UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

DEPARTAMENTO DE FARMÁCIA GALÉNICA E TECNOLOGIA FARMACÊUTICA

Identification of critical points in colloidal delivery systems for intravenous administration and intracellular delivery of nucleic acids as therapeutic agents

Andreia Alexandra Dias Mouro

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Identification of critical points in physicochemical characterization of colloidal delivery systems for intravenous administrations and intracellular delivery of nucleic acids as therapeutic agents

Andreia Alexandra Dias Mouro

This dissertation was supervised by Professor Mafalda Videira and Professor Rogério Gaspar.

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ABSTRACT

The RNA-mediated gene-silencing technology, carried out by small interfering RNAs (siRNAs), has attracted a great deal of attention as novel promising therapeutic strategy in oncology.

One of the common themes emerging from the studies on cell-specific delivery of siRNA is the need for optimizing the intracellular trafficking of the siRNA to elicit a silencing response. Polymer nanoparticles have become recognized as an efficiency strategy for oligonucleotide delivery to a specific cell population.

Among these carriers, PLGA-co-PEG nanoparticles have attracted much attention since they are assumed to meet the criteria required for successful siRNA delivery: they are sufficiently small for efficient tissue penetration and cellular uptake and offer physical protection against RNase activity as well as a favorable colloidal stability.

In this study the ability of a polymeric micelle based system for the targeting and delivery of a siRNA to breast cancer cells was proved using as model the siRNA against the Green Fluorescence Protein (GFP). The efficiencies observed during *in vitro* studies with a MDA-MB-436/GFP cell line confirmed the potential of this new delivery system but it needs further investigation.

Key Words:

Polymeric micelles, siRNA, PLGA-co-PEG, Green Fluorescence Protein (GFP).

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LIST OF ABBREVIATIONS

^{13}C-NMR	carbon 13 nuclear magnetic resonance
ApoB	apolipoprotein B
Arg	arginine
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCR5	chemokine (C-C motif) receptor 5
CD4	cluster of differentiation 4
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary (cells)
CMC	critical micelle concentration
CPP	cell-penetrating peptide
CXCR4	chemokine (C-X-C motif) receptor 4
DDS	drug delivery system
DMRIE	*N*-(1-(2,3-dimyristyloxypropyl)-*N*,*N*-dimethyl-(2-hydroxyethyl)ammonium bromide
DNA	deoxyribonucleic acid
DOPC	1,2-oleoyl-sn-glycero-3-phosphocholine
DOPE	dioleoyl phosphatidylethanolamine
DOSPA	2,3-dioleyloxy-*N*-[2(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propanaminium trifuoro acetate
DOTAP	*N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propane methysulfate
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
dsRNA	double stranded ribonucleic acid
EGFP	enhanced green fluorescent protein
EPR	enhanced permeability and retention
GC	guanine-cytosine
GFP	green fluorescent protein
gp160	glycoprotein 160
GU	guanine-uracil
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPMA	N-(2-hydroxypropyl)methacrylamide
IFN	interferon
IgG	Immunoglobulin G

kDa	kilo-Dalton
kPa	kilo-Pascal
Lys	lysine
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
N/P ratio	molar ratio of nitrogen atom content in the polymer to phosphorous atom content in the DNA
nm	nanometer
nM	nano-Molar
nt	nucleotide
ODN	oligodeoxyribonucleotide
PC	phosphatidylcholine
PCL	polycaprolactone
pDNA	plasmid deoxyribonucleic acid
PEG	polyethylene glycol
PEI	polyethylenimine
PGA	polyglycolic acid
piRNA	piwi-interacting ribonucleic acid
PLA	polylactic acid
PLGA	poly(d,l-lactic-co-glycolic acid)
Pre-miRNA	precursor miRNA
Pri-miRNA	primary miRNA
PrP	Prion protein
PrPSC	*Sc*rapie Prion protein (an abnormal isoform of PrP)
PTD	peptide transduction domain
RES	reticuloendothelial system
RISC	ribonucleic acid-induced silencing complex
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
rpm	rotations per minute
shRNA	small hairpin or short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SNALP	stable nucleic acid lipid particles
SOD	superoxide dismutase
ssRNA	single stranded ribonucleic acid

Tg	glass transition temperature
TLR	Toll-like receptors
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
UTR	untranslated region
VEGF	vascular endothelial growth factor

1. INTRODUCTION

1.1 Nanotechnology in Medicine

Nanotechnology can be defined as the "intentional design, characterization, production and application of materials, structures, devices, and systems by controlling their size and shape" in the nanoscale range, usually defined to be between 1 to 100 nm (Kim BS 2010). The dimension of the particles is not consensual; the National Nanotechnology Initiative defines nanotechnology as research and development at the atomic, molecular, or supramolecular levels in the sub-100 nm range (approximately from 0.1 to 100 nm) (Morrow KJ 2007).

Rational design of nanomedicines began almost half a century ago and more than 40 products have completed the complex journey from laboratory to routine clinical use (Duncan R 2011).

The consensus definition of Nanomedicine by the European Science Foundation's Forward Look Nanomedicine is "Nanomedicine uses nano-sized tools for diagnosis, prevention and treatment of disease and to gain increased understanding of the complex underlying pathophisiology of disease. The ultimate goal is to improve quality of life". The European Commission's Joint Research Center Report "Nanomedicine: Drivers for development and possible impacts" additionally observes that nanoparticles for medical applications are defined as particles with a size between 1 and 1000 nm (Duncan R 2011). This definition is also justified from a technical point of view because the control of the materials in nanometer size range results in new clinical properties but requires new approaches in chemistry and manufacturing techniques (Wagner V 2008).

The distribution, safety, and uptake of particles by cells and tissues will depend on the surface charge, size and hydrophobicity of the particle (Ozpolat B 2009). The optimal size is considered by most authors to be between 10 to 100 nm. If the particle size is less than 10 nm, the nanoparticles will be quickly eliminated by renal clearance (threshold < 6 nm); at sizes greater than 100 nm, the chance of the particle being captured by RES in liver, spleen, lung and bone marrow, will dramatically increase (Ozpolat B 2009, Wang X 2009). However, the size limit of less than 100 nm is rarely critical from a formulation or efficacy perspective, because the required properties (for example, increased bioavailability, reduced toxicity, use of a lower dose, improved solubility) may also be achieved with a size range greater than 100 nm (Morrow KJ 2007).

Materials exhibit unique properties at nanoscale, the changes in properties are due to increase in surface area and dominance of quantum effects which are associated with very small sizes and large surface area to volume ratio. At the nanoscale, the tensile strength, opto-electrical properties, and surface chemistry of materials become radically changed (Kim BS 2010).

Nanomaterials are being developed in order to carry diagnostic or therapeutic agents through biological barriers, these materials have regulating optical, electronic, magnetic, and biological properties, and they can be designed to have different sizes, shapes, chemical compositions, and surface chemical

characteristics (Kim BS 2010). It had be pointed that the nanocarrier should be made of a well characterized, easily functionalized and biocompatible material, that should also exhibit high differential uptake efficiency in the target cells over normal cells (or tissue); be soluble or colloidal under aqueous conditions for increased effectiveness, and exhibit an extended circulating half-life having, at the same time, a low rate of aggregation (Peer D 2007).

One of the main issues for achieving therapeutic efficacy with chemotherapeutic agents is drug resistance with P-glycoprotein being the best known and most extensively investigated. There is some evidence that nanoparticles can bypass the P-glycoprotein efflux pump, leading to a greater intracellular accumulation (Cho K 2008, Wang X 2009). The exploit of passive and active targeting can enhance the intracellular concentration of therapeutic agents in target cells and minimize toxicity in other cells (Wang X 2009).

If in one hand it is recognized that nanoparticles can offer numerous advantages as drug carrier systems, but there are still some limitations that have to be solves such as poor bioavailability, instability in circulation, inadequate tissue distribution and toxicity (Cho K 2008). Some other authors defend that nanocarriers confer some advantages over traditional drugs, such as protecting the therapeutic entity from early degradation and prematurely interaction with the biological environment, improve the absorption into a selected tissue, control the pharmacokinetic and drug tissue distribution profile, and improve intracellular penetration (Peer D 2007).

To effectively deliver the therapeutic agent to the targeted tissue and in order to enhance passive targeting, the siRNA nanocarrier can be conjugated with specific moieties, such as lipids (like cholesterol), PEG or a non-anionic surfactant. These modifications confer to the nanoparticles the ability to remain in the circulation for an extended amount of time without being eliminated and can modify the biodistribution (Cho K 2008, Ozpolat B 2009, Tan JS 2011). Another strategy is the active targeting that change the delivery of nanoparticles by using targeting ligands guiding the nanocarrier via specific ligand-receptor interactions at the target cell surface (Tan JS 2011).

When nanomaterials are administered intravenously they are able to accumulate in tumors due to the EPR effect when the vasculature of immature tumors has pores smaller than 200 nm, allowing extravasion of high molecular mass molecules (> 40 kDa) into tumor tissue (Kim BS 2010, Shim MS 2010). A representation of the EPR effect is demonstrated in Figure 1.

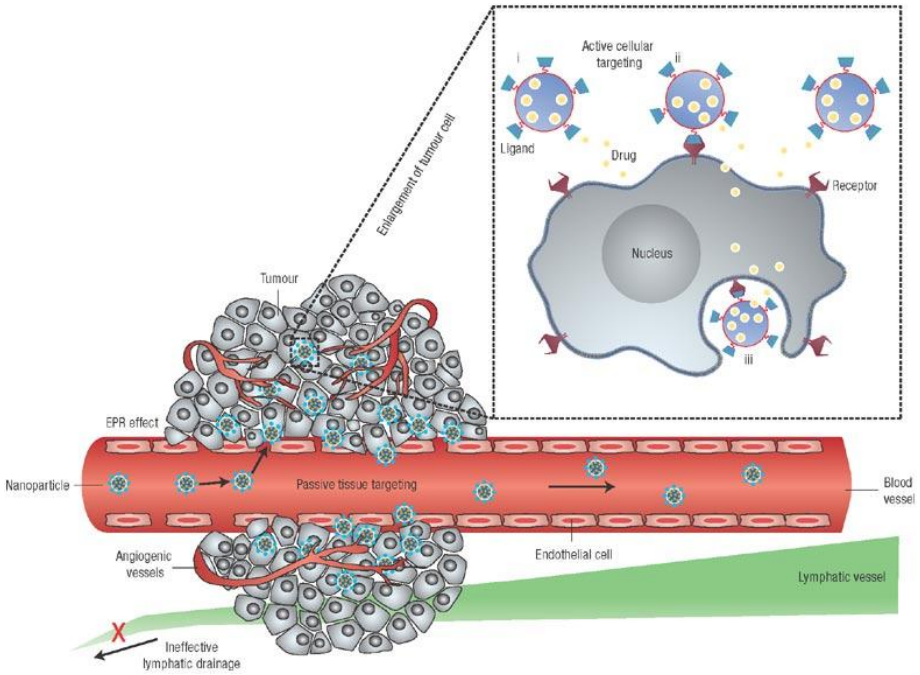


Figure 1 - Polymeric nanoparticles are shown as representative nanocarriers (circles). Passive tissue targeting is achieved by extravasation of nanoparticles through increased permeability of the tumor vasculature and ineffective lymphatic drainage (EPR effect). Active cellular targeting (inset) can be achieved by functionalizing the surface of nanoparticles with ligands that promote cell-specific recognition and binding. The nanoparticles can (i) release their contents in close proximity to the target cells; (ii) attach to the membrane of the cell and act as an extracellular sustained-release drug depot; or (iii) internalize into the cell. Reproduced from Peer D 2007.

1.1.1 Surface particle characteristics

As mentioned above, the surface of nanoparticles is an important factor determining their life span and fate during circulation relating to their capture by macrophages and interaction with biological barriers. Having a hydrophilic surface is important to nanoparticles to escape from macrophages, so the particles can be coated with a hydrophilic polymer, such as PEG or nanoparticles can be readily prepared from block copolymers with hydrophilic and hydrophobic domains (Cho K 2008).

PEG conjugation permits the control of particle size and prevents *in vivo* particle aggregation, but the length of PEG chain has optimized for each individual delivery system because it seems to influence the stability and the protective properties (Whitehead KA 2009). PEGylation forms a barrier around nanoparticles that provides steric stabilization and protection from the physiological surroundings, since it confers "stealth" properties that prevent protein adsorption (Whitehead KA 2009, Laroui H 2011).

Surface characteristics can significantly influence the way it interacts with the target cell and other physiological molecules (Whitehead KA 2009) and they have three fundamental roles in the function of engineered nanoparticles. First, surface chemistry is known to heavily influence the process of opsonization, which ultimately dictates the RES response. Second, to achieve cellular targeting, ligands known to bind cell surface receptors of selected cells should be included in the design of nanoparticles. Third, if is desired to target only a specific cell population, the use of antibodies, aptamers, peptides and oligonucleotides grant to nanoparticles highly specific interactions (Petros RA 2010).

A common feature of all nanodevices is their large ratio of surface area to volume which is an advantage because when they are used as carriers their surface can be coated with many molecules, such as polymers or bio-recognition molecules in order to obtained an enhanced biocompatibility and selective targeting of biologic targets (Kim BS 2010).

In an *in vitro* outlook, a positively charged delivery carrier can facilitate uptake through binding to the negatively charged cellular membrane and also promotes complex formation with siRNA. *In vivo* the negatively charged serum proteins will often bind to the positively charged nanoparticle rendering it inactive (Whitehead KA 2009). One of the important parameters that allow the assessment of formulation stability is zeta potential, which accurately estimates the charge of a nanoparticle and describes the cell-nanoparticle interactions (Laroui H 2011).

1.1.2 Particle size and shape

One of the advantages of the nanoparticles is that their size can be regulated; it should be large enough to prevent the rapid leakage into blood capillaries and small enough to escape capture by RES fixed macrophages (Cho K 2008). Bearing this is mind, rigid and spherical particles with a size of about 100-200 nm have the highest ability to have an acceptable biodistribution profile with extended circulation because they are sufficiently large to avoid uptake in the liver and small enough to circumvent filtration in the spleen (Petros RA 2010).

The particle shape can be coupled to particle size, and particle geometry also has an important part in particle internalization. The optimum parameters for developing nanoparticles in terms of shape have not been determined (Petros RA 2010).

1.2 RNA Interference – the world of siRNA

The identification of most of the genes in the human genome gives the opportunity for the development of a number of potential new drug targets. At the time, pharmaceutical companies are not prepared to this great challenge. One of the main limitations is knowing which gene products are functionally involved in the pathology of a particular disease (Leung RKM 2005).

What distinguishes this mode of gene silencing from other antisense strategies, such as antisense DNA oligonucleotides and ribozymes (Aagaard L 2007), is that, though in both methods the sequence complementarity feature on the mRNA is utilized for target recognition, the RNAi mechanism has a catalytic component incorporated into it; a single siRNA molecule can silence thousands of copies of mRNA molecules. This machinery is extremely efficient in comparison with antisense technology, where a single antisense nucleic acid molecule can bind to and thereby silence only one single mRNA molecule (Tseng YC 2009). Showing a higher potency, siRNA may function with lower concentrations than conventional antisense strategies and ribozymes, and seems to be less toxic *in vivo*, two arguments that are essential for the development of a therapeutic agent (Aagaard L 2007, Pan X 2011).

Some of the advantages of siRNA strategies are mainly related with the fact that its synthesis does not require a cellular expression system, complex protein purification, and the relatively uncomplicated process (Reischl 2009).

To date, four types of anti-mRNA strategies have been identified: (i) ODNs, in which a synthetic, small, single-stranded ODN inhibits the translation of a specific gene; (ii) rybozimes, which are catalytically active RNAs that cleave single-stranded regions in RNA through trans-esterification or hydrolysis reactions; (iii) siRNA, which will be extensively explored later; and (iv) microRNAs, typically with 20-24 nucleotides length and that regulate target mRNAs post-transcriptionally (Reischl D 2009).

All these strategies have a final aim of suppressing the translation or degradation of target mRNAs (Reischl D 2009).

1.2.1 The "rise of silence"

RNAi was first observed by plant biologists in the late 1980s, but its molecular mechanism remained unclear until the late 1990s (Leung RKM 2005). In 1998, Fire *et al* (Fire A 1998) published their discovery of "potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*", a nematode worm (Gondi CS 2009), and received the Nobel Prize in physiology or medicine in 2006 for this breakthrough (David S 2010). This publication suggested for the first time that small dsRNA could play pivotal roles in gene expression, a function that was never anticipated (Grimm D 2009).

In 2001, Tuschl and co-workers (Elbashir SM 2001) opened the way to use RNAi for experiments in mammalian cells by chemically synthesizing 21 nt siRNA, describing the structural requirements for the siRNA to trigger RNAi. This created new opportunities, not only for research, but also for therapeutic treatments (Nguyen T 2008, Kurreck J 2009).

Observed in the majority of eukaryotes, RNAi is an evolutionary conserved process, highly efficient and a specific pathway that inhibits gene expression (Gondi CS 2009, Grimm D 2009).

RNAi process is a normal defense to protect and regulate the genome, acting by degradation of specific target mRNA, including RNA from viruses and transposons (Gondi CS 2009, David S 2010).

Since RNAi is an endogenous biological process, it is expected that roughly all genes can be potently suppressed by siRNA. One of the factors of great interest is that the identification of highly selective sequences occurs much faster than the discovery of small drugs. At the same time the synthesis and manufacture of siRNA is relatively straightforward (Oh YK 2009).

Despite the existence of more types of small RNAs, like shRNA, miRNA, and piRNA, siRNA was generally selected for developing therapeutic applications (Tokatlian T 2010).

1.2.2 Mechanism of RNA-mediated gene-silencing

The RNAi machinery is located in the cell cytoplasm and can be divided into 2 phases: the initiation phase (in which the effector molecules are generated) and the following effector phase – that is considered the actual RNAi mechanism (David S 2010).

In the cytoplasm of mammalian cells, during the so called *initiator phase*, an enzyme known as Dicer, a member of the RNase III family initiates the process of silencing by breaking down the long dsRNA molecules there are in the cell, to generate siRNA of 21-23/25 nt (depending on the author). The effector siRNA contain a symmetric 2 nt over-hang at the 3'-end, a 5'-phosphate, and a 3'-hydroxy group. The fragments obtained are incorporated into RISC and unwound by an RNA helicase into a ssRNA, which is followed by the degradation of sense strand ssRNA (Aigner A 2006, Oh YK 2009, Tseng YC 2009, Laufer SD 2010, Lee SY 2010). The process is briefly described in Figure 2.

The guide or antisense strand serve as a molecular template to recognize homologous mRNA and the RISC containing the guide strand is activated, and initiates the search and binding to complementary mRNA molecules (Aigner A 2006, Oh YK 2009, Laufer SD 2010).

Once siRNA binds the target mRNA by hybridization, RISC catalyses an endonucleolytical cleavage of the complementary target mRNA strand within the target site. This process leads to the generation of unprotected RNA ends, resulting in rapid degradation of mRNA. The RISC complex can be recovered for further cycles, resulting in a reduction of the specific mRNA levels and therefore decreasing the expression of the corresponding gene and protein (Aigner A 2006, Lee SY 2010).

One of the main components of the RISC complex is the Argonaute 2 protein that is responsible for mRNA degradation and ssRNA formation. This protein has about 100 kDa and contains four defined domains, N-terminal, PAZ, Mid and PIWI. Argonaute 2 has a catalytic function in RISC and facilitates the guiding of the anti-sense ssRNA strand to complementary mRNA sequences and degrades target mRNA through the PIWI domain of the Ago 2 protein, a structural homolog of RNase H. The presence of ATP is not necessary, but increases the rate of endonuclease activity. The hydrolysis reaction involved in the breakdown of the target mRNA phosphodiester backbone requires a divalent metal ion (Mg^{2+}) and releases the 5'-PO_4 and 3'-OH groups. When the guide strand is bound to RISC, this complex can go through several rounds of mRNA binding and cleavage (Oh YK 2009, Laufer SD 2010).

miRNA are endogenous noncoding RNA with generally 22, the generation of this nuclei acid begins in the nucleus where the encoded transcripts pri-miRNA are processed into pre-miRNA. They are pre-processed by a nuclear RNase III (Drosha) before being exported from the nucleus into the cytoplasm by exportin 5-RanGTP. The generation of miRNA, Due to an imperfect matching with 3'UTRs, miRNA sometimes do not lead to the cleavage of mRNA in RISC, resulting in a translational suppression (David S 2010, Pecot CV 2011).

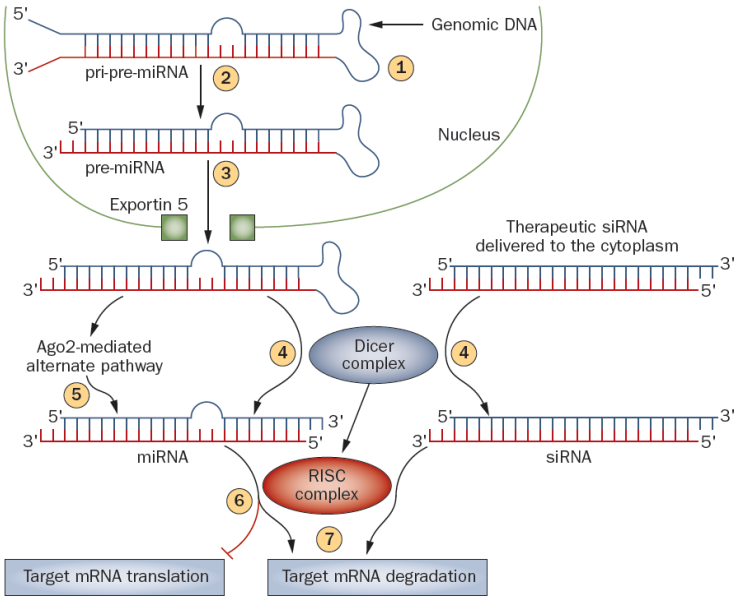


Figure 2 - Major steps in the RNAi pathway. Pri-miRNA transcribed (1) from genomic DNA is processed (2) into smaller structured pre-miRNA with short region of noncomplementarity and is transported (3) into the cytoplasm via exportin 5. Such endogenous pre-miRNA and exogenous siRNA delivered into the cytoplasm are substrates of the endoribonuclease Dicer (4), which further shortens the double strands of small RNAs to 19–21 nucleotides. This Dicer-mediated process is then associated with formation of the RISC complex and unwinding of the double-stranded RNA to facilitate the targeting activity (7) of the antisense strand (in red). An alternate pathway for pre-miRNA processing (5) that is independent of Dicer has also been described. Two outcomes for miRNA action—translational inhibition (6) and mRNA degradation (7), have been described. Target mRNA degradation is the main action of siRNA-based gene silencing. Reproduced from Czech MP 2011.

Some siRNA issues and advantages have to be pointed. They have a transient effect as a result of the decrease in their concentration into the cell, which may be due to the constant cell division and the degradation by endogenous RNases (Ramon AL 2008, Kurreck J 2009).

The degradation of the target RNA usually begins immediately after the siRNA enters the cell; however, the decrease in the amount of protein depends on its half-life. In the majority of times the target gene is not completely silenced and this is why RNAi is known as a knockdown tool (Kurreck J 2009).

Since they are natural nucleotides, they show low toxicity: when degraded by endogenous nucleases, the formed metabolites are incorporated in the cells. On the other hand, siRNA have poor ability to cross the cell membranes by free diffusion because of length (MW is near 13 to 15 kDa), their anionic charge and are repulsed by the negative charges of cells membrane and, once they enter the cytoplasm, they may not be automatically released in the right compartment (Oliveira S 2006, Ramon AL 2008, Reischl D 2009).

siRNA demonstrate limitations relative to biodistribution, especially *in vivo*: fast glomerular filtration (leading to renal elimination), non-specific organ accumulation, weak stability in physiological fluids, competitive uptake by non-target cells, degradation by nucleases, inefficient uptake by tissue cells, endosomal trapping, and the possibility of triggering an immune response and off-target effects (Oliveira S 2006, Ramon AL 2008, Li J 2009, Oh YK 2009, Lares MR 2010, Lee SY 2010). The

fundamental step for the siRNA therapeutics is to accomplish the projected targets; if not, it might injury the non-targeted cells (Lares MR 2010).

The majority of siRNA loaded nanoparticles enter the cell through endocytosis. This fact results in a limitation to siRNA efficiency, because siRNA needs to be released into the cytosol to activate the RNAi machinery. Some *in vitro* studies demonstrated that siRNA loaded nanoparticles enter more than 90% of the cells through endocytosis (Tokatlian T 2010).

After internalization, an early endosome is formed and the vesicular interior reaches an acid pH, which remains in the endosomal and lysosomal compartments, achieving a final pH of about 4.5 in the perinuclear lysosome (Tokatlian T 2010). Because of this mildly acidic medium in the endosome/lysosome some strategies using acid-responsive delivery carriers are being developed (Shim MS 2010). pH-responsive polymers, such as polymers containing protonable amines that can disrupt the endosomal membrane through the so called "proton sponge effect", or polymers that undertake hydrophilic-to-hydrophobic transitions and can induce endosome membrane lysis (Tokatlian T 2010). The studies carry out suggested the need for optimization of the intracellular trafficking of the siRNA so that it can enter cell and be present in the cytoplasm in quantities that can trigger the silencing response (Manjunath N 2010).

The unintentional silencing of non-target genes may lead to problems and potential toxicity, it is known that cells respond effectively to siRNA by different reactions depending on cell type, siRNA sequence, and intracellular location (Oliveira S 2006, Oh YK 2009). In order to overcome this question, the design and selection of potent siRNA should be cautiously performed. Some of the criteria for selecting the siRNA involve the consideration of internal repeated sequences, the secondary structure, GC content, base preference at specific positions in the sense strand and appropriate length (Oh YK 2009).

Being an RNA entity, siRNA is anionic, hydrophilic, and is unable to freely diffuse into the cell, due to the relatively large molecular weight and anionic charge (Oh YK 2009, Singh SK 2009). These issues can be an obstacle for the use of siRNA as a therapeutic due to the lack of efficient *in vivo* delivery systems. When developing the delivery system it should be considered prolonged circulation in blood stream, improved accumulation in the target tissue, and efficient intracellular transport of undamaged siRNA (Kim SH 2006).

In 2010, there was no straight evidence for an RNAi mechanism of action in humans from siRNA administered either locally or systemically has been reported (Davis ME 2010).

1.2.3 The stability issue

Naked and chemically unmodified siRNA have an extremely short half-life in blood or serum. To overcome this problem, chemical modifications are tested. The modified siRNA present a prolonged half-time in serum from few minutes for unmodified siRNA to 2-3 days, and in circulation from about 2 minutes to 49 minutes (Bruno K 2011). The improved siRNA stability by a variety of chemical modifications should be achieved without compromising the silencing ability. Some examples of

modification are the introduction of a phosphorothioate (P=S) backbone linkage at the 3'-end for exonuclease resistance, and 2' modifications (2'-O-methyl and 2'-fluoro) for endonuclease resistance (Guo J 2011). Several chemical modifications also reduced the immune stimulation otherwise increased by unmodified siRNA, but not all the modifications are tolerated with the same efficacy and specificity (Bruno K 2011). Examples of cytotoxicity were observed when the P=O link was partially or completely replaced by a P=S link. In contrast, siRNA modified with a boranophosphonate (P=B) backbone have a superior nuclease resistance than non-modified siRNA. When P=B was introduced in the sense strand or the terminal regions of the duplex it did not affect the gene-silencing activity (Guo P 2010).

1.2.4 From in vitro *to clinical trials*

siRNA is in the spotlight for the development of therapeutics because of its highly efficient and specific gene silencing. In theory, RNAi can be applicable to all classes of molecular targets, reducing or eliminating undesirable small molecules or proteins. Considering this, it is important to demonstrate that the inhibitor will only silence the expression of the proposed gene and not other distinct genes (Dallas A 2006, Lee SY 2010).

If possible, siRNA should be targeted into three levels: target the diseased tissue, target the cell for silencing the mRNA of interest, and targeted to the sub-cellular compartment, so it can interact with the RNAi machinery (Oliveira S 2006).

Synthetic siRNA were being tested in animal models for cancer, viral infection, and ocular degeneration, amongst others in order to evaluate their efficacy. The results of basic and pre-clinical tests showed positive silencing making siRNA potential candidates for therapeutic use (Li J 2009).
The silencing effect can last for up to seven days in dividing cells (where the therapeutic RNA is diluted with each cell division) and for several weeks in non-dividing cells, even *in vivo* (Peer D 2011).
The duration of the silencing seems to depend on the target organ, the target gene and the specie; in a study performed by Zimmermann TS *et al*, a siRNA against apolipoprotein B was active in mice for only a few days, while the knockdown in non-human primates was still effective after 11 days (Kurreck J 2009).

One of the main challenges for gene knockdown is to identify not just an effective sequence, but the sequence that provides the most potent silencing at the lowest possible concentration (dose) of the therapeutic agent. Finding an effective target site within an mRNA is not uncomplicated, there are some factors that have to be considered, such as evaluate if a given site is a good candidate (including the primary sequence), the accessibility of target site because of local, internal secondary structure or long range tertiary structure and steric occlusion, as many sites may be blocked *in vivo*. Most of these factors are either not known or predictable *a priori*. Part of what makes the RNAi approach so attractive is that

many sequences show a measurable knockdown of gene expression, in contrast to other AS technologies (Dallas A 2006).

Statistically, one in five sequences has been reported to be effective. The use of available algorithms assists the selection of the most potent siRNA for a specified target. These algorithms are developed considering factors like GC sequence content and the identification of a certain nucleotide at a certain position. After the analysis, a shortlist of siRNA with the best probability of success is selected (Dallas A 2006). The algorithms are not perfect and, ideally, all possible target-specific siRNA sequences should be tested in cells to guarantee that the best inhibitor(s) for a given mRNA are found (Dallas A 2006).

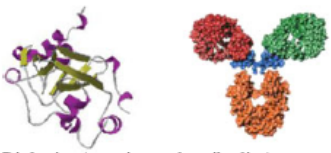
Since the RNAi mechanism are based on Watson-Crick base pairing, it is expected that undesirable side effects played no role when a target sequence that only appears once in the genome was used. In practice, it seems to be different because a single mismatch can lead to an absolute loss of silencing (Kurreck J 2009). These sequence-based targeting technology showed the ability to design precisely targeted therapeutics for almost any target sequence both coding and non-coding sequences, regardless of the function of the gene product, whether that function is known (Seyhan AA 2011).

The strong appeal of RNAi as a therapeutic is that siRNA offers many possibilities and advantages compared with traditional drugs, like the highly potency and specificity, the persistent duration of their pharmacological action (making possible to reduce the dose and frequency of administraton), the ease of design, and the quite simple and inexpensive synthesis and production (Lópes-Fraga M 2008, Seyhan AA 2011).

The best siRNA sequences that achieve gene knockdown are active at extremely low (picomolar) concentrations, it is generally possible to find such highly potent sequences for any gene (Peer D 2011, Petrocca F 2011), the use of lower doses have an impact on the amount and severity of side effects and the overall cost of treatment (Lópes-Fraga M 2008).

As can be seen on table 1, once a target has been identified, it only takes a few months to identify potent candidate siRNA molecules compared with the years typically required to identify a traditional drug, based on small molecules, proteins and antibodies (Lópes-Fraga M 2008, Petrocca F 2011). Also in table 1, it can be acknowledged that one of the principal advantages of RNAi over small-molecules and protein therapeutics are that RNAi can be used to all targets (Seyhan AA 2011). However, many problems have been found during the clinical trials and many issues have to be overcome (Seyhan AA 2011).

Table 2 - Comparison of RNAi with traditional pharmaceutical drugs. Reproduced from Seyhan AA 2011.


Small molecules	Biologics (proteins and antibodies)	RNAi
Antagonism or agonism of target	Antagonism or agonism of target	Antagonism only
Extracellular and intracellular targets	Extracellular targets	All targets, including nondruggable targets
Not all targets can be modulated selectively and potently (conformation driven)	High selectivity and potency	High selectivity and potency
No allelic specificity	Low allelic specificity	High allelic specificity
Lead ID and optimization slow	Lead ID and optimization slow	Lead ID and optimization rapid
Low cross species reactivity	Low cross species reactivity	High cross species reactivity
Easy to manufacture	Difficult to manufacture	Easy to synthesize and manufacture
Easy to deliver	Difficult to deliver	Difficult to deliver

1.2.5 The "dark side of silence"

Associated toxicity – Off-target effects

Nanoparticles can be applied in numerous disorders and diseases, which can lead to long-term treatments. So, the carrier constituents and their potential toxicity have to be carefully evaluated. Some polymers have particular toxicities, such as hematotoxicity, the ability to activate the complement system, carcinogenicity, teratogenicity, and immunogenicity (Wang X 2009). Basically, cells are controlled by dynamic actions of networks of genes interacting with each other. These complex networks may lead to the silencing of any gene by a siRNA can affect other genes, pathways and even the entire system (Seyhan AA 2011).

All drugs, with no exception to siRNA, have unintended off-target effects (Dykxhoorn DM 2006).

It is not simple to foresee the off-target gene regulation, even though sequences with a high degree of similarity to the target determined using BLAST search should be excluded (Dykxhoorn DM 2006).

Undesired off-target changes in mRNA can be investigated using mRNA microarrays, which generally show that few off-target mRNAs are reduced by more than 2-fold. However, for some genes a variation of 2-fold or less may be important and clinically significant. It is unlikely that microarray analyses will be helpful to screen and modify siRNA sequences to predict and minimize off-target effects before their clinical testing, but they can provide functional tools to direct modifications if unexplained toxicity is observed (Dykxhoorn DM 2006).

One of the suggested methods that can be used to minimize off-target effects is the chemical modification of the second residue in the active strand of the siRNA (this is considered a key residue in the seed region for endogenous microRNA activity), which possibly can suppress off-target effects with no interference in the silencing of the target gene (Dykxhoorn DM 2006).

Sequence-dependent effects of siRNA

It is usually acknowledged that siRNA duplexes with less than 30 bp do not induce antiviral IFN response (Lares MR 2010). Exogenous siRNA can also direct transcriptional gene silencing by inducing heterochromatin formation, leading to histone methylation and/or deacetylation, and ultimately DNA methylation (Lares MR 2010).

Since siRNA and miRNA have extremely similar mechanisms, is expected that siRNA can act as miRNA by cross-reactions with the endogenous miRNA pathway (Kurreck J 2009). In 2003, Jackson *et al* first revealed the miRNA-like off-target effects of siRNA, suggesting that this activity was induced in mRNA only when 7 or 8 nucleotides in 3'-UTR were matching with the 5' end of siRNA (Guo J 2011). This off-target effect was reduced when base mismatches occurred in the 5' end of the siRNA guide strand, but the gene off-targeting was restored on introduction of a new set of transcripts with 3'-UTR that were complementary to the mismatched guide strand (Guo J 2011).

These effects primarily concern the unintentional silencing of targets sharing partial complementarity with RNAi effector molecules but also include receptor-mediated immune stimulation through the recognition of certain nucleotide motifs. Among these motifs GU-rich sequences are probably the most studied, and are first presented by Judge *et al* as receptive to TLR in the endosomal pathway, being highly immunostimulatory (Martin SE 2007, Guo J 2011, Monaghan M 2011). The presence of these motifs in siRNA (e.g. 5'-UGUGU-3' or 5'-GUCCUUCAA-3') have been identified as the major contributors to this type of response and have only been observed with primary peripheral blood leukocytes and plasmocytoid dendritic cells (Wu SY 2009, Seyhan AA 2011).

Some reports indicate that siRNA can induce adverse effects *in vitro* and *in vivo*, and that the systemic administration of siRNA can activate high levels of inflammatory of cytokines such as TNF-α, interleukin-6 and IFN, in particular IFN-α (Martin SE 2007, Guo J 2011).

Unmodified siRNA can trigger a potent innate immune response, particularly when associated with delivery carriers that facilitate intracellular uptake and activation of TLRs (Lares MR 2010). siRNA can interact with TLRs on the surface of the cells or in endosomes (Seyhan AA 2011).

The siRNA may induce innate immune responses either TLR-mediated and non-TLR-mediated. TLR3, TLR7 and TLR8 are activated when they interact with nucleic acids within the endosomal and lysosomal compartments (Shim MS 2010, Pecot CV 2011). The TLRs have different patterns of expression: TLR7 is mainly expressed in plasmacytoid dendritic cells and B cells, TLR8 in myeloid dendritic cells, monocytes and macrophages, and TLR3 in mature myeloid dendritic cells. TLR3 is also expressed in lung, aorta, dermis, choroidal and umbilical vein endothelial cells (Pecot CV 2011).

The activating effects of the siRNA on endosomal TLRs are sequence dependent and motifs could be identified, leading to a strong stimulation of the immune response. This can be circumvented by avoiding the use of some motives in the siRNA (Kurreck J 2009). It was also shown that the presence of a ribose sugar backbone is essential for TLR7 recognition. Endothelial cell surface TLR3 is also

triggered by exogenous siRNA *in vivo*, leading to the production of cytokines such as IFN and IL-12 (Guo J 2011).

The TLRs are type I transmembranar proteins that are evolutionarily conserved in insects and vertebrates. In humans, 10 functional TLRs (TLR1–10) have been identified and shown to sense pathogen-derived compounds, defending the host against foreign agents. They are expressed on the cell surface or in intracellular endocytic vesicles or organelles. TLR activation by signaling pathways lead to cellular activation, increasing the antigen-presenting capacity of dendritic cells and the production of type I IFN. In immune cells, TLRs that recognize nucleic acids are only expressed in endosomes, including TLR3, TLR7/8, and TLR9, which identify dsRNA, ssRNA, and ssDNA, respectively (Sioud M 2010). The non-TLR-mediated innate immune response principally occurs through the cytoplasmic RNA sensors retinoid acid-inducible gene and dsRNA-binding protein kinase (Pecot CV 2011). IFN induction may be used concomitantly with siRNA when the activation of the immune system is used to destroy tumor cells. It has been demonstrated *in vivo* that siRNA that triggered RIG-1 activity and simultaneously IFN type I response mediate Bcl-2 gene silencing and improve the apoptosis of tumor cells in lung metastases (Lares MR 2010).

It is assumed that is not possible to achieve a completely specific RNAi application, but the unspecific effects can be minimized using appropriate designed siRNA in the lowest dose as possible (Kurreck J 2009). Another approach to decrease miRNA-like off-target effects is applying siRNA chemical modification (Guo J 2011).

Interference with endogenous miRNA

Introduction of siRNA in the cell can be a potential cause of toxicity, since it can interfere with the processing and function of endogenous miRNA (Dykxhoorn DM 2006). miRNA and siRNA pathways are quite similar, thus siRNA can function as miRNA and some studies have shown that siRNA can regulate unintended transcripts via seed complementarity in 39 UTRs (Caffrey DR 2011). These off-targets can produce false positives in siRNA screens and have the potential to cause adverse side effects in a clinical trial (Caffrey DR 2011).

When siRNA are introduced directly into the cytoplasm, they will not compete with miRNA at this stage, large intracellular siRNA concentrations might compete for limiting amounts of Dicer or RISC. It may take only about 1,000 siRNA molecules/cell to silence gene expression efficiently (a rough estimate derived from the frequencies of individual endogenous miRNA in cells). Quantitative information about the relative numbers of Dicer and RISC molecules and endogenous miRNA in different cells, together with information about the numbers of siRNA required for efficient gene silencing, would be helpful for anticipating whether this toxicity might be an issue (Dykxhoorn DM 2006).

There are a variety of ways in which off-targets might be induced, but miRNA-like binding in the 39 UTR has been projected as one of the main causes of siRNA off-targets effects. The study of Caffrey

DR *et al* revealed four key findings that are expect to be applicable to a particular subset of siRNA molecules that potently silence their intended target:

1) Reducing the concentration of a siRNA to a low effective dose without loss the silencing effect can lead to a significant reduction in the number of off-target effects (Caffrey DR 2011).

2) The authors established that one specific modification reduced the number of off-target effects for one of two siRNA, but some undesired effects still occurred when cells were treated with a relatively high dose of modified siRNA. Similar to what happens with unmodified siRNA, these off target effects were significantly reduced at lower effective doses (Caffrey DR 2011).

3) The increase of the dose from 10 nM to 25 nM promoted a rise in the number of down-regulated off-target effects that possess 39 UTR complementarity to the seed region, but also led to an even more dramatic increase in the number of off targets that lack 39 UTR complementarity (Caffrey DR 2011).

4) The utilization of a considerable high dose of siRNA (25 nM) leads to a potential phenotypic effect in the cell. Moderate induction of apoptosis with higher doses of siRNA complies with the increasing number of off-target effects observed when higher doses are applied (Caffrey DR 2011).

The findings mentioned beyond should not be generalized for all siRNA, but some authors suggest that the off target effects can be determined using a modified the nucleic acid at the lowest possible dose. The concentration of siRNA for biological applications usually ranges from 25 nM to 100 nM, although these doses are likely to increase the number of off-target effects (Caffrey DR 2011).

Some studies with miRNA suggested that they play an important role in maintaining gene activity; examples include their function as oncogenes or tumor suppressor genes (Aagaard L 2007).

Table 3 - List of off-target effects encountered in mammalian systems and steps to minimize their impact. Reproduced from Martin SE 2007.

Off-target effect	Type	Steps to mitigate
Saturation of the endogenous RNAi machinery	Sequence-independent	Use effectors at lowest possible concentration
		Use negative control effectors for comparison
Immune response	Sequence-independent	Use effectors at lowest possible concentration
Immune response	Sequence-dependent	Avoid known stimulatory motifs
		Use chemically modified effectors
		Use multiple effectors to confirm phenotypes
Silencing of unintended targets through partial complementarity	Sequence-dependent	Use effectors at lowest possible concentration
		Use multiple effectors to confirm phenotypes

Saturation of RNAi machinery

Drugs that depend on normal cellular processing to accomplish their purpose face the risk of saturating the cell pathways and therefore disturb the cellular system (Aagaard L 2007).

The exogenous introduced siRNA can saturate the cellular RNAi machinery and thus inhibit the function of endogenous miRNA, leading to the onset of toxic non-specific effects (Ebbesen M 2008, Seyhan AA 2011). Even though siRNA do not compete with miRNA at the upstream miRNA pathway,

high intracellular quantities of siRNA can contend for limited quantities of Dicer and RISC (Seyhan AA 2011).

These effects may be explained by transfection conditions (an example are lipid transfection reagents), inhibition of endogenous miRNA activity (that appears to depend on saturation of Exportin-5) or stimulation of pathways linked to the immune response (Martin SE 2007). Some studies described that massive expressed shRNA can saturate Exportin-5, inhibiting the endogenous pre-miRNA nuclear export and subsequently inducing mice lethality (López-Fraga M 2008). The siRNA do not require export from the nucleus, so their activity would not be expected to depend on Exportin-5 (Martin SE 2007). It has been shown that siRNA can activate the immune response in a sequence-independent, concentration dependent manner (Martin SE 2007). In order to overcome the non-specific effects the lowest effective dose of siRNA must be administered (Ebbesen M 2008).

1.3 Nanotechnology: a way for bridging molecular biology to clinical application

One of the possible techniques that can avoid the limitation of *in vivo* oligonucleotides delivery is local administration, but it is not always possible because the target tissue cannot be reached, or the area that needs treatment is excessively large to the administration of a local injection; some examples where it can be applied are in skin, head or neck cancer (Oliveira S 2006, Pan X 2011). We decide to develop nanoparticles so that we can achieve a systemic administration.

1.3.1 General biological barriers

Tissue level

When administered nanoparticles have to circumvent numerous organ clearance mechanisms, like the encountered in spleen and liver, which have to be compensated with the carrier to reach its target. The fenestrations in the spleen normally do not exceed 200–500 nm in size, so particles larger than approximately 200 nm must be designed to have some deformability to be able to remain in circulation (Petros RA 2010).

Several of the strategies that can be used to avoid the removal by macrophages are the pre-injection of carriers to saturate the phagocytic capacity of the RES, followed by an injection containing of carriers loaded with the therapeutic entity; changing the hydrophilicity of the carrier surface, which will reduce the rate of opsonization; use specific proteins linked to the surface of the carrier, minimizing or avoiding the complement activation; or use cell surface proteins attached to the surface of the carrier (Petros RA 2010).

Cellular level

There are two fundamental barriers at the cellular level that nanoparticles have to defeat so that siRNA can be correctly delivered: the first is the cell membrane, which prevents the diffusion of molecules larger than 1 kDa, and the subsequent trafficking and release of siRNA into the cytoplasm (Petros RA 2010, Tan SJ 2011). siRNA or its complex adheres rather to target cells via receptor-mediated specific binding, which is followed by cellular uptake (Shim MS 2010).
There are a number of different endocytic pathways to internalize substances by eukaryotic cells and facilitate the carrier internalization. These pathways include phagocytosis, macropinocytosis, clathrin-mediated endocytosis, non-clathrin-mediated endocytosis, and caveolin-mediated uptake. Each of those pathways, mostly depending on the specific siRNA carrier, drives siRNA to specific sub-cellular compartments and for an efficient activity, nucleic acid tools have to reach their cellular targets after gaining entry into the cell (Reischl D 2009, Petros RA 2010, Aliabadi HM 2012). For example, the internalization by clathrin-mediated pathway follows the traditionally traffic from endosomes to the lysosomal compartment (with a decrease of pH and exposure to degradation), while internalization via caveolin-mediated process are directed to caveosomes with a less defined fate (but may escape the aggressive conditions that may destroy the siRNA) (Petros RA 2010, Aliabadi HM 2012). In both situations, the carrier needs to escape from the endosome to gain access to the desired compartment.

Concerning clathrin-mediated endocytosis, the established maximum size for cellular uptake is 150 nm. In some *in vitro* studies, it was observed that siRNA complexed with PEI cannot complete the silencing of a gene when a size greater than 150 nm was used (Monaghan M 2011).
The conjugation of ligands to the surface of nanoparticles can influence the way of cellular internalization; some examples are folic acid, albumin and cholesterol that showed a facilitated uptake through caveolin-mediated endocytosis, while glycoreceptors seemed to promote clathrin-mediated endocytosis (Petros RA 2010).

Other endocytic pathway is macropinocytosis, a regulated process that takes up a large amount of liquid by plasma membrane at a slower speed as compared to other methods. This pathway can be triggered by incorporating cell-penetrating peptides into the designed nanoparticles (Petros RA 2010, Aliabadi HM 2012).

After the internalization of the siRNA by a cell, it must be released from the endosome and avoid entrapment and degradation (Shim MS 2010). It is not clear understood the role(s) of particle size, shape and flexibility with ligand type, density, and region-specific labeling on the nanoparticles and why cells might utilize multiple pathways for internalization of same complexes. The adoption of the different traffic pathway might be displayed as a function of the therapeutic entity dose (Petros RA 2010, Aliabadi HM 2012).

1.3.2 Administration routes

When developing a therapeutic strategy, such a gene delivery system, some pharmaceutical issues must be considered. A safe vector delivery system should own some characteristic features: it should not trigger an immune response and it should not be infectious. The delivery system should provide stability to the encapsulated or electrostatically bound nucleic acid (Pathak A 2009).
The types of target tissues and cells dictate the optimal administration route of local versus systemic delivery. siRNA can be directly applied to some tissues of the body that are easily accessible, including the eye, skin, muscle, respiratory and genital tracts via local delivery, whereas systemic siRNA delivery is the only way to reach metastatic and hematological cancer cells (Dykxhoorn DM 2006, Shim MS 2010) among other systemic diseases.

In some pre-clinical animal models local routes have been successfully tested and seem to be promising, some examples are intra-ocular, intranasal for pulmonary delivery, intra-tumor, direct cervico-vaginal and rectal mucosa administration, and direct injection into the central nervous system (Li J 2009, Shim MS 2010). Local delivery offers several advantages when compared with systemic administration, it needs lower effective doses, the formulation is simple, it shows a lower risk of inducing adverse effects, and facilitates the site-specific delivery (Shim MS 2010).

In phase I clinical studies applied to the treatment of MAD and RSV, the uptake in target tissues after intravitreal injection and intranasal application of naked siRNA, respectively, have been demonstrated. It suggests that a large amount of the cells located in the mucosal surfaces of the body can efficiently internalize siRNA, which makes it possible to think that topical siRNA therapy has the smallest obstacles to defeat (Dykxhoorn DM 2006).

When the target sites are not localized or not readily available the systemic delivery via intravenous, intraperitoneal or oral administration is commonly applied (Shim MS 2010). In comparison with other types of administration, intravenous injection makes the particles readily available to the majority of tissues. Despite its rapid action onset and bioavailability, vectors transported through intravenous injection meet some obstacles; such as the component of blood, organs of the circulatory system, epidermis and mucus cells, tissue junctions, and immune cells (Pan X 2011).
Due to the challenges associated to siRNA stability, there are a number of tissues where topical or localized therapy can be applied since they are accessible and relatively simple to transfect. Some of the examples of local delivery are mucosal membranes (intranasal, intratracheal, intravaginal and intrarectal), intraocular, transdermal, intrathecal and intratumoral (Whitehead KA 2009, Wu SY 2009, Bruno K 2011, Peer D 2011). The lung can be a good candidate to siRNA therapeutics, some studies demonstrated that direct instillation of siRNA into the lung via intranasal or via intratracheal enables direct contact with lung epithelial cells (Whitehead KA 2009).

Epithelial cells of mucosa and skin can be efficiently transfected using *in vitro* methods. However, the cornified layer of the skin is a significant barrier to transfection. One of the limitations is that siRNA delivery only occurs after abrasion, which can be an obstacle to their use in this therapeutic application (Peer D 2011). Some studies have demonstrated that female genital mucosa can be efficiently transfected and, in animal models, protection against sexual transmission of herpes virus infection was accomplished (Peer D 2011).

Oral administration presents the disadvantages of direct exposure to the particular stomach environment and it is still difficult to designed particles that remain stable in acid medium and can are capable to resist to digestive enzymes (Pan X 2011).

Long-term stable circulation of nanovectors requires protection from kidney filtration, uptake by phagocytes, aggregation with serum proteins, call down of T or B cells (unless if it was intentional), and enzymatic degradation (Pan X 2011).

The size of the siRNA-carrier complex can be adjusted through the variation of physical and chemical properties of the carrier. The tissue penetration needs the carrier to surpass the vascular endothelial barrier. Particles smaller than 100 nm tend to escape out to the interstitial space of sinusoid and trapped by hepatic parenchyma cells or Kupffer cells, this happens because hepatic sinusoid has comparatively larger gaps than the other organs. At the same time, particles larger than 200 nm can penetrate only a few tissues such as tumor sites, liver and spleen, and are rapidly removed from systemic circulation. Bringing together previous liposomes reports, the suitable size for systemic administration seems to be around 100 nm in diameter (Urakami T 2007, Pan X 2011).

Once accessing the capillary endothelium, siRNA loaded nanoparticles should target certain cells for specific cell transfection. The targeted delivery minimizes the uptake by undesired cells, which increases the therapeutic entity concentration in the target tissue and reduces adverse effects (Pan X 2011). The physiological barriers that siRNA should overcome in order to be effectively delivered are represented in Figure 3.

Summarizing, to further improve target specificity, also considering the possible adverse effects occurring when siRNA is processed by non-target cells, and allow application of siRNA for systemic treatment, several strategies can be proposed. Altogether the information can provide support to increase nuclease resistance, reduce renal excretion, specific cell uptake, promote uptake by the target cells, and guarantee the proper intracellular trafficking (Oliveira S 2006).

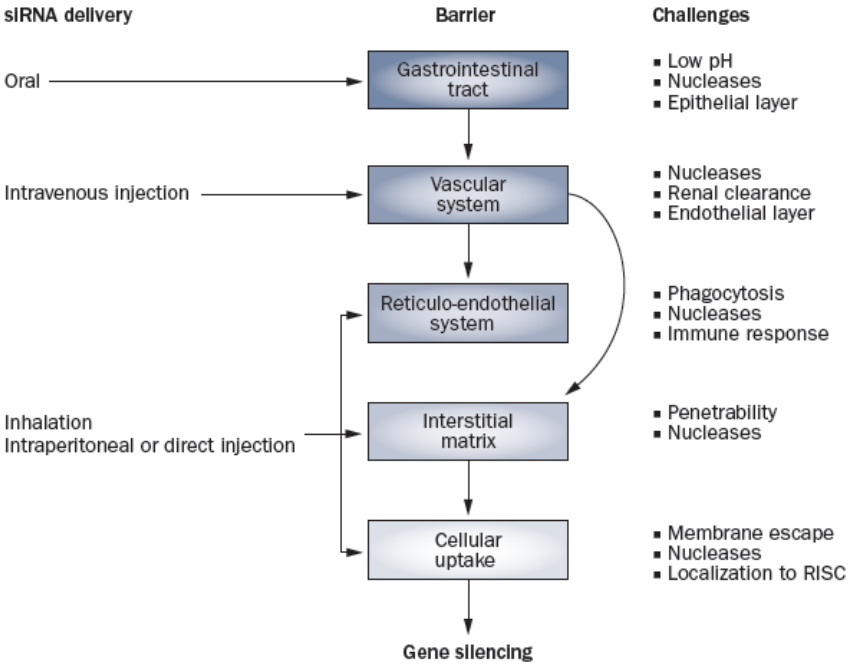


Figure 3 - Physiological barriers to successful delivery of small RNA therapeutics in humans. Reproduced from Czech MP 2011.

1.3.3 Administration via viral vectors

The strategy of using viral vectors consists in take advantage of the natural mechanism of cell infection by viruses, by the utilization of adapted viruses that are unable of replicate in order to introduce nucleic acids in a cell (Ramon AL 2008).

Viruses have proved to be the most efficient gene delivery systems. After cell binding they are capable of delivering the loaded nucleic acid into the cell in a proficient way along with nuclear localization. One of the main strengths of this delivery strategy is the efficient gene transduction, but some issues still remain, like the control over the transduced cell type, especially after systemic administration (Oliveira S 2006). Additionally, possible inflammatory reactions, immunogenicity, mutagenicity and oncogenic transformations continue to be important safety considerations for viral vectors that need to be addressed (Aigner A 2006, Oliveira 2006, David S 2010).

Plasmid vectors have demonstrated that they can be internalized in mammalian cells in vitro by simple transfection. In order to reach their ultimate aim, transcription, plasmid vectors need to enter the nuclear membrane. For cells that are actively dividing, plasmid vectors can easily enter the nucleus, because the nuclear membrane is disrupted during mitosis. In cells that are not in division phases, siRNA is a better approach than plasmids (Tseng YC 2009)

At the moment, there are four main groups of viral delivery systems used for RNAi: retrovirus, lentivirus, adenovirus and adeno-associated-virus, but other groups can be considered (Singh SK 2009, David S 2010).

Adenoviral vectors

The adenoviral vectors are DNA viruses and are most used because they can be transduced into dividing and non-dividing cells. Usually, adenovirus-mediated expression is short-lived because it does not integrate into the genome and, now, they can accommodate 30 kb of foreign nucleic acid (Reischl D 2009, Singh SK). There are three generations of these viral vectors and the use of first and second generation has been limited, since they are immunogenic and have a low loading capacity. Third generation were produced by deletion of the viral sequences, except the packaging elements and terminal repeats, resulting in lesser immunogenicity and increasing their loading capacity (López-Fraga M 2008, Singh SK 2009).

Adeno-associated virus

Adeno-associated virus are nonpathogenic, single-stranded DNA that are capable to infect several cell types, being dependent on a helper virus for proliferation, and they can hold between 3.5 to 4.0 kb of foreign nucleic acids (Reischl D 2009). Similar to the Adenoviral vectors they can infect both dividing and non-dividing cells (Leung RKM 2005, Reischl D 2009). These viruses are one of the most commonly found in the humans (Singh SK 2009), they are non-pathogenic and can also accomplish long term gene expression (Tseng YC 2009). These are probably one of the safest viral delivery vectors because, so far, they have not been associated with malignancy and demonstrated low rates of random integration into the host genome (Singh SK 2009), which can be explained by their specifically integration in a particular region of the chromosome 9 (Leung RKM 2005).
These vectors have been used to silence genes, both *in vitro* and *in vivo* (Leung RKM 2005) and do not seemed to trigger immune responses like adenoviral vectors (Singh SK 2009).

Retroviral vectors

Retroviruses are a class of enveloped viruses with a single-stranded RNA molecule that is converted in a double stranded DNA when it infects a cell, and they can carry approximately 7.5 kb (Kumar LD 2007, Reischl D 2009). Once the virus integrates the host's genome, it is expressed in the form of proteins (Reischl D 2009) and they permit the stable introduction of constructs into dividing cells (transformed and primary), which is a limitation for cancer therapy *in vivo* (Leung RKM 2005, Kumar LD 2007). Most widely used retroviral vectors are the derivate of murine stem cells virus or Moloney murine leukemia virus (Leung RKM 2005, Reischl D 2009).
One of the advantages of these vectors is their capacity to integrate into the host genome, resulting in a long-lasting expression; nevertheless they have a limited host range because they need to bind to specific receptors to enter the cells (Singh SK 2009).
For *in vivo* use, retrovirus shows some weaknesses, specifically safety concerns and limitations in efficacy (Li CX 2006). One of these virus limitations is the random genomic integration, which could

lead to many undesirable effects, like insertional mutagenesis and potential carcinogenesis (Li CX, 2006, Singh SK 2009).

Lentiviral vectors

The lentiviruses are part of the retroviruses family (Reischl D 2009) and are capable of transducing both dividing and nondividing postmitotic cells (e.g. neurons, macrophages, haematopoietic stem cells, muscle and liver cells) (Leung RKM 2005, Kumar LD 2007, Singh SK 2009).
The most widely used lentiviral vectors are HIV-based, where safety it's a major concern (Leung RKM 2005, Singh SK 2009). Currently, there are a new generation of lentiviral vectors with reduced risks because of they are in the form of replication-defective and self-inactivating vectors (Singh SK 2009).

Other viral vectors and further considerations

Some other viruses have been used, like baculoviruses that are insect viruses and do not infect mammals. With the help of their envelope proteins they can transduce mammalian cells and this attribute has been explored to DNA deliver (Singh SK 2009). Some studies using baculovirus-mediated siRNA delivery approaches has been successfully used to inhibit viral replication of some viruses, like hepatitis C and hepatitis B virus (Singh SK 2009).
Other type of used virus is Herpes virus, that is usually infectious to cells, but with the deletion of some of the genes can be used due to a significantly reduced toxicity (Singh SK 2009).

Despite the initial promise as a vehicle to deliver of siRNA, immunogenicity and other safety issues are still great concerns of using in human and viral vectors no longer appear to be the tool of first choice (Nguyen T 2008, Tseng YC 2009). In some preliminary clinical studies, these vectors triggered fatal inflammatory reactions in one patient and leukemogenesis in another, leading to a shift in the delivery approach into synthetic and less immunogenic vector systems able to be systemically administered (Patel S 2011).

1.3.2.2 Non-viral administration

Toxicity and immunogenicity concerns associated with viral vectors have led to an active interest in non-viral vectors for gene delivery. The challenge is to develop a safe and efficient vector that can deliver siRNA, or other nucleic acid, in the desired target site.

Numerous siRNA carriers have been developed to achieve the success of cytoplasmatic delivery of siRNA *in vitro* and *in vivo*. Non-viral gene delivery systems are preferred for siRNA delivery because of their important benefits: the safety (presenting a low immunogenicity and smaller frequency of integration), relatively low production costs, reproducibility and possibility of large-scale production (Oliveira S 2006, Lee SY 2010).

However, their efficiency is still low when compared to viral vectors, which can be a limiting factor (Oliveira S 2006, Ramon AL 2008, Reischl D 2009). Moreover, lower loading efficiencies, colloidal instability, poor cell internalization and endosomal escape can limit their application (Reischl D 2009).
In addition to polymers and lipids, some inorganic platforms are being used as nanoparticle drug delivery systems, such as metallic nanoparticles (gold, silver, palladium), semiconductor quantum dots, magnetic nanoparticles (iron oxide), as well as carbon-, silicon-, and calcium-based nanomaterials (Wang X 2009, Tan SJ 2011). For the purpose of this work, only polymeric nanoparticles will be deeply explored.
Nevertheless, the first generation of nanoparticles used is mainly based on liposomes and polymer–drug conjugates (Petros RA 2010) thus a brief overview is herein considered. If the final charge of complexes between carriers and siRNA are positive, it will improve the cell entry since the charge of the cell membrane is negative (Ramon AL, 2008). Generally, the colloidal carriers can transport an ample range of nucleic acids (Oliveira S 2006).

Lipid based delivery

Lipid-based delivery systems have some interesting properties, such as biocompatibility, protection from nuclease degradation, ability to entrap hydrophilic and hydrophobic therapeutic entities and allowing the systemic delivery of nucleic acids to target cells across cell membranes (Peer D 2007, Lares MR 2010). One of the advantages of using cationic lipids is that an auxiliary intracellular interaction with cellular nonionic lipids would allow the neutralization and release of siRNA (Lares MR 2010).

Liposomes are perhaps the most known delivery carrier, being self-assembling spherical particles. These particles have a membrane composed of phospholipid bilayers surrounding an aqueous compartment, in which both hydrophilic and hydrophobic agents can be stored and mediate uptake of the substances into the cells by some form of vesicular transport, for example, through endosomes. The size of liposomes can range from 25 nm to 10 µm depending on the preparation method (Kurreck J 2009, Wang X 2009, Petros RA 2010).
Depending on the method of preparation, lipid vesicles can be multi-, oligo- or unilamellar, containing many, a few, or one bilayer shell(s), respectively (Martins S 2007). Small unilamellar vesicles are surrounded by single lipid layer (25-50 nm), whereas several lipid layers surround large unilamellar vesicles (100-200 µm) (Martins S 2007). Giant unilamellar vesicles have a mean diameter between 1 µm and 2 µm (10 layers) (Martins S 2007). Multivesicular vesicles are liposomes with lots of vesicles inside, liposomes are characterized in terms of size, surface charge and number of bilayers (Martins S 2007).
Like other nanoparticulate systems, liposomes can be functionalized, adding ligands directed to cell surface receptors in order to endorse selectivity to specific cells and tissues. One of the best examples is

the preparation of "stealth" liposomes (consisting on the PEGylation of the liposome). This strategy reduces rapid recognition of liposomes by RES, thus increasing the circulation time and the systemic dose of the therapeutic entity, which is a reflection of a decrease in the clearance and/or volume of distribution (Lammers T 2008, Aliabadi HM 2012).
The siRNA-lipid complexes showed significantly improved cellular internalization and endosomal escape of the nucleic acid. Further clear progress in siRNA delivery using liposomes is the use of neutral lipids in order to deal with the toxicity of cationic lipids (Shim MS 2010). Some examples of neutral lipids are DOPC, DOPE, DSPC and PC, among others (Aliabadi HM 2012).
Although the widely use of liposomes, they present some weaknesses: they are less efficient than viral vectors, have more difficulty to cross cell membranes, and some cationic lipids can be cytotoxic and form aggregates (Ramon AL 2008).

Another approach are lipoplexes, consisting in complexes of cationic lipids (such as DOTAP, DMRIE or DOSPA) and nucleic acids, having the advantage of being a self-assembling system formed as a result of the interaction between anionic charged nucleic acids and cationic lipids (López-Fraga M 2008, Reischl D 2009, Aliabadi HM 2012). Lipoplexes have been widely used for its high transfection efficiency because of the cationic charge of lipids; however, nonpermanent electrostatic interactions can make them heterogeneous and unstable, leading to a short shelf-life (López-Fraga M 2008, Tan SJ 2011) that can be overcome by PEGylation of lipoplexes (Kurreck J 2009). PEGylation will also lead to a reduced toxicity of this delivery system (Kurreck J 2009).

A more complex siRNA formulation is SNALP, a type of specialized lipid bilayer, typically composed of multiple lipids, including neutral, cationic and PEGylated lipids (López-Fraga M 2008, Aliabadi HM 2012). These lipid formulations show high levels of delivery to the liver, low immunostimulatory response, and lower required doses for systemic delivery when compared to other lipid-based siRNA delivery systems. The lipidic mixture of cationic and helper lipids in SNALP take on an inverted hexagonal lipid structure within the liposomal membrane, facilitating the release of siRNA via fusion of the particles with the endosomal membrane (Tan SJ 2011).

Solid lipid nanoparticles are an alternative system to traditional colloidal carriers and present an efficient and non-toxic profile. They are made from solid lipids (at room and body temperature) and a stabilizer surfactant. These nanoparticles can be used to delivery therapeutic entities to mucosal surfaces, and act as proteins, peptides and vaccines carriers (Almeida AJ 2007, Ekambaram P 2012). They are sub-micron systems ranging from 50 to 1000 nm (Ekambaram P 2012).

Lipid-based systems are considered less toxic than viral systems and this can be explained by the physiological compounds that form these structures leading us to assume that they will be well tolerated by cell (Martins S 2007).

Nevertheless the toxicity of the emulsifiers must always be taking into account, as well as the toxicity and immune response that different components may trigger (Martins S 2007, Tan JS 2011). One example is cationic liposomes complexed with siRNA have demonstrated that they are able to trigger both type I and II interferon responses *in vivo* (Tan JS 2011).

Polymer based delivery

The ideal nucleic acid delivery system must be stable, be cost effective and capable of transferring the genetic material into the target site (Pathak A 2009). The choice of polymers and the formulation should be able to achieve greater complexation and endosomal escape and should not influence the biocompatibility and the stability of the delivery system (Singha K 2011). Some biodegradable polymeric nanoparticles have shown advantage over liposomes by presenting an increased stability and the capacity to achieve an extended release (Muthu MS 2009).

Polymers, which are the most widely used materials in drug delivery vehicles, fall into two major categories: natural and synthetic. Generally, they are non-immunogenic and highly versatile (Patel S 2011). As we will see, the capacity for self-assembling that some polymers exhibited is of extreme importance due to the well-known production process simplicity (Blanco E 2009). They can used to develop different nanosystems, some examples are polyplexes, polymeric micelles, nanoplexes, nanocapsules, alginate nanogels (Martimprey H 2009) and dendrimers (Cho K 2008). These examples are represented in Figure 7.

Cationic polymers can electrostatically complex with the nucleic acids forming nanosized complexes named polyplexes (Martimprey H 2009, Reischl D 2009, Guo P 2010).

Polymeric micelles were self-assembly colloidal dispersions obtained after adding amphiphilic copolymers with the nucleic acid by electrostatic interactions and were normally more stable than polyplexes, having a smaller size (Martimprey H 2009, Reischl D 2009).

Other kind of polymeric delivery carriers were nanoplexes, which can be defined as matricial systems with the nucleic acids bound on their surface (Martimprey H 2009).

Nanocapsules were vesicular carriers composed of an aqueous core, where the nucleic acid is loaded, bounded by a thin polymer cover (Martimprey H 2009).

Dendrimers were hyperbranched molecules, having a regular pattern and repeated units and a central core with an internal cavity that can be used to transport the therapeutic entity (Cho K 2008).

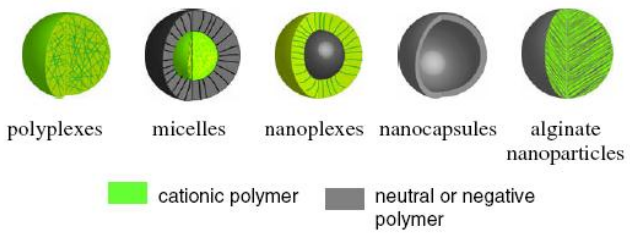


Figure 4 - Structure of the different polymeric nanocarrier systems. Reproduced from Martimprey H 2009.

A large number of polymers have been widely examined; natural, such as heparin, dextran, albumin, gelatin, alginate, collagen and chitosan, and synthetic, like PEG, PGA, PLA, PCL and HPMA (Wang X 2009). siRNA and other nucleic acids are simply complexed with cationic polymers via attractive electrostatic interactions (Shim MS 2010).

The resulting structures can be polymeric nanoparticles (polymer-drug conjugates), polymeric micelles (amphiphilic block copolymers) or dendrimers (hyperbranched polymeric structures) (Cho K, 2008).

Some of the differences between ationic polymeric nanoparticles and cationic lipids-based systems are the higher capability of polymers to form a complex with negatively charged siRNA, the hydrophobic moiety and water solubility (Ilardurya CT 2010).Herein we describe other examples, like the PEI-based nanocarriers that offer several advantages including high transfection efficiency and endosomal escape; the chitosan nanoparticles that have shown to be highly effective and safe for *in vivo* delivery of siRNA when administered in mice through intranasal and intravenous administration; and the use of atelocollagen for *in vivo* siRNA delivery resulted in increased cellular uptake and prolonged release of genes and oligonucleotides (Ozpolat B 2009).

a) PEI

Polyethylenimines are a series of synthetic and non-invasive polymers, recognized as an efficient non-viral nucleic acid carrier and allowing permanent gene expression in the target region without undesirable expression in other issues. PEI is available in branched or linear forms and a wide range of MW, and had demonstrated high transfection efficiency under *in vitro* and *in vivo* conditions. The polymer possesses a high cationic charge that is generated by protonation of the amino groups, making it pH dependent (Aigner A 2006, Zhang S 2007, Ilardurya CT 2010).

PEI is one of the most extensively investigated synthetic polymers for nucleic acid delivery *in vitro* and *in vivo*, due to their membrane destabilization potential, high charge density and ability to provide safeguard to the endocytosed nucleic acid from enzymatic degradation (Pathak A 2009, Shim MS 2010).

This polymer is very potent in transfection with its uniquely high buffering ability at the endosomal pH, the so called “proton sponge effect”, which releases nucleic acids into the cytoplasm. It seems to be a promising non-viral carrier for siRNA delivery *in vivo*, but its high toxicity and limited biodegradability need to be suitably controlled and reduced (Shim MS 2010).

The increase of the quantity of PEI and the increase of N/P ratio from 2 to 20 results in a significantly decrease in the particle size from >1000 nm to 100-200 nm, with simultaneous reduction in the polydispersity index. Complexes formed at low N/P ratios in the range of 2 to 5 tend to aggregate due to hydrophobic interactions, as well as Van der Waals forces. In contrast, higher N/P ratios reduce aggregation as a result of electrostatic repulsion, which contributes to the stabilization of complexes under physiological salt conditions (Ilardurya CT 2010).

Some studies have demonstrated the effectiveness of PEI carries, in 2004 Ge Q *et al* found that PEI-delivered nucleotides were distributed preferentially to the lungs following intravenous injection, showing PEI-siRNA is effective to treat influenza virus; in the same year Urban-Klein *et al* demonstrated that PEI-siRNA could be applied intraperitoneally to achieve a systemic therapeutic effect (Zhang S 2007).

Although the good results obtained, the most important problem with the use of PEI is the marked cytotoxicity due to induction of cell death by either necrosis or apoptosis (Ozpolat B 2009).

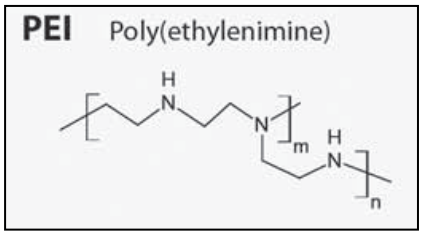


Figure 5 – Molecular formula of PEI. Reproduced from Tan JS 2011.

b) Chitosan

Chitosan is a mucopolysaccharide closely related to cellulose, it is is obtained by deacetylation of chitin, the major compound of exoskeletons in crustaceans and is digested by chitinases after oral administration (Nagpal K 2010).

The main advantages of chitosan are the low production costs and simple preparation methods of nanoparticles, the fact of being biodegradable and biocompatible, having low toxicity and low immunogenicity, suitability to prepare nanoparticles for controlled release, and the high positive charge that allows the formation of complexes with negatively charged nucleotides by electrostatic interaction (Katas 2006, Nagpal K 2010). The properties of chitosan are dependent on the MW, degree of deacetylation and viscosity. Generally, chitosan with lower MWs and lower deacetylation, exhibit greater solubility and faster degradation (Nagpal K 2010, Wang JJ 2011).

Katas and Alpar may be the first group to investigate the use of chitosan to deliver siRNA *in vitro*. The MW of chitosan is an important parameter for an efficient RNAi silencing, an example is the study of Liu *et al* which indicated that chitosan molecules 5–10 times the length of siRNA could form stable complexes, but chitosan with a low MW could not complex and compact siRNA into stable particles, with the formation of aggregates and having almost no silencing (Zhang S 2007, Mao S 2010).

Howard *et al* indicated an increase in size of the nanoparticle at lower N/P, nanoparticles formed at N/P 71 measured 181.6 nm but increased to 223.6 nm at N/P 6 (Mao S 2010).

Techaarpornkul *et al* investigate the cytotoxicity of chitosan/siRNA complexes and the results showed that the different formulated chitosan/siRNA complexes did not affect the viability of cells. So they can assume that chitosan/siRNA formulation were safe (Techaarpornkul S 2010).

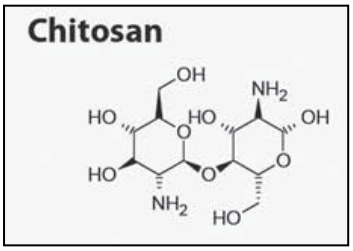


Figure 6 – Molecular formula of Chitosan. Reproduced from Tan JS 2011.

c) Poloxamers

Poloxamers, also known as Pluronics, are amphiphilic copolymers composed of poly(ethyleneoxide) and poly(propylene-oxide) (Artzner F 2007, Yallapu MM 2010). They were initially used in pharmaceutical industry as surfactants, emulsifying agents, solubilizing entities, dispersing components, and for controlled release (Artzner F 2007, Patel HR 2009). More recently some studies demonstrated their capacity to self-assemble and originate micelles with the increase of temperature or polymer concentration (Artzner F 2007). The easily forming micelle drug carriers can frequently have a 40 nm diameter (Yallapu MM 2010).

d) Other polymers and polymeric delivery strategies

Atelocollagen was the first biomaterial introduced as a gene delivery system (Aigner A 2008) and it is a biocompatible polymer which is liquid at 4ºC and a gel at 37ºC (Masiero M 2007).
The size of the complexes formed between atelocollagen and negatively charged nucleic acid molecules is determined by the ratio between both components, with complex sizes being in the range of 100 to 300 nm at low concentrations, which allows cellular uptake (Aigner A 2008). In contrast, at higher concentrations the complexes stay longer in some places and make atelocollagen act like a depot for sustained release (Aigner A 2008). Atelocollagen can also increase cellular uptake and nuclease resistance (Masiero M 2007), protect siRNA and allow *in vivo* delivery (Aigner A 2008).

Alginate, a negatively charged polysaccharide extracted from brown algae, is another biological polymer generally employed in pharmaceutical industry due to its low toxicity and low immunogenicity. Upon addition of divalent calcium ions into an alginate mixture under certain conditions, alginate nanoparticles are formed as a result of ionic crosslinking between the polysaccharide chains, and have been used for gene delivery. PEI–alginate nanostructures have also been assembled as a platform for siRNA delivery, resulting in up to 60% suppression in protein expression *in vitro* (Tan JS 2011).

Cyclodextrin-based polycations (CDP) can deliver therapeutic molecules ranging from small molecule drugs, plasmids, and recently, siRNA. CDP present 50 nm to 150 nm in diameter, and are composed of

cationic polymers with funnel-shaped cyclodextrin molecules integrated into the polymer strand. As the individual components of CDPs were known to be relatively non-toxic, they were designed specifically as highly non-toxic and non-viral vectors (Guo P 2010).
Some authors claim that CDP protects RNA from serum, so modified nucleotides are not required. On of most surprising characteristics of this strategy is that the resulting siRNA complex, even when siRNA containing an immune stimulatory sequence is included, does not produce immune stimulation like the one seen with other siRNA–lipid complexes (Guo P 2010).

Cell-penetrating peptides (CPPs) are cationic and/or amphipathic peptides that are able to facilitate the translocation of a conjugated therapeutic entity across the plasma membrane, making them as an effective and non-toxic mechanism for drug delivery. These peptide sequences were also termed Trojan horse peptides (Bolhassani A 2011).
The two fundamental types of CPP are the cationic, composed by short amino acid sequences rich in arginine, lysine and histidine, which were responsible for the cationic charge of the peptide; and the amphipathic, which have lipophilic and hydrophilic ends that takes control in the penetration of the plasma membrane (Bolhassani A 2011). The mechanism for the CPP-facilitated cellular uptake is unknown (Bolhassani A 2011), but it seems to be receptor and energy-independent, but in certain cases, entry in the cell can be somewhat mediated by endocytosis (Reischl D 2009).
The large charge at physiological pH excludes the passive diffusion of CPPs across the lipid bilayer. Despite the controversy surrounding the translocation mechanism, the cell entry property of these peptides makes them an attractive carrier for genes, proteins, and other types of drugs and imaging entities (Bolhassani A 2011).
One of the CPPs advantages is the lack of toxicity when compared with other delivery systems, such as liposomes and polymers (Bolhassani A 2011).

e) PLGA and PLGA-PEG

PLGA is one of the first polymers approved by the US Food and Drug Administration for human use (Tan JS 2011) and particles including PLGA are extensively studied as therapeutic delivery vehicles due to their biodegradable and biocompatible (Xie H 2010).
PLGA is one of the most successfully used biodegradable polymers because its hydrolysis origins two metabolite monomers, lactic acid and glycolic acid (Danhier F 2012). The two monomers formed are endogenous and can be metabolized without problems by the body via the Krebs cycle. Considering this, a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications (Tan SJ 2011, Danhier F 2012).
The degradation rate of PLGA depends on a diversity of parameters that include the lactic acid/glycolic acid ratio, MW, and the shape and structure of the polymer matrix (Singha K 2011). PLGA having

different compositions of lactic acid and glycolic acid have been commercially developed and are being investigated for a broad spectrum of biomedical applications (Singha K 2011).

siRNA-loaded PLGA nanoparticles have attracted much attention since they are assumed to meet the criteria required for successful delivery and show great promise in *in vitro* gene silencing: they are sufficient small for efficient tissue penetration and cellular uptake; the siRNA can be entrapped into the PLGA matrix; present high stability; offer physical protection against RNase activity as well as a favorable colloidal stability of the system; demonstrate an easy cellular uptake by endocytosis; have a favorable safety profile; and the nanoparticles obtained have sustained-release characteristics (Cun D 2011, Singha K 2011, Tan JS 2011, Muthu MS 2009).

As previously referred, particle size is one of the key features in determining biodistribution because it influences circulating half-life, cellular uptake and biodistribution. The kinetic aspects of therapeutic entities release are also strongly influenced by particle size (Xie H 2010). Application of particles in the range of approximately 100 nm are likely to be advantageous because they are internalized by cells at rates 15 to 250 fold greater than micron size particles (Xie H 2010).

Cun *et al* demonstrate the possibility to increase the loading efficiency without using cationic co-adjuvants to more than 60–70% of biologically active siRNA by choosing the optimized formulation parameters without compromising the particle size and the negative particle zeta potential. By statistical experimental design, the most important parameters were found to be the concentration of PLGA and the w/o phase ratio. siRNA was detectable on the surface of the nanoparticles and suffer a first burst release followed by a continuous triphasic release (Cun D 2011).

The combination between PLGA with PEG (denominated PLGA copolymer or PLGA-co-PEG) is mainly used for the preparation of "stealth" polymeric nanoparticles (Muthu MS 2009).

PLGA-co-PEG copolymers are amphiphilic (Jeong B 1999), hydrophilic PEG occupies the outer shell region and hydrophobic PLGA constitute the inner core in order to decrease free energy of hydration (Jeong B 2000).

In 2009, Saltzman WM and co-workers reported that siRNA pre-complexed with low MW, natural polyamines demonstrated high encapsulation efficiencies into PLGA nanoparticles (up to 40% with spermidine), achieving sustained silencing of gene expression by up to 60% after topical application to the vaginal mucosa in mice (Tan JS 2011).

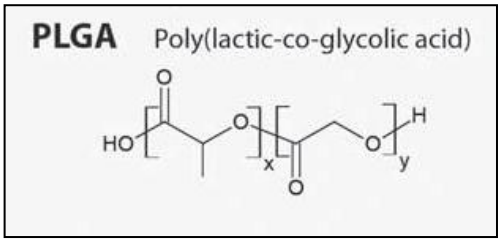


Figure 7 – Molecular formula of PLGA. Reproduced from Tan JS 2011.

Polymeric micelles, self-assemblies of block copolymers, have a reasonably narrow size distribution in the range of 10 nm to 100 nm and they can be used as a targeted drug delivery carrier for various therapeutic entities (Muthu MS 2009). One of the best properties of PLGA-co-PEG is the capacity of form self-assembly micelles by dissolve the polymer in an organic solvent and evaporate the solvent by increasing the temperature, under pressure conditions in a relatively simple equipment.

1.4 Therapeutic applications – siRNA as "active substances"

RNAi has become a promise in biomedical investigation and as a novel therapeutic for great range of diseases due to the potent and sequence-specific gene silencing (Pan X 2011). siRNA can be designed to silence potentially any gene, expanding the universe of targets possibly to the entire genome but the challenge of find an optimal delivery method for specific sites of the genome without affecting any normal genes in the cell is still in attendance (Underwood L 2010, Petrocca F 2011).
RNAi-based therapeutic agents are developed to degrade the target RNA and prevent the expression of the associated undesirable protein necessary for disease initiation or progression (Reddy LS 2007). The siRNA may be able to target a single mutation in the genome which is causing a disease. Once the target has been located, the siRNA has the potential to silence the single mutation, which possibly will eliminate the disease (Underwood L 2010).

This new approach can be used and have been tested in diverse fields of Medicine. Some of the applications are in cardiovascular and cerebral vascular diseases (some examples are congenital heart disease (Tang Y 2007), hypertension (Tang Y 2007), atherosclerosis (Reddy LS 2007, Tang Y 2007), cardiac hypertrophy (Tang Y 2007), myocarditis (Tang Y 2007) and heart failure (Tang Y 2007)); metabolic diseases, like diabetes (Rondinone CM 2006, Czech MP 2011), hypercholesterolemia (Davidson BL 2011, Tiemann K 2009), obesity (Rondinone CM 2006, Czech MP 2011); ophthalmic and retinal diseases, like wet age-related macular degeneration (Urakami T 2007, Fougerolles AR 2008, Davidson BL 2011); pulmonary diseases, where some targets are respiratory syncytial virus (Fougerolles AR 2008), parainfluenza virus, severe acute respiratory syndrome, the common cold, lung cancer (Pan X 2011), cystic fibrosis, asthma (Davidson BL 2011, Pan X 2011), kidney disorders (Davidson BL 2011) and chronic obstructive pulmonary disease (Leung RKM 2005, Davidson BL 2011); cancer; infectious diseases; and neurodegenerative disorders (Grimm D 2006, Gonzalez-Alegre P 2007, Wu SY 2009).

To siRNA properly function as a therapeutic agent, it is needed a great understanding of the molecular events essential to the appearance of the disease. The pathogenesis has implicated specific gene products for which silencing can lead to the disappearance of the disease. RNAi have to be explored in the previously described scenario as a potential therapeutic strategy and, at least, three major groups of

human diseases fall in this profile: cancer, infections and neurodegenerative disorders (Gonzalez-Alegre P 2007). In the context of this work we will explore these three potential applications in more detail.

1.4.1 Cancer

Cancer is a disease characterized by anomalous cell repair, differentiation, proliferation, and cell to cell/tissue/host interaction; it is a result of an accumulation of genetic and epigenetic alterations (Wang Z 2011). The genetic alterations command the malignant behavior of tumor, contributing for uncontrolled cell proliferation, invasion of adjacent tissues, development of metastasis starting in the primary tumor, neovascularization and capacity to resist to chemotherapeutic drugs (Guo P 2010). For the tumor be able to growth, it needs a constant supply of nutrients. Metastases with a diameter bigger than 2 mm are dependent on blood supply and, therefore, induce a rapid and defective angiogenesis. Blood vessels inside a tumor are often incomplete, with capillaries having an increased permeability and ending directly inside the tissue, other characteristic is the poor lymphatic drainage (Ogris M 2002, Peer D 2007).

Within a tumor there are different areas that have to be distinguished: the outer core, usually well vascularized and simply accessible via the bloodstream, adjacent to the inner core is the semi-necrotic and necrotic regions, here the tumor cells don't have available sufficient nutrients and oxygen, becoming necrotic. The area of interest for a therapeutic agent is frequently the outer region, where the cells are actively dividing (Ogris M 2002).

Nanomedicines have the potential to revolutionize cancer diagnosis and therapy (Peer D 2007) as it brings new expectations for cancer treatment. This approach grants new properties to the therapeutic agent (improved stability, adapted pharmacokinetics, lower toxicity) and targets directly the tumor (Seigneuric R 2010).

Due to the EPR effect the nanocarriers can extravasate into the tumor via the permeable vessels, which is an advantage relatively to free drugs that diffuse non-specifically. The nanocarriers also accumulate in the tumor tissues because of the lack of lymphatic drainage, allowing the release of the therapeutic agent in the core of tumor (Peer D 2007).

One of the biggest problems of chemotherapy is the relative lack of specificity which affects the tumor but also normal tissues, causing adverse side-effects. *In vivo* data allows estimating that from out of 100,000 molecules administered intravenously; only 1 to 10 in fact will reach the desired target (Seigneuric R 2010).

In tumor cells, proto-oncogenes have normally to be activated by various mechanisms, producing oncogenes that operate in a dominant way. There are two important abnormalities in cancer cells: the deregulation of cell cycle, resulting in uncontrolled growth, and the resistance to death, as a result of anomalies in proteins that intervene in apoptosis (Reddy LS 2007). siRNA have been designed to target

dominant oncogenes, deregulated oncogenes, and viral oncogenes involved in the appearance of tumoral diseases (Oh YK 2009).

To selectively target cancer cells without harm normal cells, siRNA should be designed to target a gene specifically involved in the growth or survival of the tumor cell (Reddy LS 2007). A considerable number of preclinical studies with siRNA have presented positive outcomes by silencing genes (Wang Z 2011).

In vivo studies have also shown favorable outcomes by RNAi targeting of components critical for tumor cell growth, invasion and metastasis, angiogenesis, apoptosis, cell cycle regulation, cell senescence, and chemoresistance (Ogris M 2002, Rao D 2009, Guo J 2011, Wang Z 2011).

Some examples of RNAi mediated cancer therapies applicable with non-viral delivery include: direct killing of tumor cells (with enzyme or pro-drug therapy); inhibition or reversion of angiogenesis via VEGF-signaling; inhibition of tumor survival and inducing apoptosis via inhibiting Wnt pathway, NF-κB pathway, EGFR, Her-2/neu, telomerase, MDM2 and p53, or Bcl-2; inhibit tumor cell metastasis or migration; induce chemoprotection (in bone marrow); stimulate anti-tumor immunity (cytokines or vaccines); enhance radio- or chemo-sensitivity by inhibiting MDR1. The combination of siRNA therapy that inhibits more than one pathway, and simultaneous silencing, demonstrate an enhanced anti-tumor effect and could be an important advance for cancer therapy (Ogris M 2002, Tseng YC 2009, Guo J 2011).

Additionally, siRNA can be used in combination with chemotherapy or radiation therapy to inhibit tumor growth (Wang Z 2011).

Table 3 – Ongoing clinical trials for RNAi-based drugs. Reproduced from Petrocca F 2011.

Phase	Route	Disease	Target	Delivery Agent	Company	Year Initiated
I	Systemic	Liver cancer	KSP/VEGF	Liposome	Alnylam Pharmaceuticals (Cambridge, MA)	2009
I	Systemic	Solid tumors	RRM2	Nanoparticle	Calando Pharmaceuticals (Pasadena, CA)	2008
I	Systemic	Solid tumors/lymphoma	HIF-1α survivin	LNA oligonucleotide	Santaris Pharma (Hoershoelm, Denmark)/Enzon Pharmaceuticals (Bridgewater, NJ)	2009
I	Systemic	Solid tumors	PKN3	Liposome	Silence Therapeutics (London, United Kingdom)	2009

Abbreviations: RNAi, RNA interference; KSP, kinesin spindle protein; VEGF, vascular endothelial growth factor; RRM2, ribonucleotide reductase subunit 2; HIF-1α, hypoxia-inducible factor 1; LNA, locked nucleic acid; PKN3, protein kinase N3.

1.4.2 Infectious diseases

Diseases caused by viruses and bacteria continue to be main causes of death globally and the emergence of resistant strains are an increasing concern (Reddy LS 2007). Many deaths also result from bacterial infections and some important examples are pneumonia and sepsis. The ability of RNAi to inhibit the replication or cellular uptake of viruses and other infectious agents has been demonstrated in cell culture studies and, therefore, holds potential for future treatments of human patients (Reddy LS 2007).

The use of siRNA can be used to try of turn off virus gene expression. For viruses that have high mutation rates, there are two possible methods of improving siRNA therapeutic potential. Targeting the viral pathways might be a better approach than targeting the virus directly, because if the virus changes

due to a mutation, the siRNA will still silence the targeted host genes because the pathways used by the virus do not change. The second method involves targeting the cofactors used in the viral pathways, since they also remain the same (Underwood L 2010).

HIV

Synthetic siRNA have been used to target several early and late HIV-encoded RNAs in cell lines and in primary hematopoietic cells including the TAR elements, tat, rev, gag, env, vif, nef and reverse transcriptase (Reddy LS 2007).

Targeting the virus directly represents a substantial challenge for clinical applications, because the high viral mutation rate will lead to mutants that can escape being targeted. RNAi-mediated downregulation of the cellular cofactors required for HIV infection is an attractive alternative or complementary approach. Cellular co-factors such as NF-B, the HIV receptor CD4 and the co-receptors CXCR4 and CCR5 have been successfully downregulated by RNAi, resulting in the inhibition of HIV replication in numerous human cell lines and in primary cells, including T lymphocytes and hematopoietic-stem-cell-derived macrophages. There are drawbacks in targeting cellular HIV co-factors because non-infected cells will inevitably be targeted as well, leading to toxicities that are similar to those observed with the current ant-retroviral drugs. Viral targets will to be included in any successful strategy using RNAi. These targets should be sequences that are highly conserved throughout the various clades to ensure efficacy against all viral strains (Reddy LS 2007).

Silencing host receptors with critical biological functions, such as CD4, may result in unacceptable side effects. Targeted delivery of siRNA to viral-infected cells then becomes an essential supplement to this approach. For example, siRNA conjugated to an anti-gp160 antibody was delivered specifically to a mouse melanoma cell line stably expressing HIV gp160, which mimics the antigen presented by HIV-infected cells (Guo P 2010).

The delivery of siRNA to HIV-mediated cells is also a challenge. The target cells are primarily T lymphocytes, monocytes and macrophages. As synthetic siRNA do not persist for long periods in cells, they would have to be delivered repeatedly for years to effectively treat the infection (Reddy LS 2007).

Hepatitis Virus

Although a vaccine is available for hepatitis A and B virus, treatment options for chronically infected patients are limited and particularly ineffective in case of hepatitis C infection. HBV is an excellent candidate for therapeutic RNAi, as its unusually compact genome with lack of redundancy results in very limited sequence plasticity and prevents the virus from evading from RNAi by mutation. Thus, ideally, a single siRNA can potentially target multiple viral transcripts simultaneously and efficiently inhibit not only viral gene expression, but also DNA replication, because HBV amplifies through a RNA intermediate (Reddy LS 2007). Both HBV and HCV have been targeted *in vitro* and *in vivo* by siRNA. The potential targets for RNAi-mediated gene inhibition include specific viral genes necessary

for viral infection, pre-treating donor livers to prevent re-infection in chronically infected HBV or HCV liver transplant patients and regulating the host response to chronic viral infection (for example, by modulating viral replication within hepatocytes or altering the inflammatory or fibrotic sequel of infection). Proof of principle was provided by Song and co-workers using high-pressure tail vein injection in mice to target mRNA encoding Fas, a cellular death receptor and a key mediator of T-cell-mediated apoptosis in virally infected hepatocytes. In this study, the first to demonstrate RNAi efficacy successfully in an animal model of disease, anti-Fas RNAi not only protected mouse hepatocytes from apoptosis, but also saved the majority of animals from death by the fulminant hepatitis normally induced by injection of anti-Fas antibody (Pellish RS 2008).

Other applications

Some studies have been made in other diseases. Sepsis continues to be the leading cause of mortality in patients that enter the intensive care unit and independent autopsy studies showed marked sepsis-induced apoptotic depletion of CD4+ T cells and B cells (Brahmamdam P 2009). Another target are flaviviruses, including dengue virus, Japanese encephalitis virus, yellow fever virus and West Nile virus, that don't have any available tharapeutic at the time (López-Fraga M 2008). Numerous other diseases caused by parasitic protozoans, helminths and insect vectors, and micobacterial infections (such as tuberculosis) are targets to siRNA therapeutics (López-Fraga M 2008).

Another important aplication can be in the prion disease, also designated as transmissible spongiform encephalopathies, which include Creutzfeldt–Jakob disease, kuru, fatal familial insomnia and Gerstmann–Sträussler–Scheinker syndrome in humans. These diseases, so far, don't have any available therapies (White MD 2009, Aguzzi A 2010). Main pathological features of these disorders are the amyloid aggregation of PrPSc and spongiform degeneration of neurons, astrocytosis and neuronal loss (White MD 2009). White and co-workers achieved *in vivo* silencing of PrP via RNAi, and resultant inhibition of PrPSc replication in cell culture (White MD 2009).

1.4.3 Neurodegenerative disorders

Some important neurodegenerative disorders that didn't have treatment yet are Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, and the polyQ repeat disorders like Huntington's disease and spinocerebellar ataxias (Grimm D 2007, Reddy LS 2007). Whereas precise genetic mutations are responsible for a small percentage of cases of Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, all cases of Huntington's disease result from mutations in the huntingtin protein, in addition, each disorder is characterized by the dysfunction and death of specific populations of neurons (Reddy LS 2007). In this particular group of disorders, one of the biggest challenges is achieving safe and efficient delivery of therapeutic RNAi agents to neurons, which are highly specialized, post-mitotic cells protected by the blood–brain barrier (Gonzalez-Alegre P 2007).

The chronic nature of many brain disorders also means that sustained delivery of an RNAi reagent, or its repeated administration, will be required to achieve a long-term benefit (Gonzalez-Alegre P 2007).

Alzheimer's disease

Alzheimer's disease is characterized by dementia, with a progressive loss of memory and the ability to execute some tasks, impaired communication and identification of people and objects (Ross CA 2004). The most affected cells in Alzheimer's disease hippocampal and cortical neurons, involved in learning and memory processes (Reddy LS 2007) conjugated with synaptic pathology and altered neuronal connections (Ross CA 2004). Being the most common neurodegenerative disorder, Alzheimer's disease pathology is characterized by the formation of two types of protein aggregates in the brain: amyloid plaques (Aβ peptide) and intracellular neurofibrillary tangles (tau protein), that have been the targets on the developing therapeutics. It is acknowledged that the number of amyloid plaques in brain didn't have a direct association with the degree of dementia (Aguzzi A 2010). Another target is BACE1 that cleaves the amyloid precursor protein in the generation of amyloid-β peptide, post-mortem studies realized in brains of patients with Alzheimer's disease demonstrated increased amounts of BACE1 but not of its mRNA, which points out a post-transcriptional regulation of the protein in the presence of the disease (Salta E 2012).

Parkinson's disease

Parkinson's disease is one of the most common neurological disorders, second only to Alzheimer's disease, and approximately 2% of people develop this disease during their lifetime (Manfredsson FP 2006, Maraganore DM 2011).

The disease is associated with progressive loss of dopamine-producing neurons in the *substantia nigra pars compacta* that control body movements (Manfredsson FP 2006, Reddy LS 2007), which leads to reduction in brain dopamine levels and hence occurrence of motor dysfunctions (Wong HL 2012). This causes tremors even at resting, slow and rigid movements, and postural instability (Ross CA 2004).

A hallmark pathological feature of this disease is the presence of intra-cytoplasmic inclusions in the form of fibrillar aggregates, termed Lewy Bodies, in the dopaminergic neurons that contain α-synuclein; the Lewy Bodies are highly immunoreactive to components of the proteasomal machinery such as ubiquitin and proteasomal subunits (Manfredsson FP 2006, Salta E 2012). The α-synuclein is the only parkin locus gene for which the pathogenesis has been clearly linked to an over-expression mechanism, being amenable to gene silencing therapeutic strategies (Maraganore DM 2011).

It have been realized that neurotrophic factors could be utilized to invigorate the quiescent brain cells for increased production of dopamine (Wong HL 2012) and that the one of the therapeutic approaches can be targeted brain delivery of siRNA that silence α-synuclein expression (Maraganore DM 2011).

Huntington's disease

Huntington's disease is one of nine neurodegenerative diseases that are caused by expansion of CAG triplet repeat sequences within the protein coding regions of otherwise unrelated genes (Maxwell MM 2009). This disease is caused by a single mutation, described as the expansion of the CAG repeat in the gene encoding for the polyglutamine in the N terminus of huntingtin protein (Ross CA 2004, Maxwell MM 2009).

Clinical features of Huntington's disease include involuntary movements (chorea) and cognitive, behavioral, and psychological changes (Maxwell MM 2009).

The novel toxic properties of mutant huntingtin protein are believed to reside in N-terminal proteolytic fragments containing the expanded polylutamine tract, and mutant huntingtin fragments of various lengths, when expressed as transgenes, are sufficient to cause a progressive neuropathological phenotype in rodents. Several Huntington's disease mouse models have been generated to date. It is supposed that a reduction of mutant huntingtin protein levels would confer a significant therapeutic benefit in Huntington's disease. This theory is supported by studies conducted in mouse models that permit regulated and reversible expression of the mutant transgene, the observations made provide a key proof of concept to support the therapeutic potential of RNAi in this disease (Maxwell MM 2009).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is an age-dependent paralytic disorder resulting from selective death of motor neurons in the brain and spinal cord (Reddy LS 2007, Guo P 2010). Progressive muscle weakness and atrophy can be ultimately fatal, usually within five years after the onset of the disease. Currently, there are not an effective therapeutic (Guo P 2010).

A great fraction of familial cases are caused by a dominant mutation in the gene encoding SOD1, the exact molecular mechanisms of disease pathogenesis are not explained yet, but evidence implicates anomalous toxic properties of the mutant protein. It is believed that agents able to reduce the levels of mutant protein will reduce the toxicity and slow the disease development (Miller TM 2006, Guo P 2010). It's supposed that these cases could be susceptible to a gene-silencing therapeutic and in 2005 three different approaches demonstrated a beneficial effect in ALS model mice by the use of viral-encoded siRNA designed to reduce mutant SOD1 accumulation (Miller TM 2006).

1.5 The new regulatory paradigm

To use the potential of nanotechnology for drug delivery, full attention is needed to safety and toxicological issues (Jong WH 2008). Having different physicochemical properties than micron-sized particles, the administration of nanoparticles can result in a changed body distribution, penetration of blood brain barrier, and triggering of blood coagulation pathways (Jong WH 2008). Costigan S evaluate the evidence for toxicity of nanoparticles used in healthcare products and pointed four possible

mechanisms of toxicity: chemical toxic of one of the nanoparticle components, toxicity as a result of degradation products, toxicity attributed to nanoparticles endocytosis, and membrane lysis possibly originated by chemical toxicity (Costigan S 2006).

For the new developed technology reach the market it has to be approved by the competent health authorities. Similar to all other drug and medical device manufacturers, one of the requisites for a nanocarrier to be used as a therapeutic is the compliance with Good Manufacturing Practices. However, the novelty of manufacturing presents unique challenges to companies seeking to comply with such regulations (Nijhara R 2006).

One of the issues for the nanomedicine manufacturers is the scale-up and current absence of suitable manufacturing standards for nanotechnology materials and components can be an issue to overcome as briefly as possible. The exceptional characteristics of nanoscale materials will also lead to a revolution in quality control measurements and techniques. It is expected that the adoption of widespread standards for manufacturing and quality control will contribute to a persistent expansion of nanomedicine (Nijhara R 2006).

Small molecular drugs usually also have numerous adverse effects and, at least, the same safety standards should be applied to the preclinical development of RNAi applications (Kurreck J 2009).

2. AIM

A careful characterization of the potential toxicity of both the polymer and the final nanoparticle is critically important (Wang X 2009). Further testing and investigation of the mechanisms of siRNA-based therapeutics is clearly worth the time and effort because clinically useful siRNA-based products will contribute significantly to the future of medicine (Lares MR 2010).

Until today, the biggest obstacle for use siRNA as a new class of drugs has been the delivery (Seyhan AA 2011) and it is imperative that siRNA reach the cytoplasm of the target cell to become effective and induce silencing (Mao S 2010). Antisense drugs have been struggled with the same problem for over two decades (Seyhan AA 2011). An ideal carrier for siRNA should be able to bind and condense siRNA, provide protection against degradation, specifically direct siRNA to target cells, facilitate its intracellular uptake, escape from the endosome/lysosome into cytosol, and finally promote efficient gene silencing (Mao S 2010). In this thesis we try to develop a nanoparticle DDS that can deliver siRNA to the cells.

The major objective in the development of a DDS is the possibility to reach the market. Simple methods have to be used in order to allow the scale-up process so that the DDS can be produced in large quantities. The success of a medicine also depends on its vehicle and administration form. The hydrodynamic injection used in most of the proofs of concept of siRNA efficacy is not suitable for human use and is necessary to find out a vehicle that leads to a siRNA administration with the minimum of adverse effects and the maximum of efficacy. Other important issue is the compliance of the patient to the new DDS.

The nanotherapeutic system developed in this work is polymeric micelles. Usually this nanosystem has a size from 10 nm to 100 nm, has a spherical and supramolecular core-shell structure, and is composed of amphiphilic di- or tri-block polymers (Blanco E 2009). The use of polymeric micelles is not new; it was first reported by Ringsdorf and coworkers for cancer treatment (Ringsdorf H 1975, Gros L 1981). In this work the fundamental aim is to develop polymeric micelles.

To selecting an appropriate material, two criteria are required for successful nanoparticle-mediated siRNA delivery: first, that the particles are small enough to penetrate tissue barriers and be taken up by cells, and second, that large, stable quantities of siRNA can be entrapped and released. In this work, we work with polymers that can form micelles that later can incorporate nucleic acids. The three polymers tested were PLGA-co-PEG, Pluronic® and Soluplus®.

3. MATERIALS AND METHODS

In this chapter is described the preparation and characterization of nanoparticles, using different polymers, for the administration of siRNA. The methods that are used are simple and don't require complex equipments, which is important in a scale-up process of industrial production.

3.1 Materials

Acetonitrile HPLC Grade was purchased from Fisher Scientific. Ethanol absolute for analysis and Dichloromethane for analysis was purchased from Merck. Tween 80® type analysis was purchased from Riedel-deHaën, Allied Signal Inc. Methanol multisolvent HPLC grade was purchased from Scharlan. Phosphate Buffered Saline (PBS), pH 6.8 and pH 7.4. Water was purified by reverse osmosis (Milli-Q, Milipore®).

Pluronic® F 127 (ethylene oxide/propylene oxide block copolymer, MW of approximately 12.6 kDa) was obtained from BASF®, Soluplus® (copolymer comprised of polyethylene glycol, polyvinylcaprolactam and polyvinylacetate, the average MW is in the range of 90 kDa-140 kDa) was provided by BASF®, Resomer® RG 502 (Poly(D,L-lactide-co-glycolide) with a MW of about 7 kDa-17kDa) and Resomer® RGP t 50106 (Poly[(D,L-lactide-co-glycolide)-co-PEG] triblock) were purchased from Boehringer Ingelheim. Control siRNA duplex GFP (jellyfish) SR-CL020-005 was ordered from Eurogentec SA (siRNA negative control duplex is made of two complementary strands and dTdT 3' overhangs in antisense and in sense strand). GeneRuler™ 1kb DNA Ladder was purchased from Fermentas®. We also used alamarBlue® Cell Viability Reagent from invitrogen™ and Jurkat (Human T-cell lymphoblast-like) cells for this assay. The MDA-MB-231/GFP cell line was obtained from Cibbim-Nanomedicine, Barcelone, Spain, the culture medium used for this cells was D-MEM enriched with fetal bovine serum (FBS), Non-Essential Amino Acids (NEAA), L-glutamine and Pen-Strep.

The equipments used in this work were an Ohaus® Pioneer PA214 analytical four decimal place laboratory balance, an IKA® vortex 4 basic Model V4B, the rotary evaporator was an IKA® Rotary Evaporator RV10-Basic with HB-10 Bath, Eppendorf® Centrifuge 5430, Megafuge 1.0R Heraeus Instruments, Selecta Ultrasound-H, NanoDrop® NP-1000 Spectrophotometer, Autosizer 4700 from Malvern® Instruments IV, UK, Vivaspin® 2 Centrifugal Concentrator and Nanosep® centrifugal device 3K and 10 K.

3.2 Methods

3.2.1 Preparation of nanoparticles

The nanoparticles were obtained using the solvent evaporation method. The temperature and time that the films remained in the rotary evaporator varied and had to be adjusted through the time of work, the fixed variables were the rotation of the balloon, approximately 185-245 rpm, and the pressure, about 80 kPa.

i) Preparation of micelles of PLGA and PLGA-co-PEG

PLGA/PLGA-co-PEG micelles were prepared with an organic phase of dichloromethane. In the initial stage of the work were used a mixture composed by 8 mg of Resomer® RG 502 (PLGA) and 8 mg of Resomer® RGP t 50106 (PLGA-co-PEG) in 1 ml of dichloromethane was used. The mixture was placed in a 25 ml balloon and remained about 12 to 15 minutes in rotary evaporator.

In a second phase, in order to adjust the ideal quantity of PEG in the formulation, the formulations showed in table 4 were prepared.

Table 4 – Quantities of polymers PLGA and PLGA-co-PEG and solvent dichloromethane used in the first experimental formulations.

Formulation	Quantity of PLAG (mg)	Quantity of PLAG-co-PEG (mg)	Quantity of dichloromethane (µl)
1	1	1	400
2	1	2	400
3	1	3	400
4	3	3	600
5	2	4	600
6	1.5	4.5	600

ii) Preparation of micelles of different polymers

For the evaluation of the behavior and characteristics of other polymers we selected to work with Resomer® RGP t 50106 (PLGA-co-PEG), Pluronic® F 127 and Soluplus®. The solvent used for the development of PLGA-co-PEG micelles was acetonitrile and for Pluronic® F 127 and Soluplus® micelles a solution of methanol:ethanol (1:1) was used. The first developed formulations was presented in the table 5, all the formulations were made in triplicate.

Table 5 – Quantities of polymer and solvent used in the formulations used to test different polymers.

Formulation	Quantity of polymer (mg)	Quantity of solvent (ml)	Time for solvent evaporation (minutes)
PLGA-co-PEG	20	5	20
Pluronic	20	5	25
Soluplus	20	5	30

iii) Optimization of micelles

Micelles of Soluplus® were prepared using 20 mg of polymer plus 5 ml of methanol:ethanol (1:1) solution, and the balloon was placed 30 minutes in rotary evaporator.
Different quantities of PLGA-co-PEG were used as described in the table 6. Each formulation was prepared in duplicate.

Table 6 – Quantities of PLGA-co-PEG and solvent used in the optimization of these polymer formulations.

Formulation	Quantity of polymer (mg)	Quantity of acetonitrile (ml)
1	20	5
2	5	3
3	2	1

After this optimization, replications of the films of Soluplus® and PLGA-co-PEG were carried out in the same conditions.

3.2.2 Rehydration of the films – plain and loaded with siRNA micelles

Rehydration of the films obtained after solvent evaporation were performed with different solutions, depending on the type of micelles or in order to optimize the rehydration.
The siRNA stock solution had a concentration of 50,000 nM and the final concentration used was 250 nM.

i) Preparation of micelles of PLGA and PLGA-co-PEG and micelles of different polymers (Pluronic® F127 and Soluplus®)

We worked with triplicates, so one of the films was rehydrated with 1 ml of purified water and the other two films were rehydrated with a solution of siRNA. After add the solvent, the balloons were vortexed for about 15 minutes.

For each solution 100 µl were transferred for ultrafiltration in Nanosep® 10 K and centrifuged in Eppendorf® Centrifuge 5430 for 30 minutes at 6000 rpm. In some cases the ultrafiltration was made in Nanosep® 3 K. When it was not possible to develop all the techniques in the same day, the solutions were stored at 2ºC – 8ºC.

The micelles obtained were rehydrated according to table 7.

Table 7 – Type and quantities of rehydration solution used in each formulation.

Formulation	Quantity of rehydration solution
Pluronic F127 20 mg	2 ml purified water
Soluplus A 20 mg	2 ml purified water
Soluplus B 20 mg	2 ml PBS pH 6.8
PLGA-co-PEG 20 mg A	4 ml heated PBS pH 6.8
PLGA-co-PEG 20 mg B	4 ml of heated PBS pH 6.8:Tween 1.5% (1:1)
PLGA-co-PEG 5 mg A	4 ml of heated PBS pH 6.8:Tween 1.5% (1:1)
PLGA-co-PEG 5 mg B	6 ml of heated PBS pH 6.8:Tween 1.5% (1:1)

Table 8 presents the plain polymeric micelles formulations and in table 9 are the loaded siRNA micelles that were prepared.

Table 8 – Type and quantities of rehydration solution used in the different formulations for assembly of plain micelles.

Formulation	Quantity of rehydration solution
Soluplus 2 mg	2 ml PBS pH 7.4
PLGA-co-PEG 2 mg	2 ml of heated PBS pH 6.8:Tween 1,5% (1:1)

Table 9 - Type and quantities of rehydration solution used in the different formulations for assembly of siRNA loaded micelles.

Formulation	Quantity of rehydration solution
Soluplus 2 mg	2 ml PBS pH 7.4 with 250 nM siRNA
PLGA-co-PEG 2 mg	2 ml of heated PBS pH 6.8:Tween 1.5% (1:1) with 250 nM siRNA
PLGA-co-PEG 1 mg	10 ml of heated PBS pH 6.8:Tween 1.5% (1:1) with 250 nM siRNA

3.2.3 Nucleic acid quantification

The NanoDrop® NP-1000 Spectrophotometer was used to determine the quantity of siRNA in all samples (supernatant). First, the sample retention system of the equipment was always cleaned with purified water. Then 1 µl of the sample was pipetted into the cuvette. The assays are made in triplicates and a blank assay was also performed. The results of the sample concentration are given in ng/µl based on absorbance at 260 nm, since nucleic acids absorb light at this wavelength.

3.2.4 Characterization of the nanoparticles

3.2.4.1 Particle size distribution

The particle size and particle size distribution (mean diameter and the polydispersity index) was determined in the Autoziser® by dynamic light scattering, also known as photon correlation spectroscopy, because this technique has a detection range from 0.3-0.6 nm to 10 µm, covering the

estimated dimension of the nanoparticles obtained. The samples without visible turbidity were placed directly in universal, disposable Malvern® cuvettes. Sample with turbidity were previously diluted with purified water. The analysis was performed at a fixed scattering angle of 173º at a temperature of 25ºC. For each sample, the mean diameter of three determinations was calculated; therefore the values reported are the mean ± standard deviation of at least three runs of the formulation.

3.2.4.2 Surface charge (zeta potential)

The zeta potential of the 2 mg PLGA-co-PEG rehydrated with 2 ml of heated PBS pH 6.8:Tween 1.5% (1:1) was also measured in the Autosizer®. The measurements were made in triplicate.

3.2.4.3 Particles morphology by TEM

Transmission Electronic Microscopy was employed to determine the shape and surface morphology of the produced polymeric micelles. This assay was performed by external services.

3.2.5 Cellular viability

PLGA-co-PEG micelles loaded with siRNA solution (at 1:5 and 1:10 dilutions) are added to Jurkat cells. Each well of the plate was filled with 50 µl of the suspension (equivalent to 100,000 cells). For the Soluplus solution, the procedure was the same. alamarBlue® reagent was added directly to each well and the plates are incubated for 8 hours and 24 hours at 37°C, and the fluorescence was measured at 600 nm wavelength.

3.2.6 Agarose gel electrophoresis

The agarose gels were made in a concentration of 1% (w/v) in Tris/Borate/EDTA running buffer and with 1 µg/mL ethidium bromide. The siRNA bands stained with ethidium bromide were detected on a UV transilluminator.

3.2.7 Cell culture and GFP silencing

This assay was performed by a member of iMed team.

4. RESULTS AND DISCUSSION

Non-viral vectors have the advantage of low immune response that enables repeated administration and the capability of large production with acceptable costs (Lv H 2006). In this work our aim is to develop polymeric micelles.

TEM

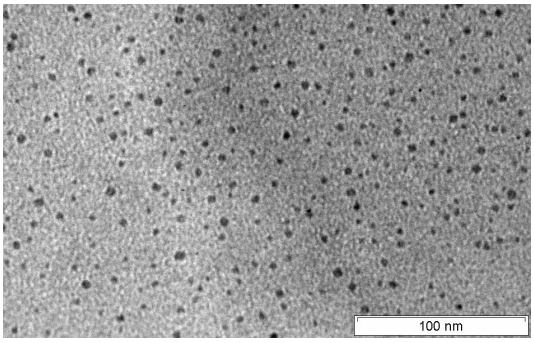


Figure 1 – Image of PLGA-co-PEG micelles obtained by TEM.

The 2 mg of PLGA-co-PEG rehydrated with 2 ml of PBS:Tween formulation micelles were examined by transmission electron microscopy (TEM). The image above (Figure 8) demonstrates that the micelles present an almost uniform size distribution and the particles have diameters of approximately 10 nm or less.

Optimization of polymeric micelles

One of the identified problems was the removal of the film after solvent evaporation. The formed film was sometimes gel-like and it was one of the issues that had to be solved. The first parameters changed were the time of evaporation, the rehydration time, and the film during the vortex process. Some of the formulations presented hair-like structures in suspension; this might be due to hydrolyses of PLGA in its monomers of glycolic acid and lactic acid. Later, it was decided to rehydrate the films with the solution containing the surfactant Tween 80 since it contributes to the removal of the film.

During the development of the formulations, it was noticed that Pluronic® micelles where unstable and due to lack of time they were not optimized.

After testing different volumes of rehydration solution, it was identified that 2 ml was the ideal volume one because the film was removed without difficulty.

CMC

The micelles formation process can be described as a delicate equilibrium between attractive and repulsive forces. The CMC is the concentration at which formation of micelles was achieved and this value can be used to evaluate the thermodynamic stability of these particles. Polymeric micelles are colloidal dispersions prepared from amphiphilic copolymers that usually present particle sizes between 5 nm to 100 nm (Reischl D 2009). The CMC is very low for polymeric micelles, typically on the order of 10^{-6} to 10^{-7} M, resulting in stable structures that are not easily dissociable *in vivo* (Blanco E 2009).

According to the literature, the CMC value of PEG-co-PLGA was around 2 mg/L (Lee SH 2010) to 6 mg/L (Ashjari M 2012).

The first attempts to develop the polymeric micelles didn't work because the CMC was not reached. In the starting formulations the quantity of polymer used was very high and therefore solvent was quickly consumed, preventing the formation of micelles. One of the initial tasks was the identification of polymer quantity that enabled the accomplishment of nanomicelles.

siRNA quantification

The optimized micelles of PLGA-co-PEG and Soluplus® were rehydrated with a siRNA containing solution. After siRNA loaded micelles preparation a device for ultrafiltration was used to separate the extremely small particles. The supernatant obtained after the ultrafiltration was analyzed in Nanodrop®, the results showed in table 10 were obtained by spectrophotometry. Three measurements were made for each formulation.

Table 10 – Determinations of siRNA concentrations obtained in Nanodrop®.

Formulation	Mean of siRNA concentrations (ng/µl) ± Standard deviation
Soluplus®	1.23 ± 0.15
PLGA-co-PEG	0.12 ± 0.09

Since the determinations were made in the supernatant, it can be assumed that both results demonstrate that possibly the siRNA was incorporated into the micelles, because the quantity of nucleic acid detected by this technique is considerably low with Soluplus® and the value obtained with PLGA-co-PEG is near zero.

Particle size and polydispersity index

The results obtained for all other formulations are presented in table 11.

Table 11 – Determinations of size (Z-average diameter) and polydispersity index obtained in Autosizer® 4700.

Formulation	Z-average diameter (nm) Mean of samples ± SD	Polydispersity index Mean of sample ± SD
Pluronic 20 mg in 4 ml of purified water	43.44 ± 3.3	0.667 ± 0.013
Soluplus in 2 ml purified water	60.3 ± 1.0	0.055 ± 0.015
Soluplus in 2 ml PBS pH 6.8	67,4 ± 0.9	0.027 ± 0.019
PLGA-co-PEG 20 mg in 4 ml PBS:Tween 1.5%	154.4 ± 75.0	0.325 ± 0.023
PLGA-co-PEG 5 mg in 4 ml PBS:Tween 1.5%	11.8 ± 0.9	0.251 ± 0.047
PLGA-co-PEG 5 mg in 6 ml PBS:Tween 1.5%	80.1 ± 44.4	0.213 ± 0.044
PLGA-co-PEG 1 mg in 10 ml PBS:Tween 1.5%	11.07 ± 0.11	0.256 ± 0.005

The Z-average diameter is the mean diameter based on the intensity of scattered light and is sensitive to the presence of aggregates and/or large particles. Bearing this in mind, we can see that all the formulations are in the desired nanometer scale, but exists a better variation in PLGA-co-PEG formulations. The results obtained with Soluplus® are consistent, even when two different solvents were used to rehydrate the film.

The polydispersity index is a measure of distribution of sizes in the micelles sample; a polydispersity index of 1 indicates large variations in particle size and a value of 0 means that size variation is absent. A small polydispersity index indicates that populations are homogeneous.

Analyzing the results obtained, the value of polydispersity index for Soluplus® micelles is closer to zero than the value found for PLGA-co-PEG micelles. The polydispersity index of Pluronic® is not comparable to the values obtained with the other polymers, being the result closer to one than to zero.

The mathematic models used by Autosizer® allow the acquirement of size distribution graphics. For the main formulations, the graphics are presented in the images 9 to 16.

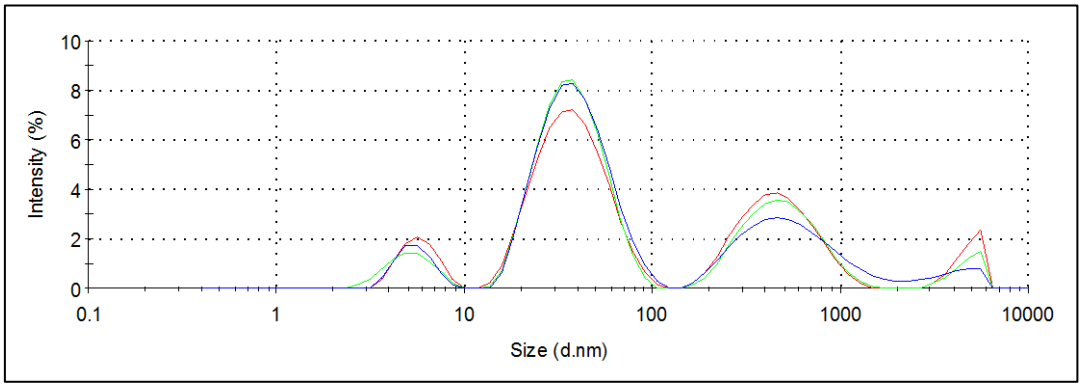


Figure 9 - Graphic size distribution versus intensity for Pluronic® rehydrated with purified water formulation.

The graphic is consistent with the values presented in table 11. The sample is extremely heterogeneous, with several populations (peaks) of micelles with very different sizes.

In the literature, Pluronic® F 127 is characterized as a nonionic surfactant composed of polyoxyethylene-polyoxypropylene copolymers in a concentration ranging from 20% to 30%.

Micellization occurs in diluted solutions of block copolymers in selected solvents above the CMC, at a given temperature (Escobar-Chávez JJ 2006). It was shown that Pluronic® block copolymer micelles can interact with membrane efflux pumps chemosensitising chemotherapy-resistant cancer (Gaspar R 2009). This properties and the potential for using the polymer to develop a DDS influence the choice of this polymer, but contrary to expected, the formulations demonstrated to be unstable and showed a profile of sizes that were not the desired, so it was decided not to continue using Pluronic® F 127. With more time, some improvements may be done and the formulation may be interesting to be developed in the future due to the favorable properties of this polymer.

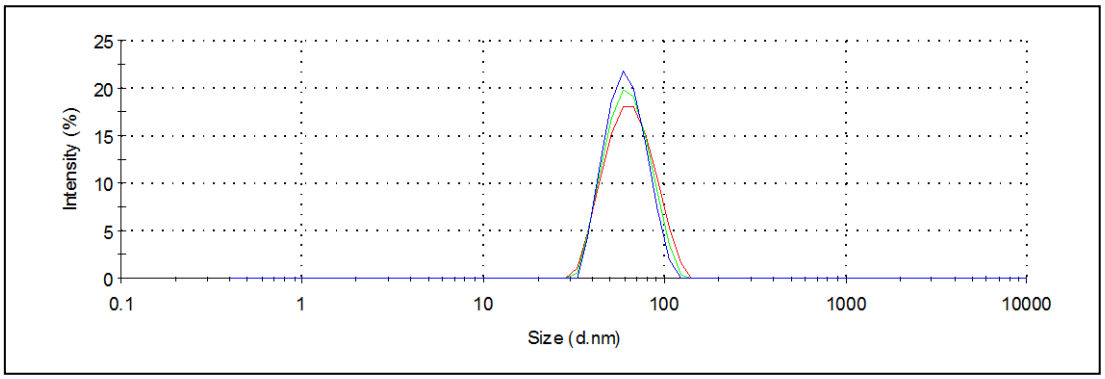


Figure 10 – Graphic size distribution versus intensity for Soluplus® rehydrated with purified water formulation.

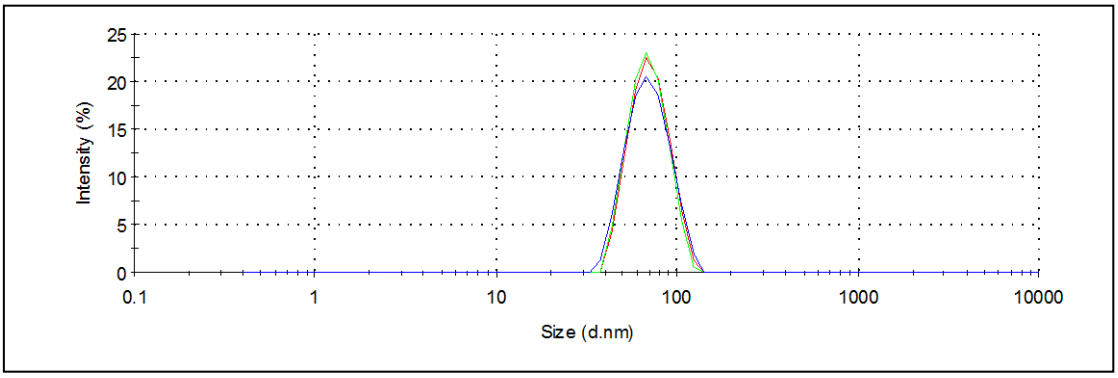


Figure 11 - Graphic size distribution versus intensity for Soluplus® rehydrated with PBS pH 6.8 formulation.

Accordingly to the technical information (Technical Information of Soluplus®), Soluplus® was a polymer of interest for this work because the polymer itself presents two advantages for utilization as a colloidal delivery system: when in higher polymer concentrations it may result in a cloudy or turbid aqueous solution, due to formation of colloidal micelles; and the presence of PEG in the chemical structure of the polymer.

The evaluation of the graphics allows to conclude that Soluplus® has only one population (peak), which is consistent with the values obtained for the polydispersity index.

However, the particles size was not the desired one and the assay of cellular viability was not favorable, so it was decided to optimize only the PLGA-co-PEG formulation.

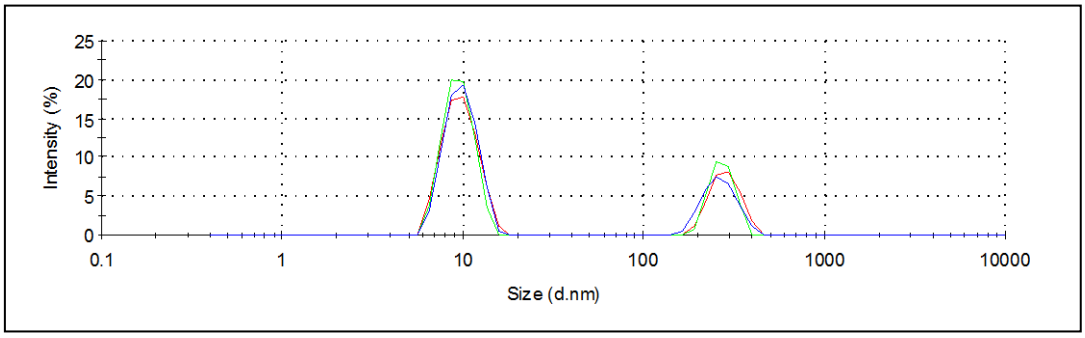


Figure 12 - Graphic size distribution versus intensity for 20 mg of PLGA-co-PEG rehydrated with 4 ml of PBS:Tween formulation.

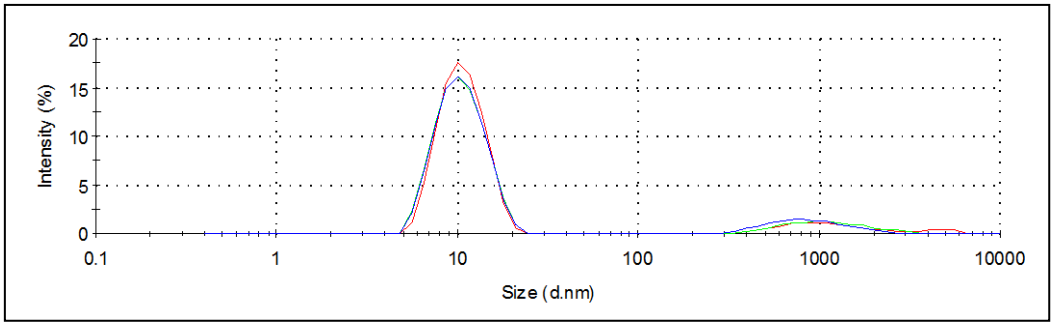


Figure 13 - Graphic size distribution versus intensity for 2 mg of PLGA-co-PEG rehydrated with 2 ml of PBS:Tween formulation.

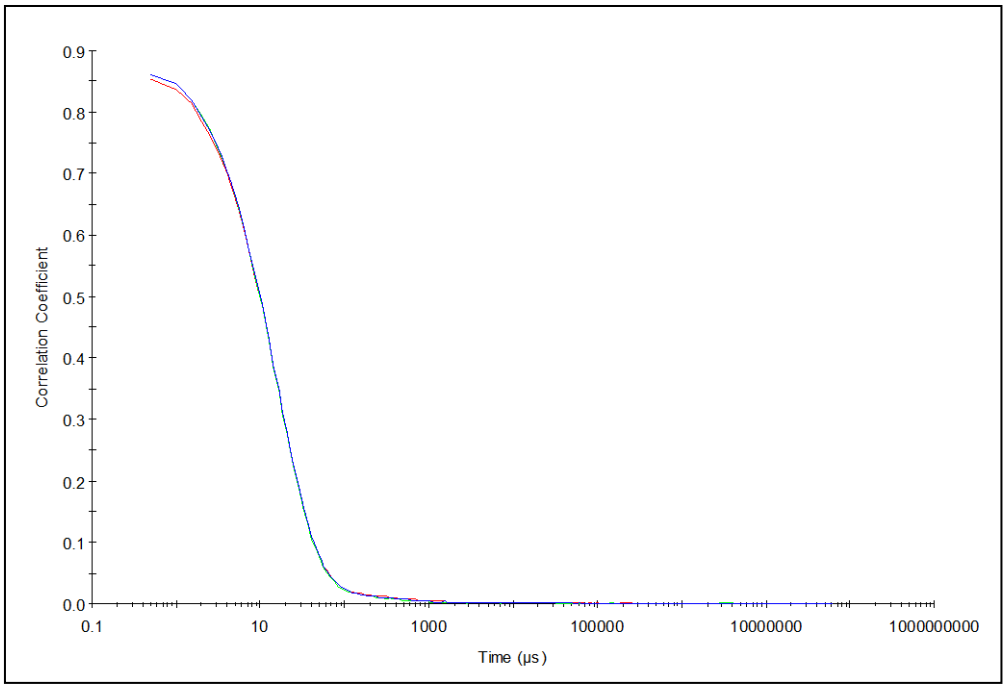


Figure 14 – Graphic of Raw Correlation Data (correlation coefficient versus time) for the 2 mg of PLGA-co-PEG rehydrated with 2 ml of PBS:Tween formulation.

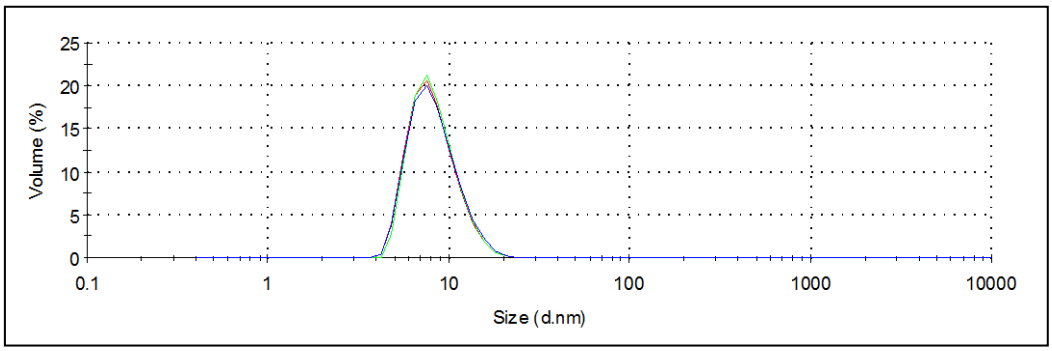


Figure 15 - Graphic of the size distribution (volume in % versus size) for the 1 mg of PLGA-co-PEG rehydrated with 10 ml of PBS:Tween formulation.

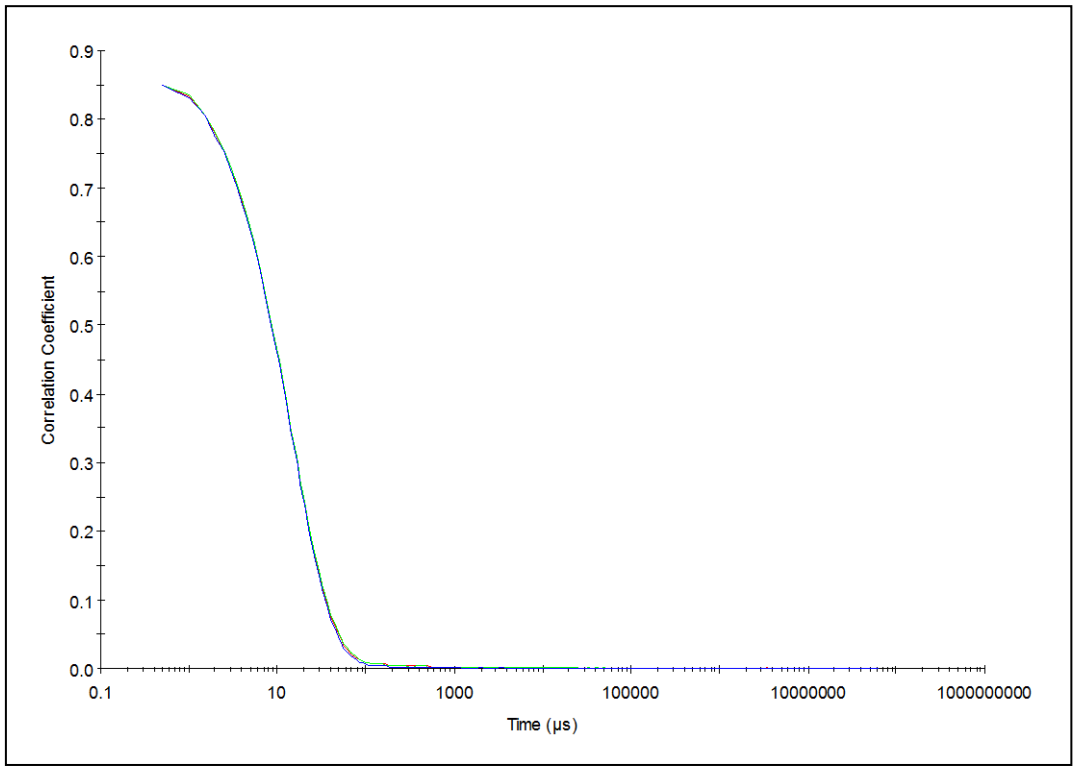


Figure 16 - Graphic of Raw Correlation Data (correlation coefficient versus time) for the 1 mg of PLGA-co-PEG rehydrated with 10 ml of PBS:Tween formulation.

The size distribution of the PLGA-co-PEG micelles exhibited a biphasic trend, the peak of micelles, with approximately 10 nm, was consistent between tests. This size is also consistent with that observed in TEM.

The second peak appears in an undesirable range of sizes and is very broad; this may be an artifact of the equipment. Trying to use a different refraction index in the equipment can be helpful to test if the second population disappears.

The formulations that gave better results were PLGA-co-PEG prepared with 1 mg or 2 mg of the polymer. As represented in the correlograms (Figures 14 and 16), the baseline is clear, which indicate that there were no oversized particles or aggregates[1].

Zeta potential

The Zeta potential data obtained is described in the table 12.

Table 12 – Zeta potential determined in the 2 mg of PLGA-co-PEG rehydrated with 2 ml of PBS:Tween formulation.

Determined Zeta potencial (mV)	Mean (mV) ± SD
-4,00	-3,54 ± 0,65
-2,80	
-3,81	

The result is near the neutrality which is an acceptable value for this type of delivery systems.

Cellular viability

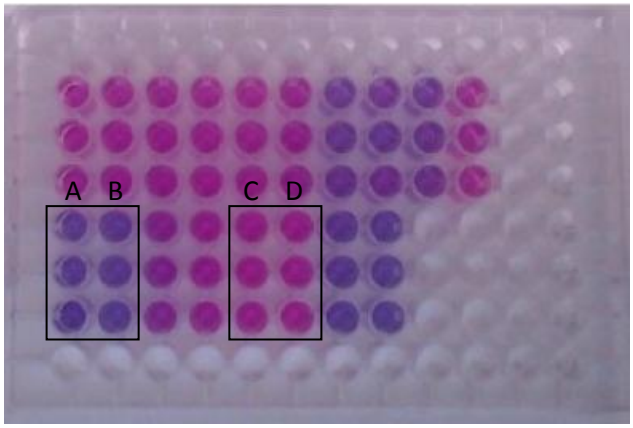


Figure 17 – Results obtained in alamarBlue® assay. The column A has 1:5 dilution of Soluplus® formulation, B has 1:10 dilution of Soluplus® formulation, C has PLGA-co-PEG + siRNA 1:5 dilution and D the same formulation in a dilution of 1:10.

The results obtained after measuring the cell viability with the alamarBlue® procedure are presented in table 13.

Table 13 – Results of cell viability obtained alamarBlue® reagent assay.

Formulation	1:5 Dilution (number of viable cells)	1:10 Dilution (number of viable cells)
PLGA-co-PEG	36572	44194
PLGA-co-PEG	39275	44244
PLGA-co-PEG	40520	44715
Soluplus	6250	6185
Soluplus	6332	5627
Soluplus	7474	6205

[1] The correlation coefficient must be between 0.5 and 1, and the formulations the values obtained were among 0.8 and 0.9.

Table 14 – Mean of the three samples and standard deviation for controls and formulations tested in cell viability assay.

Formulation	Mean of samples ± SD
Control (1:5)	32587.3 ± 4314.8
Control (1:10)	30852.3 ± 4743
PLGA-co-PEG (1:5)	26904 ± 345.9
PLGA-co-PEG (1:10)	30457 ± 2018.4
Soluplus (1:5)	6685.3 ± 684.2
Soluplus (1:10)	6005.7 ± 328.1

Comparing the obtained results for each formulation with the control cells (cells to which micelles were not added) the percentage of viable cells can be calculated. The table 15 exhibits the results of viable cells per formulation.

Table 15 – Percentage of viable cells in the two tested formulation in cell viability assay.

Formulation	% of viable cells
PLGA-co-PEG (1:5)	82.6%
PLGA-co-PEG (1:10)	98.7%
Soluplus (1:5)	20.5%
Soluplus (1:10)	19.6%

The alamarBlue® assay depends on a fluorometric/colorimetric growth indicator based on detection of metabolic activity. The method includes a redox indicator that both fluoresces and changes color in response to cell growth (Lancaster MV 1996). If the cells being test grow, their metabolic activity maintains a reduced environment that makes the indicator change to the reduced form that is red and fluorescent. With the inhibition of cell growth, the environment becomes oxidized, and the medium turns into the color blue and is non-fluorescent (invitrogen® - Technical Information).

By the simple observation of Figure 17 it can be seen that the wells where the micelles of Soluplus® are incorporated show a blue coloration, which means cellular death; with PLGA-co-PEG the wells maintain with the color pink, which identifies viable cells.

With the Soluplus® formulation it can only be obtain a final percentage of viable cells of approximately 20%. Using the PLGA-co-PEG (1:5) formulation the obtained viable cells were 82.6% and in PLGA-co-PEG (1:10) formulation were obtained 98.7%. This means that incorporation of PLGA-co-PEG micelles does not impair the viability of the cells. Considering this assay, it can be concluded that PLGA-co-PEG micelles are less toxic than the Soluplus® ones.

The decision was to proceed the study with the PLGA-co-PEG micelles, testing them in the electrophoresis and in GFP silencing assay.

Agarose gel electrophoresis

Agarose gel electrophoresis is an easy technique to separate nucleic acid fragments based on their sizes. The used dye was ethidium bromide, which intercalates between the bases of double-stranded nucleic acids and is fluorescent under UV light.

In Figure 18 it can be observed that siRNA was not free in any of the formulations, because no signal is seen when comparing the size of the siRNA used with the sign of the Ladder.

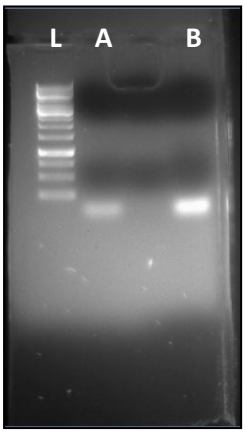


Figure 18 – Agarose gel electrophoresis 1% photography. L = Ladder 1 kb, A = sample of polymeric micelles with siRNA (filtrated in Centricon 3K), B = sample of polymeric micelles with siRNA (concentrated).

The band intensity of the concentrated sample of the non-filtered formulation is higher than that of the band of the filtrated formulation (with 3K centricon). Given the obtained signal, and considering that this electrophoresis was performed 15 days after the preparation of the polymeric micelles, it can be assumed that the siRNA did not suffer degradation. In addition, it is important to mention that the micelles were stored at room temperature and the formulation remained stable for 2 weeks.

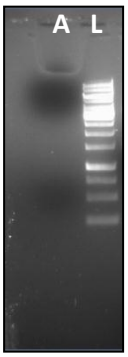


Figure 19 - Agarose gel electrophoresis 1% photography. A = sample of plain PLGA-co-PEG polymeric micelles (not loaded with siRNA), L = Ladder 1 kb.

Comparing figure 18 and figure 19, it can be seen that the blank formulation did not produce any signal. Thus, the polymeric micelles did not interfere with this particular assay.

In this case another run only with the naked siRNA should be performed in order to compare this with the results obtained in Figure 18, so that it can act like a control. Due to limited time this additional gel was not executed.

Cell culture and GFP silencing

MDA-MB-231/GFP cells were seeded in a 24-well plate (100,000 cells/well) and incubated for 24 hours. After this, polymeric micelles were incubated during 6 hours. The culture medium was replaced with a fresh one and after 48 hours cells were fixed and observed for the expression of green fluorescent protein by Flow Cytometry. The peaks integration yields the numbers of cells that no longer express GFP. The cells that continue to express GFP allow the calculation of the fraction of GFP silencing.

Each assay was executed in duplicate using 1:5, 1:10, 1:20 and 1:30 dilutions of plain (PLGA-co-PEG) and loaded with siRNA anti-GFP (PLGA-co-PEG*) polymeric micelles. The obtained results are showed in table 16.

Table 16 – Mean of the percentage of GFP negative cells.

Formulation	% of GFP negative cells
PLGA-co-PEG (1:5)	4.76%
PLGA-co-PEG (1:10)	5.23%
PLGA-co-PEG (1:20)	5.04%
PLGA-co-PEG* (1:30)	5.45%
PLGA-co-PEG* (1:5)	23.1%
PLGA-co-PEG* (1:10)	16.9%
PLGA-co-PEG* (1:20)	17.6%
PLGA-co-PEG* (1:30)	20.3%
Control	5.95%

Table 17 – Efficacy of silencing for each dilution.

Formulation	Efficacy of silencing
PLGA-co-PEG* (1:5)	4.85
PLGA-co-PEG* (1:10)	3.23
PLGA-co-PEG* (1:20)	3.49
PLGA-co-PEG* (1:30)	3.72

The obtained results for silencing are not very high. It might be due to an insufficient entry of micelles into the cells or an incorrect delivery of siRNA into the cytoplasm of the cells, failing the activation of RNAi machinery and, consequently, being unable to reduce the expression of the green fluorescent protein.

One of the parameters that can be adjusted in this assay is the contact time of nanoparticles with the cells. Better adherent cells will not suffer the washing effect when the micelles are added. This effect is negative to the micelles internalization because non-adherent cells don't internalize the expected quantity of the carrier and consequently of the siRNA. Other cellular lines can also be tested, because they can influence the acquired results.

Some of the strategies that can be adopted in the future in order to continue this work are the addition of a cationic polymer in a sufficient quantity that promote the "proton sponge effect", described earlier in the introduction of the thesis. This manipulation and adjustment of the final micelles charge will probably allow a better delivery of the siRNA in the cytoplasm and lead to an enhanced silencing effect. Optionally, the quantity of PEG may be lowered since it can be proportionally high for the quantity of PLGA.

Another approach may be using direct targeting, so that the cells entry is facilitated and the delivery of siRNA in the right sub-cellular compartment is achieved.

Often, the first choice for non-viral vectors is lipids, namely cationic lipids. Cationic lipids and cationic polymers for gene delivery may cause toxic effects *in vitro* and *in vivo*, for example, lipoplexes cause several changes to cells, which included cell shrinking, reduced number of mitoses and vacuolization of the cytoplasm (Lv H 2006). The polar and hydrophobic domains of cationic lipids may have dramatic effects on both transfection and toxicity levels (Lv H 2006). The toxicity of cationic liposomes may, in part, result from the large size of the complexes and the high positive zeta potential required for their uptake (Lv H 2006). The toxicity is normally closely associated with the charge ratio between the cationic lipid species and the nucleic acids, as well as the dose of lipoplexes administered; higher charge ratios are generally more toxic to a variety of cells (Lv H 2006). These reasons lead to try the development of polymeric micelles with different neutral or nearly neutral polymers, in order to reduce the possible toxicity.

All the polymers tested in this work proved to be inferior to PEI when comparing the results obtained with the ones described in literature. PEI was initially excluded due to the known problems, namely the interaction of the positive charged polymer with negatively charged serum proteins (like albumin), lipoproteins or IgG, and erythrocytes; the precipitation in clusters and adherence to cells surface (Lv H 2006, Bruno S 2010).
One of the concerns when using synthetic polymers for siRNA delivery *in vivo* is dose-dependent toxicity upon systemic administration (Lv 2006, Shim MS 2010). PEI and poly(L-lysine) were shown to trigger necrosis and apoptosis in a variety of cell lines (Shim MS 2010). There are some measures that may reduce the toxicity of these molecules, for example adding co-lipids or copolymers like DOPE or PEG (Lv H 2006); or by removing excess (uncomplexed) cationic polymers (Shim MS, 2010).

One of the main reasons for the choice of PLGA-co-PEG was supported by the following: PLGA has been used in pharmaceutical industry with success, and drug products containing this polymer have been approved for parenteral use by regulatory authorities around the world, some examples are

Sandostatin LAR (octreotide), Zoladex (goserelin), Neutropin (somatropin), Atridox (doxycycline), Risperdal Consta (risperidone) and Vivitrol (naltrexone).

The first approach performed in this work was an attempt to achieve the optimum quantity of PEG needed to add to PLGA in order to make stable and workable polymeric micelles. At that time, the commercial mixtures of PLGA-co-PEG were not available. Although some different combinations were tried, the films formed after the solvent evaporation was not of good quality. Later it was decided to work only with PLGA-co-PEG, with the formulations already available in the market.

5. CONCLUSION AND FUTURE PERSPECTIVES

The objective of this thesis was the development and optimization of polymeric micelles that could properly delivery siRNA since this is a bottleneck to this nucleic acid utilization as a therapeutic agent. Three different polymers were tested in order to determine their stability, and capacity for incorporation and delivery of the nucleic acid in the cytoplasm of cells.

Because of the potency and selectivity of siRNA, this became the method of choice for silencing specific gene expression in mammalian cells. Control of disease associated genes makes siRNA an attractive choice for future therapeutics. Basically every human disease caused by expression of one or more genes should be amenable for RNAi-based therapeutics (Mao S 2010).

After testing the three polymers, it can be concluded that Pluronic® does not form micelles that are needed for the incorporation of nucleic acids and that Soluplus® seems to be a potential alternative in the first stages, but the developed micelles were abandon due to the demonstrate cytotoxicity. Despite the obtained results, both polymers should be further studied and the assays repeated. With more time, the optimization and possible utilization of Pluronic® and Soluplus® micelles as a delivery system may be possible.

The PLGA-co-PEG micelles developed in this work were shown to have no cytotoxic effects in the cell line tested. For future applications, it is important to have in mind that these micelles can potentially cross the blood brain barrier due to their size of approximately 10 nm. Despite this, the micelles obtained are within the desired range (10 nm to 100 nm).

The stability of the obtained nanoparticles needs to be studied, in terms of physical and chemical stability. Physical stability can be determined by the increase in size and formation of aggregates and chemical stability can be determined by the degradation of the polymer and of the siRNA.

The next steps may be the *in vivo* studies in animal models, in order to conduct histopathology assays and determinate the biodistribution of the polymeric micelles in the different tissues and organs. Another important study to be made in the future is evaluating the *in vivo* knockdown efficiency in an animal model.

It would be important to find a method that can help in the determination of siRNA content in micelles. This is an important issue because, like any other therapeutic entity, siRNA will need to be quantified by a reproducible and validated method so that the delivery strategy can be accepted by the Regulatory Agencies.

The method used in this research project is a considerably simple method for obtaining polymeric micelles. The obtained PLGA-co-PEG micelles hold great potential for delivery of siRNA therapeutics in the future, but warrant further research.

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