (Singh and Lillard Jr., 2009; Mohanraj and Chen, 2006; Bamrungsap et al, 2012).

So, in view of drug targeting by means of nanocapsules, it is necessary to diminish opsonization and to prolong the circulation oh nanoparticles *in vivo*, which is succeeded by (a) surface coating of nanocapsules with addition of hydrophilic polymers and/or hydrophilic surfactants and, (b) formulation of nanocapsules with their biodegradable copolymers with hydrophilic segments such as PEG, polyethylene oxide and polysorbate 80 (Tween 80) (Shimanovich et al, 2014).

The zeta potential of nanoparticle is commonly used to characterize the surface charge property of nanoparticles. It reflects the electrical potential of particle and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above 30mV have been shown to be stable in suspension as the surface charge prevents aggregation of the particles. The zeta potential is also used to determine whether a charged active material is encapsulated within the center of the nanocapsules or adsorbed onto the surface (Khanbabaie and Jahanshahi, 2012; Shimanovich et al, 2014; Bamrungsap et al, 2012).

1.3.3.4. Drug Loading

Ideally, a successful nanoparticle system should have a high drug-loading capacity thereby reduce the quantity of matrix materials for administration. Drug loading can be done by (a) incorporating at the time of nanoparticles production – incorporation method or, (b) absorbing the drug after formation of nanoparticles by incubating the carrier with a concentrated drug solution – adsorption/absorption method (Khanbabaie and Jahanshahi, 2012; Shimanovich et al, 2014)

Drug loading and entrapment efficiency very much depend on the solid-state drug solubility in matrix material or polymer which is related to the polymer composition, the molecular weight, the drug-polymer interaction and the presence of end functional groups (ester or carboxyl). For small molecules, studies show the use of ionic interaction between the drug and the matrix materials can be a very effective way to increase the drug loading (Khanbabaie and Jahanshahi, 2012; Shimanovich et al, 2014).

1.3.3.5. Drug Release

To achieve a successful nanoparticle systems both drug release and polymer biodegradation are important consideration factors. Generally, drug release rate depends on (a) solubility of drug, (b) desorption of the surface bond/adsorbed drug, (c) drug diffusion through the nanoparticle matrix, (d) nanoparticle matrix erosion/degradation process, (e) combination of erosion/diffusion. So, solubility, diffusion and biodegradation of the matrix materials govern the release process. If the nanoparticle is coated bye polymer, the release is then controlled by diffusion of the drug from the core across the polymeric membrane. The membrane coating acts as a barrier to release, therefore, the solubility and diffusivity of drug in polymer membrane becomes determining factor in drug release (Mudshinge et al, 2011; Singh and Lillard Jr., 2009; Mohanraj and Chen, 2006).

Lots of methods can be used to study the *in vitro* release of the drug, for example (a) side-by-side diffusion cells with artificial or biological membranes, (b) dialysis/reverse dialysis bag diffusion technique, (c) agitation followed by ultracentrifugation/centrifugation, (d) ultra-filtration or centrifugal ultra-filtration techniques. Usually the release study is carried out by controlled agitation followed by centrifugation but the dialysis technique is generally preferred due the time-consuming nature and technical difficulties encountered on the agitation method (Singh and Lillard Jr., 2009).

2. Materials and Methods

2.1. LNA – AON

The short (16-mer) antisense DNA oligonucleotide targeting exon 51 in dystrophin transcript – 5'-AGGAAGATGGCATTTC-3' – was purchased from Exiqon. It contains a fully phosphorothioate modified backbone and 60% LNA-modified nucleotides, with two LNA-modified nucleotides at the 3'-end and at the 5'-end. From previous (unpublished) experiments from our group, that compared different sequences and lengths of LNA-AONs, this LNA-AON was selected for this study because it presented the greatest capability of inducing skipping of exon 51 in myoblast derived human cell lines.

2.2. pEGFP-NLS amplification and purification

The reporter construct used in this study is pEGFP-NLS (Enhanced Green Fluorescent Protein) constructed by Calado and coworkers. This reporter gene has a Nuclear Localization Signal (NLS) (the SV40 large T-antigen NLS with the sequence PKKKRKV) which is an amino acid sequence that allows signal the a protein for import into a cell nucleus so the fluorescent signal we see on the microscope has a specific pattern (A. Calado et al, 2000). The bacterial strain used for amplification was *Escherichia coli* DH5α cells (Invitrogen) which is the most frequently used *E. coli* strain for routine cloning applications. *Escherichia coli* DH5α bacterial strain was transformed under Kanamycin (600 ng/µL) selection. Plasmid DNA from a 200mL bacterial culture was purified with Genopure Plasmid Midi Kit (Roche) according manufacturer instructions and further purified with UltraPure™ Phenol:Chloroform: Isoamyl Alcohol (Invitrogen, Cat. No. 15593-031, Lot. No. 2725C279).

The final concentration of the pEGFP-NLS was quantified by spectrophotometry, NanoDrop™ 1000 (Thermo Scientific, model: ND-1000) and the result was 571.8 ng/µL in a total of 100µL.

2.3. BSA Nanocapsules

The reporter construct pEGFP-NLS was encapsulated on BSA nanocapsules by Gonçalo Bernarde's laboratory at the Department of Chemistry, University of Cambridge (Shimanovich et al, 2014).

The stability of the BSA nanocapsules was assessed (after one month) by electrophoresis analysis. Naked plasmid, pEGFP-NLS/BSA nanocapsules and BSA nanocapsules (1µg each) were run for 60 minutes at 80 V on 1% agarose gel on TAE 1x buffer. The results were compared with a similar experiment after protein hydrolysis with proteinase K (Qiagen) for 10 minutes at 55ºC (according to manufacturer's instruction). The gel was photographed using gel documentation ChemiDoc™ XRS+ (Bio-Rad, model: XRS+) and digital images were obtained with Image Lab software (Bio-Rad).

In order to visualize pDNA encapsulated on the nanocapsules, pEGFP-NLS/BSA nanocapsules were added to poly-L-lysine coated glass coverslips and incubated with 1 µg/ml nucleic acid stain

Hoechst 33342 (Invitrogen, Cat. No. H3570) for 10 minutes at RT and with protein stain Coomassie® Brilliant Blue R-250 (Bio-Rad, Cat. No. 161-0400) for 10 minutes at room temperature. Coverslips were mounted on microscopy slides with mounting medium (Vectashield, Vector Laboratories, Cat. No. H-1000).

2.4. Cell culture

Human Embryonic Kidney 239 cells (HEK 293, ATCC® CRL-1573™) were maintained in complete medium consisting of in Dulbecco's Modified Eagle Medium (DMEM medium) (Gibco®, Cat. No. 41966-029) 10 % FBS (Fetal Bovine Serum, Gibco®, Ref. 10270-106) at a humidified atmosphere of 5 % CO_2 at 37ºC. For microscopy experiment cells were grown exceptionally on glass coverslips coated with poly-L-lysine (0.01% solution, Sigma-Aldrich®, Lot. RNBD4661).

2.5. *In vitro* transfection

2.5.1. pEGFP-NLS transfection.

In the transfection experiment, HEK 293 cells were plated in 6 well plates at a density of $2x10^5$ cells/well. Cells were transfected 1 day after being plated with Lipofectamine®3000 (Invitrogen, Ref. L3000-001, Lot. 1660194) according to manufacturer instructions. 0.067µg of plasmid DNA conjugated to 1µL of transfection reagent was added per well to 1.5mL of culture medium. Cells were incubated for 24 – 48 hours before subsequent analysis. The transfection of 0.013µg of plasmid DNA conjugated with 1µL of transfection reagent per well was also tested with good results. Negative control was done with no DNA.

2.5.2. pEGFP-NLS/BSA nanocapsules transfection.

In the transfection experiment with pEGFP-NLS/BSA nanocapsules, the equivalent of 0.067µg plasmid DNA, conjugated to 1µL of transfection reagent was added to each well. Negative control was the correspondent amount of BSA empty nanocapsules. Transfection with pEGFP-NLS/BSA nanocapsules was also tested eliminating the transfection reagent. In this case, in the same conditions 0.067µg of plasmid DNA with no transfection reagent was added per well. As a control naked pEGFP-NLS plasmid was used.

2.6. *In vivo* experiments

2.6.1. Animals

The exon 52-deficient X chromosome-linked muscular dystrophy mice (mdx52) were gently provide by Shin'ichi Takeda from National Center of Neurology and Psychiatry, Japan. These mice have been backcrossed to the C57BL/6J (WT) strain for more than eight generations (Aoki et al, 2012); WT C57BL/6J mice were purchased from Charles River. All animal care and experimental procedures were reviewed and ethically approved by the IMM Animal Ethics Committee and

Portuguese competent authority for animal protection, Direcção Geral de Alimentação e Veterinária, Lisbon, Portugal.

2.6.2. LNA injection

To evaluate the capacity of the LNA-AON in restoring dystrophin production *in vivo*, 13-week-old *mdx52* mice were intravenously injected once in tail vein with 10mg/kg of LNA-AON (treated) or saline solution (control). The mice were sacrificed 11 weeks after injection with Eutasil (CEVA Santé Animale, France). *Tibialis anterior* muscle was isolated immediately, snap frozen in liquid N_2-cooled isopentane and stored at -80ºC for immunohistochemistry analysis.

2.6.3. pEGFP-NLS/BSA nanocapsules injection

To evaluate the *in vivo* transfection efficiency three hundred microliter of the pEGFP-NLS/BSA nanocapsules (containing 20µg pEGFP-NLS) was intravenously injected once in tail vein of 4 mice. Two mice were injected with three hundred microliter of BSA nanocapsules solution (negative control). At 48h following injection, mice were sacrificed with Eutasil (CEVA Santé Animale, France). Liver and lungs were collected. A portion of each liver was collected, snap frozen in liquid N_2-cooled isopentane and stored at -80ºC for immunohistochemistry analysis. The remaining liver and the lungs were kept in PBS until being processed for flow cytometry analysis.

2.7. Flow cytometry

Prepared single cell or particle suspensions are necessary for flow cytometry analysis. The suspension of cells or particles is aspirated into a flow cell where, surrounded by a narrow fluid stream, they pass one at a time through a focused laser beam. The light is either scattered or absorbed when it strikes a cell. Absorbed light of the appropriate wavelength may be re-emitted as fluorescence if the cell contains a naturally fluorescent substance or one more fluorochrome-labeled antibodies are attached to surface or internal cell structures. Light scatter is dependent on the internal structure of the cell and its size and shape. Light and/or fluorescent scatter signals are detected by a series of photodiodes and amplified. The resulting electrical pulses are digitized and the data is stored, analyzed and displayed through a computer system. The end result is quantitative information about every cell analyzed.

2.7.1. Preparation of cells from *in vitro* culture

Approximately $1x10^6$ transfected culture cells were washed with PBS, trypsinized and re-suspended in 1ml of DMEM supplemented with 10 % FBS. The supernatant was removed after centrifugation at 1000rpm for 5 minutes at Eppendorf Centrifuge 5804 (Eppendorf) and cells were re-suspended in 500µL of DMEM supplemented with 10% FBS, in order to achieve approximately 2 million cells per millilitre for flow cytometry analysis.

2.7.2. Isolation of cells from mouse tissue

The harvested mouse organ (liver or lungs) was pressed against a nylon mesh cell strainer of 70µm (Falcon, Ref. 352350) mounted on top of a 50mL Falcon tube and PBS was added to facilitate this process. The dispersed cells collected at the bottom of the tubes were diluted and centrifuged for 5 minutes at 1000 rpm on Eppendorf® Minispin® (Eppendorf).

2.7.3. Flow cytometry analysis

Flow cytometry analysis were performed using FACSCalibur cytometer (BD Biosciences) which is equipped with two excitation lasers (488nm and 635nm) and four fluorescence channels: green (FL1), yellow (FL2) and red (FL3 and FL4). About 300µl of cell suspension samples were analysed to detect GFP signal, using laser 488 and detector FL1 for *in vivo* and *in vitro* experiments. Signal acquisition was accessed using CellQuest™ software (BD Biosciences). A gate of 10 000 viable cells was selected. The quantification analysis was performed with FlowJo 8.7 Software (Tree Star, Inc. 1997-2012).

2.8. Fluorescent Immunodetection

Immunohistochemistry is a technique that allows to identify tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context.

Indirect method involves an unlabelled primary antibody (first layer) which react with tissue antigen, and a labelled secondary antibody (second layer) react with primary antibody. This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second layer antibody can be labelled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called indirect immunofluorescence method.

2.8.1. Preparation of cells from *in vitro* culture

Cells grown on coverslips and transfected were fixed with 3.4% PFA (Paraformaldehyde) for 10 minutes RT, washed with PBS, mounted on slides and observed by fluorescence microscopy.

2.8.2. Immunohistochemical analysis of dystrophin expression

To access dystrophin expression, ten-micrometer thick transversal frozen sections of *tibialis anterior* from C57BL/6J wild type, m*dx52* negative control and *mdx52* treated mice with 10mg/kg LNA-AON were fixed with cold acetone for 10 minutes, permeabilized with 0.5% Triton X-100 on PBS, at room temperature for 10 minutes, incubated with blocking solution (1% BSA, 0.05% Tween 20) for 30 minutes in humid chamber, labelled with a rabbit polyclonal anti-dystrophin antibody (Abcam®, Cat. No. ab85302), diluted 1:100 in blocking solution, for 60 minutes and a secondary antibody anti-rabbit tetramethylrhodamine (TRITC) – conjugated affinity pure donkey (Jackson Imunoresearch

Laboratories, Cat. No. 711-025-152), diluted 1:200 in blocking solution, for 60 minutes in humid chamber. The cellular nucleus was counterstained with DAPI (Sigma-Aldrich, Cat. No. D9542-5MG) with a final concentration of 1µg/ml, by 10 minutes incubation at RT. Coverslips were then mounted with antifade mounting medium (Vectashield, Vector Laboratories, Cat. No. H-1000).

2.8.3. Immunohistochemical analysis of GFP expression from *in vivo experiments*

To evaluate the transfection efficiency of pEGFP-NLS/BSA nanocapsules *in vivo,* ten-micrometer thick transversal frozen sections of liver from *mdx52* mice intravenously injected on tail vein with pEGFP-NLS/BSA nanocapsules (containing 20µg pEGFP-NLS) and 300µL of BSA nanocapsules solution (negative control) were fixed with 3.7% PFA on PBS for 10 minutes at room temperature, permeabilized with 0.5% Triton X-100 on PBS for 10 minutes, incubated with blocking solution (1% BSA, 0.05% Tween 20) for 30 minutes in humid chamber, labelled with a rabbit polyclonal anti-GFP antibody, Alexa Fluor® 488, (Termofisher, Cat. No. A-21311), diluted 1:200 in blocking solution, for 60 minutes and with a TRITC antirabbit secondary antibody (Jackson Imunoresearch Laboratories, Cat. No. 711-025-152), diluted 1:200 in blocking solution, for 60 minutes in humid chamber. The cellular nucleus was counterstained with DAPI (Sigma-Aldrich, Cat. No. D9542-5MG) with a final concentration of 1µg/ml, by 10 minutes incubation at RT. Coverslips were then mounted with antifade mounting medium (Vectashield, Vector Laboratories, Cat. No.H-1000).

2.9. Fluorescence Microscopy

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime. Ultraviolet (UV) light of a specific wavelength is produced by passing light from an UV-emitting source through the exciter filter. The filtered light illuminates the sample, which emits fluorescent light when illuminated by the UV light. Visible light emitted from the sample is then filtered through a barrier filter that does not allow reflected UV light to pass. Fluorescence microscopy allows the precise location of intracellular components labeled with specific fluorochromes and can reveal the presence of fluorescing material with exquisite sensitivity.

Digital images from immunohistochemical analysis of dystrophin expression were captured using LEICA DM5000B Widefield Fluorescence Microscope (Leica Microsystems) with 40x dry objective (objective type: PL FLUOTAR), using Filterset Red (515-560nm) for dystrophin detection.

Digital images from transfected HEK293 cells were captured using LEICA DM5000B Widefield Fluorescence Microscope (Leica Microsystems) with 40x dry objective (objective type: PL FLUOTAR), using Filterset blue (320-400nm) for DAPI detection and green (500-550nm) for GFP detection. This microscope has a monochrome CCD camera for fluorescence image acquisition and IrfanView as image acquisition software.

Digital images from immunohistochemistry analysis of GFP expression from *in vivo* experiments were captured using a Zeiss LSM 710 Confocal Point-Scanning Microscope (Carl Zeiss MicroImaging) with 400x magnification using the lasers Diode 405-30 for DAPI detection and DPSS 561-10 for GFP detection. Digital images were analysed with the software Image J and LSM 5 Image Browser (Zeiss).

Digital images of BSA nanoparticles encapsulating pEGFP-NLS were captured with 63x oil objective (model: Plan-Apochromat) using a Zeiss LSM 710 Confocal Point-Scanning Microscope (Carl Zeiss MicroImaging).

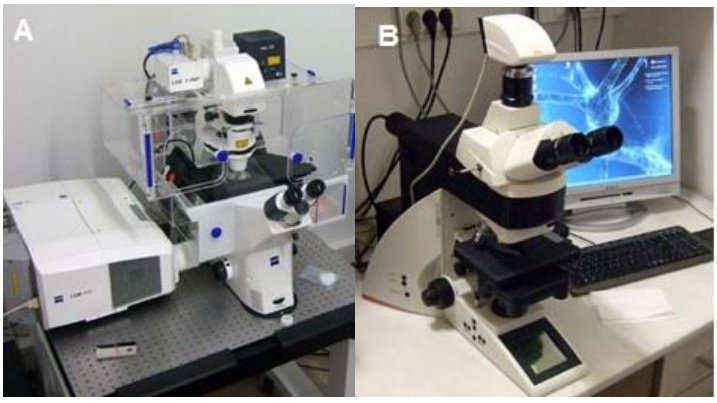

Figure 10 – (A) Zeiss LSM 710 Confocal Point-Scanning Microscope; (B) LEICA DM5000B Widefield Fluorescence Microscope

3. Results

3.1. LNA – AON to treat DMD in animal model

The majority (~65 %) of Duchenne Muscular Dystrophy (DMD) patients carries a deletion of one or multiple exons on X chromosome of *DMD* gene and these deletions cluster in hotspot regions (70 % of the deletions are located between exon 43-55). The skipping of exon 51 would be beneficial to the largest group of patients (~13 %). *mdx52* mouse model was generated with the purpose of studying the therapeutically application of inducing skipping of exon 51 since this strain has a deletion of exon 52. Concerning to therapeutics, using of antisense oligonucleotides (AONs) has showed proof of concept restoring the reading frame of *DMD* gene by exon skipping (Aartsma-Rus, 2010; Aoki et al, 2012). Recent publications suggest the use of LNA-AONs to induce exon skipping (McClorey et al, 2015; Obad et al, 2011). In our group, a class of AONs, LNA-modified oligonucleotides, is being tested to induce skipping of exon 51, to restore the reading frame of *DMD* gene. Exon skipping was successfully achieved *in vitro*, in cultured myoblasts. Therefore, this LNA-modified oligonucleotides was tested on *mdx52* mouse model.

In our experiment, *mdx52* animals were intravenously injected once in tail vein with 10mg/kg of LNA-AON (treated) or saline solution (negative control). 11 weeks after injection, mice were euthanized and *tibialis anterior* muscle was collected. C57BL/6J mice muscles were also collected to be used as a positive control. Immunohistochemical staining of muscle cryosections was accessed and dystrophin production was evaluated through fluorescence microscopy

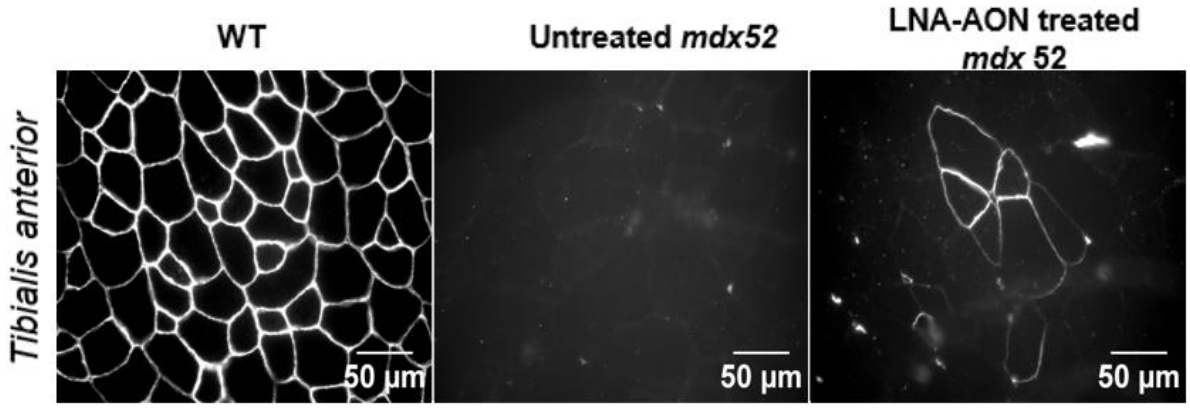

Figure 11 – Immunohistochemical findings in *tibialis anterior*. Dystrophin immunolabeling in muscle fibers. Representative fields of transversal sections from C57BL/6J wild type, untreated *mdx52* mice and LNA-AON (10mg/kg) treated *mdx52* mice, labelled with a rabbit polyclonal anti-dystrophin antibody, demonstrating absence of dystrophin in *mdx52* untreated mice and restoration of dystrophin in a group of muscles fibers after treatment with 10mg/kg of LNA-AON, 1 injection analysed 11 weeks after injection.

Immunohistochemistry of C57BL/6J mouse *tibialis anterior* muscle section revealed dystrophin protein localized at the sarcolemmal membrane of muscle fibers as expected, being used prospectively as our positive control. *Tibialis anterior* muscle section from untreated *mdx52* mouse dystrophic model do not show any dystrophin protein since the protein is completely absent in this

mouse model. In *tibialis anterior* sections from LNA-AON treated *mdx52* mice, we could detect some muscle fibers with a positive stain for dystrophin protein. This results suggest a reversal of the phenotype for the production of DMD *in vivo* correctly localized at the sarcolemma. However when compared to *tibialis anterior* from C57BL/6J mouse model the number of corrected fibers is very low.

3.2. Nucleic acid nanoparticles to study delivery in an animal model

The low number of dystrophin positive fibers detected on muscle of LNA-AON treated animals shows that the exon skipping was achieved although it suggested that new delivery systems should be tested to improve delivery efficiency. AONs should be efficiently delivered into tissues and cells in order to reach their target and carry out their functions. However, several major obstacles, namely optimization of nontoxic effective doses, improvement of the delivery systems, distribution to all affected tissues, achievement of a sustainable therapeutic effect and development of an administration strategy suitable for lifelong treatment, still remain to be overcome. One of the major obstacles to efficiently delivery of therapeutic nucleic acids are nucleases. Nucleases are enzymes present at the surface mucosal and in the blood stream which are capable of degrading nucleic acids. Many nucleic acid carriers (liposomes, polymers and peptides) have been proposed to help nucleic acids to overcome those obstacles, but their clinical applicability is plagued by a lack of cell specificity and difficult in drug release. Recently, the focus turned to nanotechnology as a potential delivery system (Falzarano et al, 2014; Chou et al, 2011; Shahbazi et al, 2012; Pichon et al, 2001; Miller, 1998).

Transfection of foreign plasmid DNA or RNA into host cell nucleus to modify, change or silken gene expression is a challenging task. The cellular nuclease enzymes constitute the major obstacle in this task as they can degrade naked plasmid in the cytoplasm before entry into the nucleus leading to low transfection efficiency (Wagh et al, 2014).

A class of materials that has emerged as being particularly promising to help nucleic acid delivery is that of biocompatible protein nanocapsules, such albumin nanocapsules (Devulapally and Paulmurugan, 2014, Shimanovich et al, 2014). Albumin is non-toxic and degradable *in vivo*, so the nanoparticles generated by using it are easily adaptable to the human body (Elzoghby et al, 2011; Jun et al, 2010). Levemir® and Abraxane® are two albumin-based drug delivery systems already approved and commercially available used to control high blood sugar in adults and children with *diabetes mellitus* type 1 and 2 and for the treatment of metastatic breast cancer, respectively (Elsadek et al, 2011).

We proposed to test the efficiency of BSA nanocapsules, produced by Gonçalo Bernardes' laboratory on encapsulating and in an animal model. A well-known genetic construct, pEGFP, was selected as a reporter gene since it is fluorescent. We chose this reporter gene since it has a Nuclear Localization Signal (NLS) allows signal the protein for import into a cell nucleus so the fluorescent signal we see on the microscope has a specific pattern. The efficiency delivery of BSA nanoparticles *in vivo* was accessed on analysing lung and liver. As several studies suggest these organs are usually

primary targets for gene incorporation (Kodama et al, 2014; Kurosaki et al, 2014). Besides, coated or uncoated nanoparticles have a tendency to accumulate in the liver (Khanbabaie and Jahanshahi, 2012).

3.2.1. *In vitro* transfection of nucleic acids nanoparticles to establish analysis techniques

Initially, the transfection efficiency of naked plasmid was tested in Human Embryonic Kidney 293 (HEK293, ATCC® CRL-1573™).

HEK293 cells were transfected with two different amounts of pEGFP-NLS (0.013µg and 0.067µg). 48h after transfection the coverslips placed ate the bottom of each well were mounted and slides were observed under fluorescent microscopy. The quantification method of transfection efficiency was cell counting through merging of phase contrast and fluorescence images (Figure 12, A). A total of 641 cells were counted for transfection with 0.013µg plasmid, resulting in an average of 8.61 % GFP positive cells (S.D. 1.49). Concerning to cells transfected with 0.067µg of plasmid DNA, a total of 2944 cells were counted, resulting in an average of 14.21 % (S.D. 5,04) positive cells for GFP (Figure 12, B).

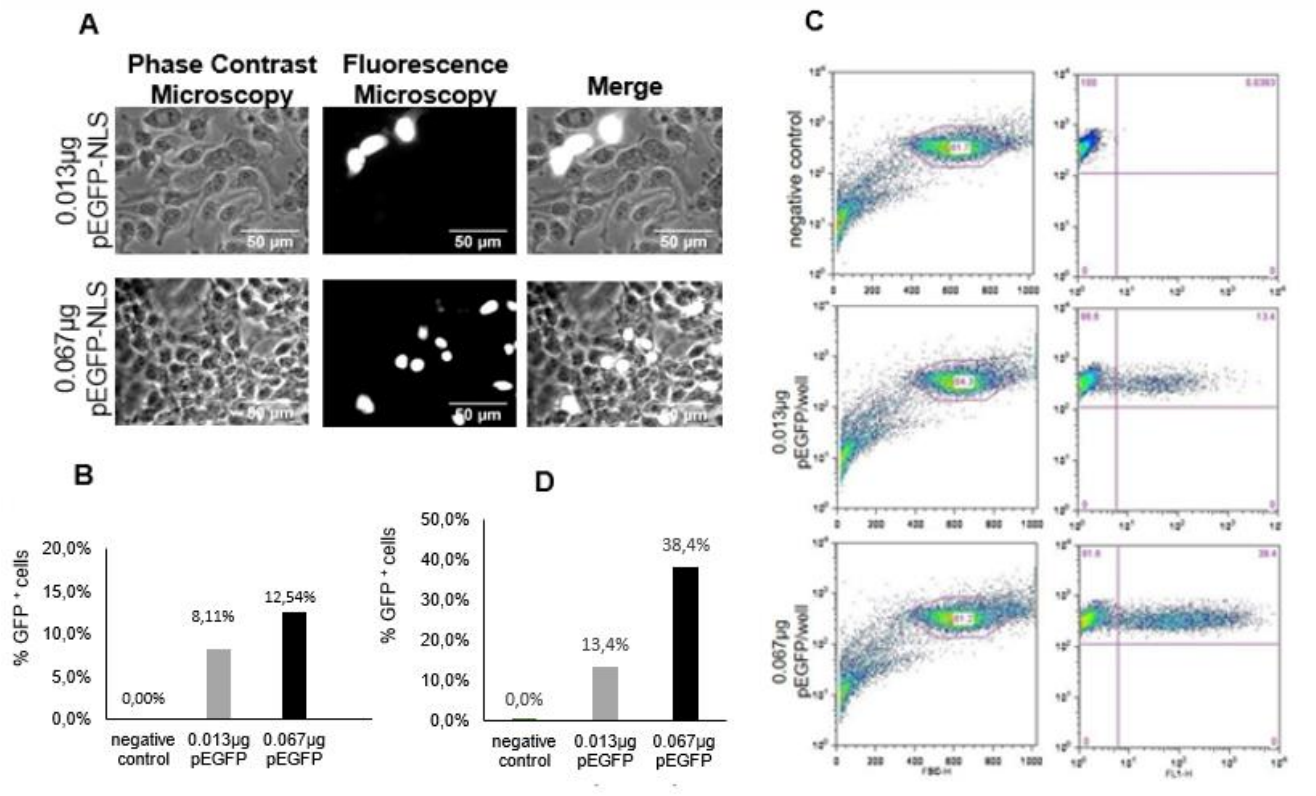

Figure 12 - Transfection efficiency on HEK 293 cells transfected with 0µg pEGFP-NLS/well, 0.013µg pEGFP-NLS/well and 0.067µg pEGFP-NLS/well. A – Fluorescence and phase contrast microscopy of the transfected HEK 293 cells as a 400x magnification; B– Results of microscopy analysis through cell counting (total of 2944 cells counted), C – Representative flow cytometry plot of viable cells and GFP$^+$ cells; D - Results of flow cytometry analysis approximately 10 000 detected events for each experiment.

In flow cytometry (Figure 12, C), 10 000 events were collected and analysed inside a gate of viable cells (set to every experiments) for each transfection experiment using Cell Quest. Data analysis was done with FlowJo software. In negative control, 61.2% of all events were considered as viable cells and GFP expression was then accessed inside that gate. As expected, no positive cells for GFP were found, consisting with results from fluorescence microscopy. For HEK293 cells transfected with 0.013µg and 0.067µg, the same gate was established, resulting in 63.9% and 60.8% of viable cells respectively. Concerning to GFP expression, results show that transfection was more efficiently for cells transfected with 0.067µg pEGFP-NLS (38.4 %), although transfection efficiency with 0.013µg pEGFP-NLS was also successfully achieved (13.4 %) (Figure 12, D). Thus, this experiment shows that a cell line is efficiently transfected by low concentrations of plasmid DNA, such as 0.013µg. The amount of pEGFP-NLS selected for the following experiments was 0.067µg.

Results obtained by fluorescent microscopy corresponds to results from flow cytometry, so, for the following experiments transfection efficiency was only evaluated by flow cytometry analysis.

Then, HEK293 cells were transfected with pEGFP-NLS encapsulated in BSA nanocapsules to set positive and negative controls before experiments *in vivo*. In the first experiment with nanocapsules, transfection efficiency of pEGFP-NLS/BSA nanocapsules were tested with and without Lipofectamine® 3000 (transfection agent). To compare efficiencies, cells were also transfected with naked plasmid with Lipofectamine® 3000. The results were analysed with fluorescent microscopy and flow cytometry.

The fluorescent microscopy images suggest that transfection with naked pEGFP-NLS and pEGFP-NLS/BSA was correctly achieved *in vitro*. It is possible to see some fluorescent cells (positive cells for GFP) in both experiments using transfection agent (Lipofectamine® 3000). Transfection of BSA nanocapsules without Lipofectamine® 3000 had the same result as the negative control, once there were not found any positive cells (Figure 13, A). By flow cytometry analysis, the expression of GFP was analysed and quantified in approximately 10 000 viable cells for each experiment. In negative control, 69.2 % of all events were considered as viable cells and GFP expression was then quantified inside that gate. Consisting with microscope images, no fluorescent cells were detected. The 2 experiments using Lipofectamine® 3000 revealed rates of transfection very similar. An average of 5.28 % and 6.3 % of GFP positive cell for transfection with naked plasmid and with BSA nanocapsules, respectively (3 separated measures were done for each experiment) (Figure 13, B). To determine whether the difference between the rates of transfection with naked plasmid and nanocapsules, a statistical Kruskal-Wallis analysis with Dunn's multiple comparisons test was accessed with Graphpad. This test compared the means of 4 experiments and turns out that the difference between cells transfected with naked plasmid and with BSA nanocapsules is not significant (Figure 13, C).

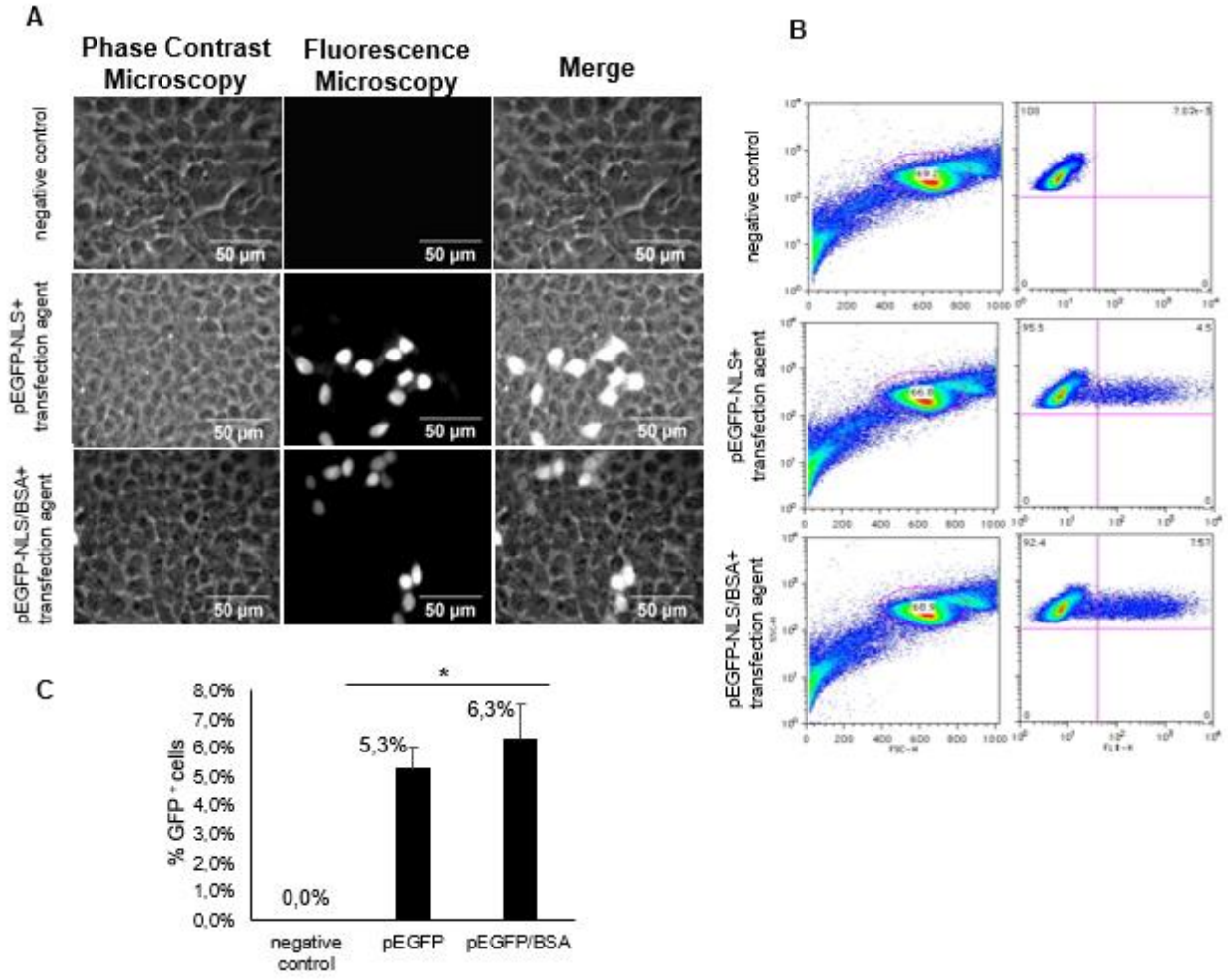

Figure 13 - Transfection efficiency on HEK 293 cells transfected with 0.067µg pEGFP-NLS per well, 0.067µg pEGFP-NLS/BSA per well, 0µg pEGFP-NLS/well with and without transfection agent. A – Fluorescence and phase contrast microscopy of the transfected HEK 293 cells as a 400x magnification; B– Representative flow cytometry plot of viable cells and GFP^+ cells; C - Results of flow cytometry analysis in approximately 10 000 detected events for each experiment. *****: with transfection agent

A second experiment was performed to access transfection efficiently of naked plasmid without transfection agent in HEK293 cells. Images from fluorescence microscopy show a positive transfection for cells transfected with pEGFP-NLS/BSA nanocapsules conjugated to 1µL of Lipofectamine® 3000. Both experiments without transfection agent, revealed no positive cells for GFP (Figure 14, A). Flow cytometry analysis confirms this results. From a selection of approximately 10 000 viable cells, an average of 10.2 % (S.D. 0.02) of GFP positive cells (Figure 14, C).

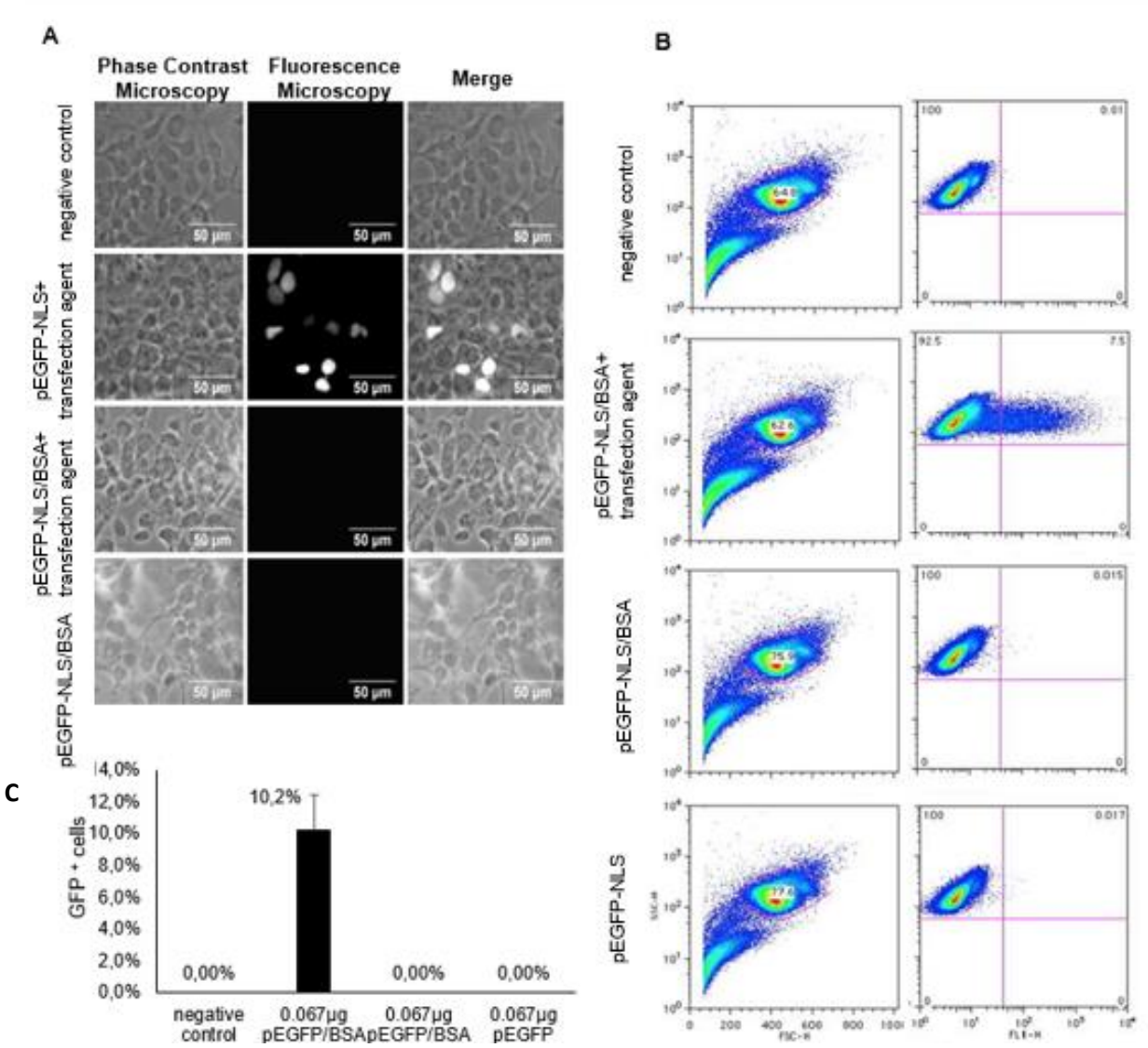

Figure 14 - Transfection efficiency on HEK 293 cells transfected with 0.067µg pEGFP-NLS/BSA per well with and without Lipofectamine® 3000, 0.067µg pEGFP-NLS per well without Lipofectamine® 3000 and 0µg pEGFP-NLS/well. A – Fluorescence and phase contrast microscopy of the transfected HEK 293 cells as a 400x magnification; B– Representative flow cytometry plot of viable cells and GFP^+ cells; C - Results of flow cytometry analysis in approximately 10 000 detected events for each experiment, resulting in an average of 10.2 % (S.D. 0.02) of GFP positive cells.

The results in HEK293 cells shows that transfection was not efficiently when is absence of transfection agent, however results *in vitro* not always reflect results *in vivo*. So, we proposed to test transfection injection in *mdx52* mouse model.

3.2.2. *In vivo* injection

For *in vivo* experiments, *mdx52* mice were intravenously injected once in tail vein with three hundred microliter of pEGFP-NLS/BSA nanocapsules solution, containing 20µg pEGFP-NLS and with three hundred microliter of empty BSA nanocapsules solution 48h after injection mice were euthanized and lung and liver collected. Ten-micrometer thick transversal frozen sections of liver from injected animals were processed to immunohistochemical evaluation and were then observed through

fluorescence microscopy. The remaining collected liver and lungs were processed for flow cytometry analysis.

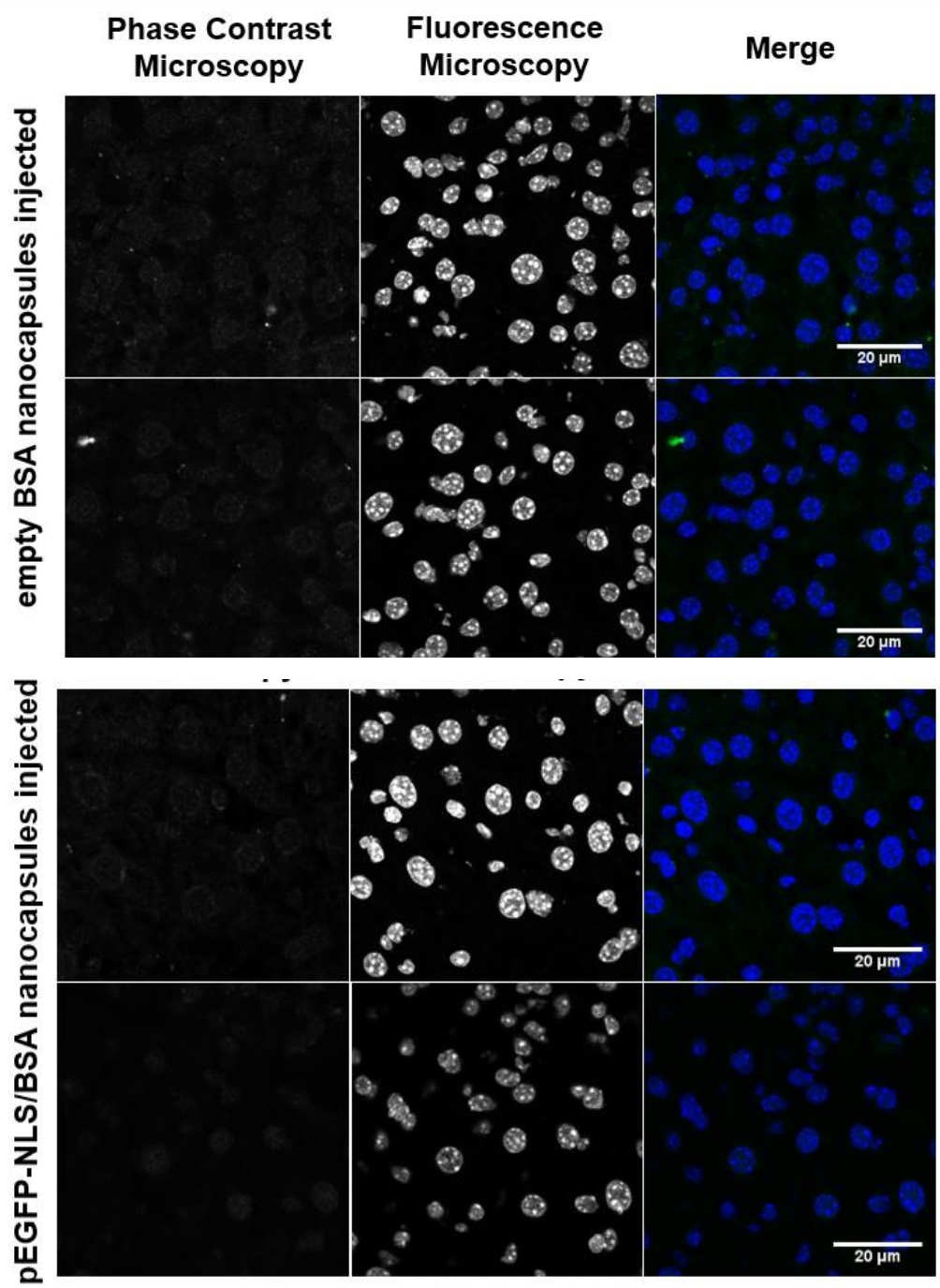

Figure 15 – Immunohistochemical analysis by fluorescent microscopy of *in vivo* GFP gene expression by BSA nanoparticles loading EGFP pDNA. DAPI stained nucleus and GFP fluorescence are showed as blue and green, respectively in merge images. (A) Digital images from liver of *mdx52* mouse model injected with empty BSA nanocapsules solution labelled with primary antibody anti-GFP Alexa 488, secondary antibody TRITC and nuclear staining DAPI. (B) Digital images from liver of *mdx52* mouse model injected with pEGFP-NLA/BSA solution labelled with primary antibody anti-GFP Alexa 488, secondary antibody TRITC and nuclear staining DAPI.

The plasmid selected to test *in vivo* transfection efficiency is a well-known genetic construct, pEGFP, and it was used as a reporter gene since it is fluorescent. This reporter gene has a NLS, so

the fluorescent protein signal we see on microscope has a specific pattern. Frozen sections of liver from injected mice with empty BSA nanocapsules and with BSA nanocapsules encapsulating pEGFP were labelled with primary antibody anti-GFP Alexa 488, secondary antibody TRITC and DAPI for nuclear staining. Fluorescent microscopy images from animals injected with negative control solution (Figure 15, A) do not show positive cells for GFP in the green channel. Nuclear staining with DAPI was successfully achieved and so it is possible to see blue fluorescence for DAPI in the nuclei of liver cells. Merging images from green channel and DAPI shows that no GFP positive signal was found at cells nuclei. The same results were found for animals injected with pEGFP-NLS/BSA nanocapsules (Figure 15. B). We expected to see green signal inside cells nuclei, but merging images of green channel and DAPI suggests that pEGFP was not expressed, so we do not seen GFP positive signal inside cell nuclei.

Transfection efficiency of pEGFP-NLS/BSA nanocapsules in liver and lung of injected *mdx52* mouse model was also accessed by flow cytometry analysis.

48 hours after injection, isolated cells of liver and lung from mdx52 mouse model were collected and analysed by flow cytometry for GFP+ cells. The transfected cells were used as negative and positive controls to evaluate in vivo transfection efficiency (Figure 16, A). Concerning to liver of animals injected with empty BSA nanocapsules and animals injected with pEGFP-NLS/BSA nanocapsules (Figure 16, B), a gate of approximately 10 000 viable events were selected for both of them. Inside the defined gate no GFP fluorescence was detected by cytometer, as there are no significant differences between results for animals injected with empty BSA nanocapsules and animals injected with pEGFP-NLS/BSA nanocapsules. The same results were found in lung of animals injected with empty BSA nanocapsules and with pEGFP-NLS/BSA solution (Figure 16, C).

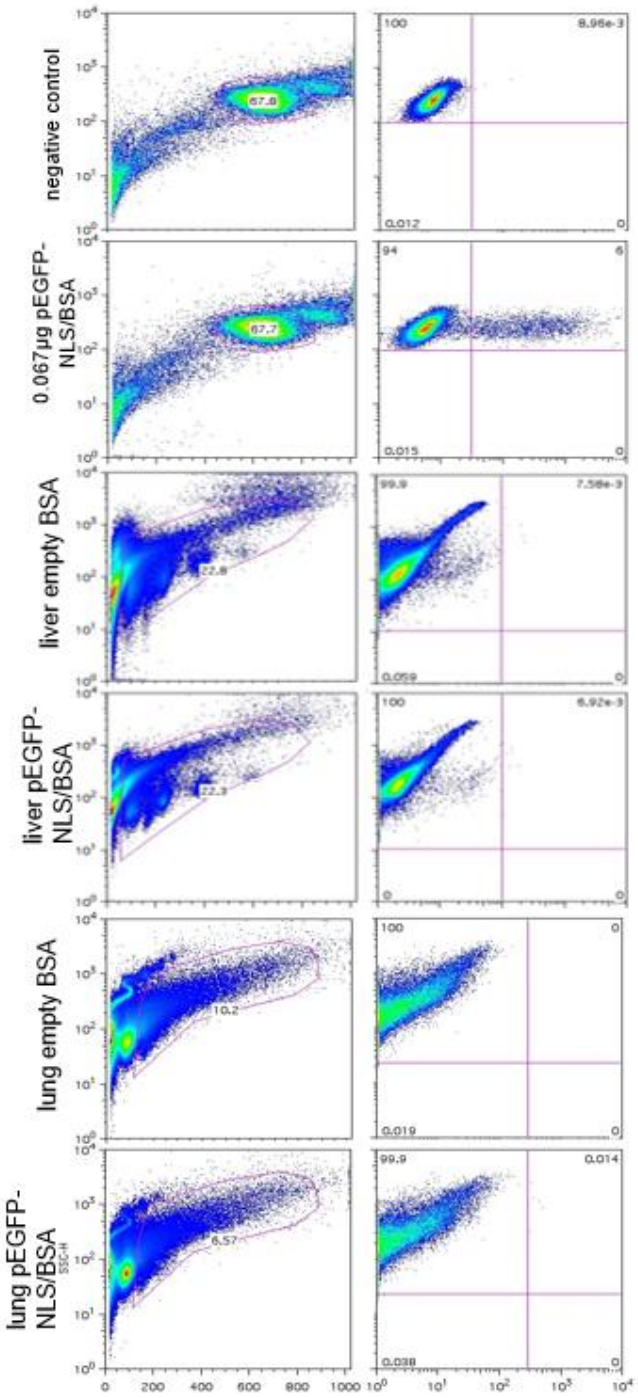

Figure 16 - Flow cytometry analysis using acquired with Cell Quest showing the % of viable cells and GFP positive cells in approximately 10 000 events; (A) Transfected HEK293 cells as negative and positive control; (B) Results in liver of *mdx52* mouse model injected with empty BSA nanocapsules solution and with pEGFP-NLS/BSA solution were both approximately 0.00%; (C) Results in lung of *mdx52* mouse model injected with empty BSA nanocapsules solution and with pEGFP-NLS/BSA solution were both approximately 0.00%.

3.3. BSA nanocapsules stability assay

Based on results obtained *in vivo*, the stability of BSA nanocapsules was finally accessed by electrophoresis. 1µg of naked plasmid, pEGFP-NLS/BSA nanocapsules and BSA solution were run for 60 mins at 80V on 1 % agarose gel on TAE 1x buffer. As a positive control 1µg of naked plasmid was run in parallel. As a negative control also an equivalent amount of BSA empty nanoparticles.

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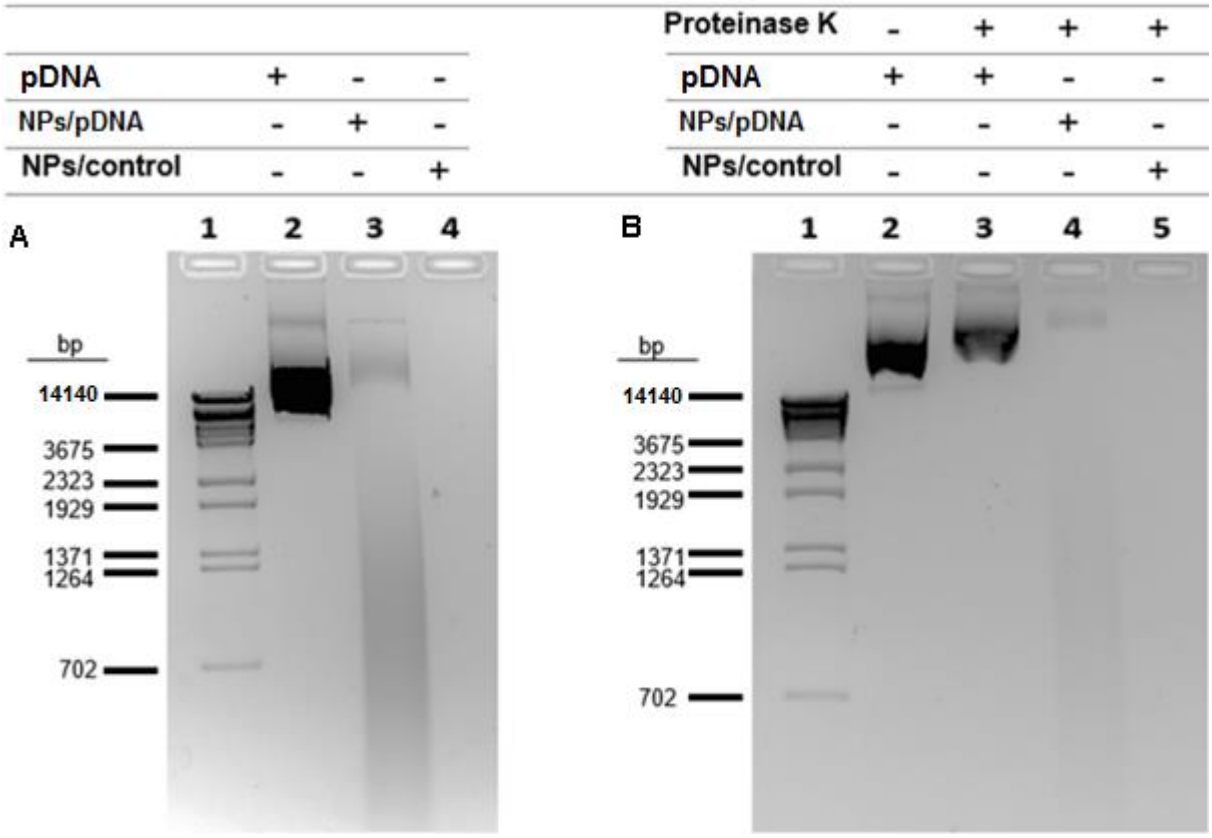

Figure 17 – Agarose gel electrophoresis results of naked plasmid DNA, pEGFP-NLS/BSA nanocapsules and BSA nanocapsules solution after (A) and following Proteinase K digestion (B). Samples were run on a 1 % gel for 60 minutes at 80 V

It can be seen from Figure 17 (A) that the pEGFP-NLS encapsulated in BSA nanocapsules is degraded once it does not show a perfect band as the naked plasmid but most of the observed DNA has a smeared pattern. The BSA nanocapsules does not reveals a band as expected from a negative control. We hypothesised that pDNA encapsulation could be changing the pattern of migration and that the smeared pattern could be due to the fact of pDNA be encapsulated. Thus, we assumed that by digestion of the proteic capsule the released pDNA would show a migration pattern similar to the one of the naked plasmid DNA. Naked plasmid DNA was also digested with proteinase K as a control for pDNA degradation in this assay.

The result obtained for the analysis of the pEGFP-NLS/BSA nanocapsules is the same as in the previous assay, confirming that the smeared pattern was due to pDNA degradation (Figure 17, B).

Images of nanoparticles highlighted by Hoechst 33342 were also obtained through fluorescence confocal microscopy. Hoechst 33342 nucleic acid stain is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to double-stranded DNA (dsDNA). It was possible to see the proteic nanoparticle in phase-contrast (Figure 18, A) overlapping with the fluorescent signal of the nucleic acid stain (Figure 18, B and C). The size of visualized nanoparticles is variable but less than 1µm (Figure 18, D).

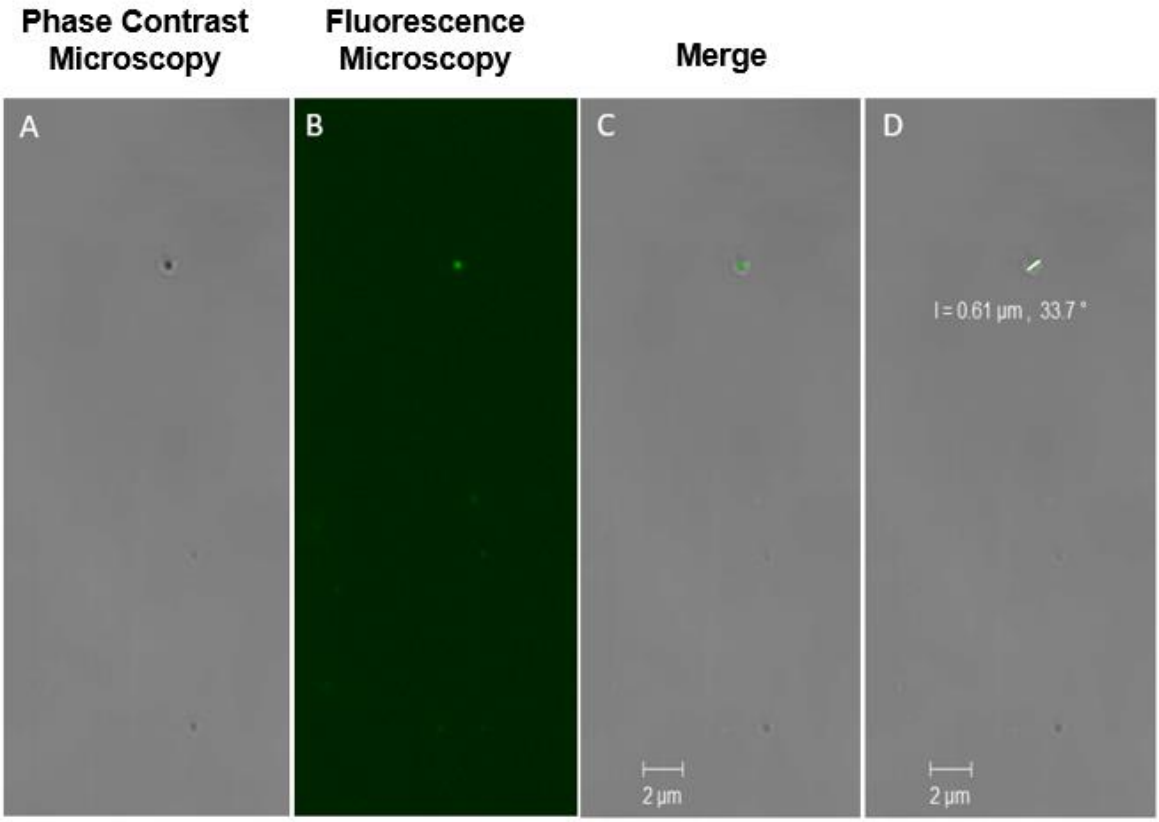

Figure 18 – Encapsulated pDNA on BSA nanoparticles can be visualized by fluorescence microscopy. pEGFP-NLS/BSA nanocapsules were stained with Coomassie blue and with Hoechst 33342 for protein and nucleic acid stain, respectively, and imaged on the confocal fluorescence microscope. The protein capsule can be seen on the phase contrast image overlapping with a blue fluorescent signal from Hoechst that is here intentionally depicted in green to better visualization on the image. The same field is illustrated in (A) Phase-contrast; (B) fluorescence and (C) merging of former images.

4. Discussion

Antisense oligonucleotides (AONs) have conceptually a great potential for the treatment of various diseases, such as the X-linked inherited Duchenne Muscular Dystrophy (DMD). Conversion of an out-of-frame transcript into an in-frame transcripts that codes for a functional protein is the goal of the antisense-mediated exon skipping strategy for DMD. Inducing the skipping of specific exons to restore the reading frame of *DMD* gene has been successfully applied in cultured muscle cells from DMD patients and in the *mdx* mouse model (Aartsma-Rus et al, 2004). Modulation of splicing using AONs is an attractive strategy for the treatment of DMD however relatively few studies have used LNA AONs compared to those using AONs based on other chemistries. Locked nucleic acid (LNA) is an artificial nucleic acid derivative that was synthesized by Wengel and coworkers in 1999 and has a methylene bridge that binds the 2' O to 4'C which improves duplex stability to complementary DNA or RNA by enhancing base stacking properties. LNAs are nuclease-resistant, nontoxic and have the highest affinity for complementary DNA and RNA yet reported for any DNA analog. Given these properties, we proposed to test the capacity of induce skipping of an LNA-AON, – 5'-AGGAAGATGGCATTTC -3' –, in *mdx52* mouse model. After mice injections, dystrophin expression in *Tibialis anterior* muscle were analysed by fluorescent microscopy. The obtained results revealed dystrophin positive muscle fibers, suggesting a reversal of the phenotype for the production of dystrophin protein. Thus, using of LNA-AONs to induce exon skipping as an approach to treat Duchenne Muscular Dystrophy is a promising strategy, however, the systemic delivery to the muscles has proved challenging.

Controlled delivery of therapeutics agents, such drugs or nucleic acids, 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. Over the last two decades, nucleic acids have emerged as versatile therapeutics agents, due to their ability to target and interfere with the flow of genetic information from DNA to protein (Falzarano et al, 2014). However, transfection of foreign DNA or RNA into a host cell nucleus to modify, change or silence gene expression still a challenge. The cellular nuclease enzymes constitutes the major obstacle in this task as they can degrade nucleic acids in the cytoplasm before entry into the nucleus leading to low transfection efficiency. A possible solution to overcome this barrier consists in trapping such therapeutic agent in a specialized delivery vehicle able to protect nucleic acids from external threats (Wagh et al, 2014). Liposomes, polymers and cell-penetrating peptides have all been proposed as such vehicles, however lack of cell specificity and difficulties in controlling drug release plagued their clinical application. Is this requirement that leads researchers to explore new strategies and new solutions in the field of delivery systems (Falzarano et al, 2014). Nanotechnology emerges has a potential and promising solution to overcome such issues, by the development of nanoparticles (1-100nm) as vehicles to controlled delivery of therapeutic agents. Protein made nanoparticles such as the ones derived from Serum Albumin have emerged as being particularly promising to heal drug delivery. Albumin is biocompatible, non-toxic and degradable *in vivo*, so the nanoparticles generated are easily adaptable to the human body. Recently published papers suggest an improvement in cellular uptake of nucleic acids, such AONs and plasmid

DNA, when using nanoparticles as a carrier (Rimessi et al, 2009; Ferlini et al, 2010; Kurosaki et al, 2011). Moreover, recent studies show a significantly improvement in dystrophin restoration in mice treated with nanoparticles encapsulating AON with respect to those treated with naked AON (Rimessi et al, 2009; Ferlini et al, 2010).

In this study we proposed using bovine serum albumin (BSA) nanoparticles as a vehicle to *in vivo* delivery of nucleic acids. As a reporter we chose a DNA construct, a plasmid coding for GFP containing an NLS, in order to access the functional uptake of the encapsulated nucleic acid to tissue cells in a mouse model. The Nuclear Localization Signal on GFP protein was chosen with the aim of accumulate fluorescent signal in a confined localization within the transfected cells, conferring it specificity.

It is described in literature that a target organ for DNA systemic delivery is the liver (e.g. Rimessi et al, 2009; Tomoaki K. et al, 2011) and also the lungs (Tomoaki K. et al, 2011; Kodama Y., et al, 2014) and we chose those organs to evaluate the uptake and effective expression of our reporter plasmid. In order to quantitatively evaluate transfected cells in mouse liver and lungs a flow cytometry method was chosen (Caiado et al, 2013). To evaluate correct localization and patterns of GFP fluorescence in tissues a fluorescence histochemical method was elected.

Before animal experiments the chosen analysis tecnhiques, fluorescent microscopy and flow cytometry, were tested and set. HEK293 cells transfected with pEGFP-NLS were used to optimize flow cytometry and microscopy analysis. BSA nanoparticles encapsulating pEGFP-NLS were produced by Gonçalo Bernardes' laboratory, at Cambridge, and tested by *in vitro* transfection. HEK293 cells transfected with pEGFP-NLS/BSA nanoparticles were analysed through fluorescent microscopy and flow cytometry to evaluate transfection efficiency. We also would expect an efficient *in vitro* transfection of HEK293 cells only by adding DNA loaded-nanoparticles without any transfection agent based on published papers that show successful transfection experiments with nanoparticles loading pDNA *in vitro*. Kurosaki et al obtained high transgene efficacies by transfection B16-F10 cells with pDNA/PEI complexes (Kurosaki et al, 2014; Kurosaki et al, 2011). Moreover, Rafiee et al analysed by flow cytometry the transfection efficiency of Chi/Alg nanoparticles loading pEGFP-N1 in HEK293 and obtained 42 % of GFP expression (Rafiee et al, 2015). However, in our experiment, fluorescent microscopy and flow cytometry analysis of HEK293 cells transfect with pEGFP-NLS/BSA nanocapsules revealed that transfection and GFP expression was not achieved. Despite this results, we proposed to test transfection efficiency *in vivo* once results obtained *in vitro* do not always reflect results *in vivo.* Thus, *in vivo* experiments were carried out in the *mdx52* mouse model. Animals were intravenously injected with pEGFP-NLS/BSA nanoparticles and with empty BSA nanoparticles as a control and 48h hours later liver and lungs were collected. Transfection efficiency was evaluated in liver and lung through immunohistochemical analysis with specific antibodies targeting GFP protein and by flow cytometry. Recently published data suggests high transgene efficiency in the liver, spleen and lung of mice injected with pDNA/PEI nanoparticles (Kurosaki et al, 2014). Also, Kodama et al, obtained high transfection efficiency *in vivo* after injected mice with pDNA-DGL complexes (Kodama et al, 2014). Besides this documented results, our experiment with *mndx52* mouse model injected with

pEGFP-NLS/BSA nanocapsules solution did not resulted in satisfactory transfection rates neither by fluorescent microscopy or flow cytometry analysis. Based on this results, we proposed to evaluate our nanoparticles through electrophoresis and results suggests that pDNA encapsulated in BSA nanoparticles was degraded as it shows a smeared band instead of a perfect one in images of gel documentation.

Some possible reasons for results obtained *in vitro* and *in vivo* might be related with lack of a complete characterization of BSA nanoparticles used in this experiment for example in terms of ability to protect plasmid DNA from degradation. The concentration of pDNA encapsulated on nanoparticles should also have been confirmed.

Near the end of our experiment, a paper was published on *International Journal of Nanomedicine*, by Qian et al, reporting a novel hybrid protein-lipid polymer nanocapsule as an effective and nontoxic drug delivery and imaging carrier. In their study Hela cells were transfected with nanocapsules composed by BSA and poly lactic-co-glycolic acid (PLGA) carrying a pEGFP-NLS plasmid as a reporter gene. After 1h incubation with the nanocapsules, the cytosol of Hela cells showed green fluorescent signal in the cell nucleus, suggesting successful intercellular delivery of EGFP-NLS (Qian et al, 2015).

In conclusion, it will be interesting to repeat the experiments in a more controlled way, however we suggest that the methodologies used are correct. Future investigations should invest in nanocapsules directed target to increase the efficiency delivery of nucleic acids. Nanomaterials are certainly very appealing as nucleic acids vehicles and show great promise in this regard.

5. Conclusions and Future Perspectives

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. 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 improve its pharmacokinetics. Biocompatible nanomaterials have been manly used as drug delivery systems. One of the most important features that has the potential to be developed on nanocapsules for therapeutic delivery is the targeting capacity: drugs or nucleic acids should only interfere with the desired cells without harmful effects to healthy tissues. Concerning to Duchenne Muscular Dystrophy, antisense oligonucleotides (AONs) have shown promising results. However, delivery to target tissue (striated muscle) has proved challenging.

The ability to target nanoparticles to specific tissues, cells and subcellular components will greatly impact their performance as delivery vehicles for molecular contrast agents, detection probes and drugs, for biomedical applications. However, the ability to deliver nanoparticles to their subcellular targets and to control their trajectory within the cell is still very poorly understood. As well, quantitative descriptions on the kinetics, amount, mechanism and trajectories of nanoparticle uptake and trafficking are lacking. These obstacles interfere with the success of clinical utility and benefit of nanoparticle-based technologies. Thus, fundamental studies must continue in order to better understand how nanoparticles interact with molecules, organelle and cellular structures, and to be able to identify causative relationships between physiochemical properties of engineered nanoparticle with cellular responses.

Concerning to protein capsules, a remarkable development occurs in the last decade. Indeed, it is now possible to assemble protein capsules of defined sizes and with pre-defined functionalities. Also, the availability of a protein carrier for surface modifications in combination with active targeting by the protein capsules would enhance the therapeutic effect of delivered agents without increasing their toxicity.

Continuous advances in synthetic chemistry and material chemistry will help realizing such novel classes of nanoparticles and nanoscale systems with integrated functionalities and properties. For example, multifunctional nanoparticles probes are recently being developed to overcome limitation inherent in single component platforms such as multimodal contrast agents comprised of magnetic nanoparticles coupled with either optical or radiolabelled probes that allow medical imaging in two modes. In fact, Qian and colleagues developed this year (Qian et al, 2015), a biodegradable double nanocapsule, made of BSA and PLGA, as a novel multifunctional carrier for drug delivery and cell imaging.

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 non-invasive skin and oral administration.

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