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Mestrado em Biofísica e Bionanossistemas

Trabalho efetuado sob a orientação da
Professora Doutora Maria Elisabete Cunha Dias Real Oliveira
e co-orientação da
Professora Doutora Andreia Ferreira de Castro

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DECLARAÇÃO

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Abstract

RNA interference (RNAi) has been found to be an important biological strategy for gene silencing. This pathway can be used as a gene therapy approach by using synthetic short interfering RNA (siRNA) molecules to promote the silencing of undesirable target genes. However, the delivery of nucleic acids into the cells is a very inefficient process due to several extracellular and intracellular barriers. Several physical, chemical and biological strategies have been developed to promote the delivery of nucleic acids into cells. Although viral vectors have been reported to be the most efficient nucleic acid delivery systems, they trigger the immune system and promote high levels of toxicity.

Non-viral vectors, though not as efficient as their viral counterparts, appeared as a safer method for therapeutic gene delivery. In this field, cationic liposomes emerged as one of the most promising and widely used non-viral gene carriers. Recent studies from our group have established a novel liposomal formulation for siRNA delivery, based on the cationic lipid dioctadecyldimethylammonium bromide (DODAB) and the *helper* lipid monoolein (MO). This liposome formulation has promoted efficient gene silencing in a human non-small cell lung carcinoma cell line (H1299).

In this project, we aimed to improve the silencing efficiency of DODAB:MO (2:1) liposomes, by promoting the co-encapsulation of siRNA with additional anionic components. Poly-L-glutamic acid (PG1 or PG2) or non-coding plasmid DNA (pDNA) were added to siRNA suspension and encapsulated within DODAB:MO (2:1) liposomes. The systems obtained were compared with lipoplexes containing only siRNA. Lipoplexes were characterized in order to understand the differences caused by addition of the anionic components. The results obtained during this project suggest that the addition of either pDNA or PG molecules to siRNA/DODAB:MO lipoplexes results in systems with similar physicochemical properties, namely size and surface charge. Nevertheless, some improvements in the siRNA encapsulation efficiency, cellular internalization and cytotoxicity were obtained when compared to siRNA lipoplexes. Additionally, lipoplexes co-encapsulating siRNA and pDNA or PG have promoted higher EGFP gene silencing efficiency, suggesting that co-encapsulation of siRNA with an additional anionic cargo can improve silencing efficiency of our liposomal formulation.

Resumo

Desde a sua descoberta, o RNA de interferência (RNAi) tornou-se uma estratégia biológica importante para o silenciamento de genes. Esta via pode ser usada como uma abordagem de terapia genética, utilizando *short interfering* RNA (siRNA) para promover o silenciamento de genes indesejáveis. No entanto, a entrega de siRNA em células é um processo muito ineficiente devido a várias barreiras extracelulares e intracelulares. Várias estratégias físicas, químicas e biológicas têm sido desenvolvidas para promover a transferência de ácidos nucleicos para as células. Vários estudos mostram que, vetores virais são sistemas de entrega eficazes, contudo, desencadeiam resposta imunitária e promovem níveis elevados de toxicidade.

Os vetores não virais, embora não tão eficientes como os seus homólogos virais, aparecem como um método mais seguro para a entrega de siRNA. Neste campo, os lipossomas catiónicos tornaram-se num dos vetores não virais mais promissores e amplamente utilizados. Estudos recentes do nosso grupo estabeleceram uma nova formulação lipossomal para a entrega de siRNA, com base no lípido catiónico brometo de dioctadecildimetilamónio (DODAB) e o lípido adjuvante monooleína (MO). Esta formulação de lipossomas foi capaz de promover o silenciamento genético de forma eficiente numa linha celular humana de células não-pequenas de carcinoma pulmonar (H1299).

Neste projeto, procuramos melhorar a eficiência de silenciamento de liposomas DODAB:MO (2:1), através da promoção da co-encapsulação de siRNA com componentes aniónicos adicionais. Poli-glutamato (PG1 ou PG2) ou DNA plasmídico não codificante (pDNA) foram adicionados à suspensão de siRNA e encapsulados em liposomas DODAB:MO (2:1), e os sistemas obtidos foram comparados com lipoplexos contendo apenas siRNA. Os lipoplexos foram caracterizados de modo a compreender as diferenças causadas pela adição dos componentes aniónicos. Os resultados obtidos durante este projeto sugerem que a adição de moléculas de pDNA ou PG a lipoplexos compostos por siRNA e lipossomas DODAB:MO (2:1) resulta em sistemas com propriedades físico-químicas semelhantes, mais especificamente, o tamanho (Z-average) e a carga superficial (ζ-potential). No entanto, foram obtidos algumas melhorias na eficiência de encapsulação de siRNA, internalização celular e citotoxicidade destes sistemas, quando comparado com lipoplexos com apenas siRNA.

Além disso, lipoplexos onde o siRNA é co-encapsulado com pDNA ou PG, promoveram maior eficiência de silenciamento do gene EGFP em linhas celulares 293T/GFP-puro, sugerindo que a co-encapsulação de siRNA com uma carga aniónica adicional pode melhorar a eficiência de silenciamento da nossa formulação lipossomal.

Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
pDNA	Plasmid deoxyribonucleic acid
siRNA	Small interfering ribonucleic acid
dsRNA	Double-stranded ribonucleic acid
shRNA	Short hairpin ribonucleic acid
EGFR	Epidermal growth factor receptor
SUV	Small unilamellar vesicles
LUV	Large unilamellar vesicles
GUV	Giant unilamellar vesicles
MLV	Multilamellar vesicles
MVV	Multivesicular vesicles
P	Packing parameter
DOPE	Dioleoylphosphatidylethanolamine
DOPC	Dioleoylphosphatidylcholine
CHO	Cholesterol
MO	Monoolein
PEG	Polyethylene glycol
CR	Charge ratio
DODAB	Dioctadecyldimethylammonium bromide
DODAC	Dioctadecyldimethylammonium chloride
Tm	Phase transition temperature
CVC	Critical vesicle concentration
ILAs	Inter-lamellar attachments

PG	Poly-L-glutamic acid
HEPES	Hydroxyethylpiperazine ethane sulfonic acid
MTT	(3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide))
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
EDTA	Ethylenediamine tetraacetic acid
q-PCR	Quantitative real-time polymerase chain reaction
siEGFP	siRNA anti green fluorescence protein
siEGFR	siRNA anti epidermal growth factor receptor
siSCRAMB	Scrambled siRNA
DLS	Dynamic light scattering
RT	Room Temperature
PDI	Polydispersity index
LDV	Laser dopller velocimetry
DTS	Dispersion technology software
NBD-PE	1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-7-nitrobenzofurazan
HBSS	Hank's balanced salt solution
GFP	Green fluorescence protein
dNTPs	Deoxynucleotide triphosphates
Cq	Quantification cycle
GADPH	Glyceraldehyde 3-phosphate dehydrogenase

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I - Introduction

1. Gene Therapy

The concept of gene therapy, first proposed in 1972 by Friedmann and Roblin [1], can be defined as the attempt to correct genetic disorders by adding exogenous nucleic acids to the target cells, in order to enhance or repress the expression of specific nucleotide sequences [2], [3].

1.1 Gene Silencing therapy

Gene silencing is a type of gene therapy defined as the interruption, or suppression, of the expression of a gene at the transcriptional or translational levels. In the mid 80's a post-transcriptional method was reported and defined as antisense therapy. The antisense technology uses homologous RNA, DNA or chemically altered nucleic acid sequences that hybridize with target mRNA transcripts, inhibiting their expression. This technique finds its hurdle due to the high molecular weight of the antisense nucleic acids, which rises several problems in the delivery process of the therapeutic agents [4], [5].

In the early 90's, nucleic acid molecules with three-stranded, or triple-helical structure (triplex DNA) were used to directly target the transcriptional regulation of gene expression. Triplex formation occurs when DNA or RNA oligonucleotide binds to homopurine region of DNA, these triplex forming oligonucleotides can be used to block the transcription of specific genes, [4], [6]. However there are many obstacles to triplex DNA therapy, such as, low cellular uptake, complexity of triplex structures formation and difficulty to find long uninterrupted homopurine sequences to promote stable triplex binding [6]. In the late 90's a new gene silencing approach was discovered by Fire et al. [7]: the RNA interference (RNAi) mechanism.

1.2 RNA interference mechanism

The RNA interference mechanism was first described in *Caenorhabditis elegans* in 1998 by Fire et al. [7], when the authors found that the introduction of RNA into cells could be used to interfere with the function of an endogenous gene. RNA (Fig.1) is a critical pathway naturally used by cells to control gene expression, and to provide protection against genetic damage induced by virus or mutations. In a simplified way, when a threat to the integrity of the genome occurs, a double-stranded RNA (dsRNA) is

broken into short pieces of 21 to 25 nucleotides, called short interfering RNA (siRNA), by the ribonuclease III enzime Dicer. This siRNA triggers the activation of an RNA-induced silencing complex (RISC), that targets specifically the homologous messenger RNA (mRNA) for enzymatic degradation [4], [8]–[11]. The main components of the RISC complex are the Argonaute 2 proteins, responsible for mRNA degradation. The fact that siRNA molecules have perfect homology to the target mRNA sequences makes this mechanism specific and efficient, because, unlike antisense and triplex approaches, dsRNA activates a normal cellular process leading to a highly specific mRNA degradation.

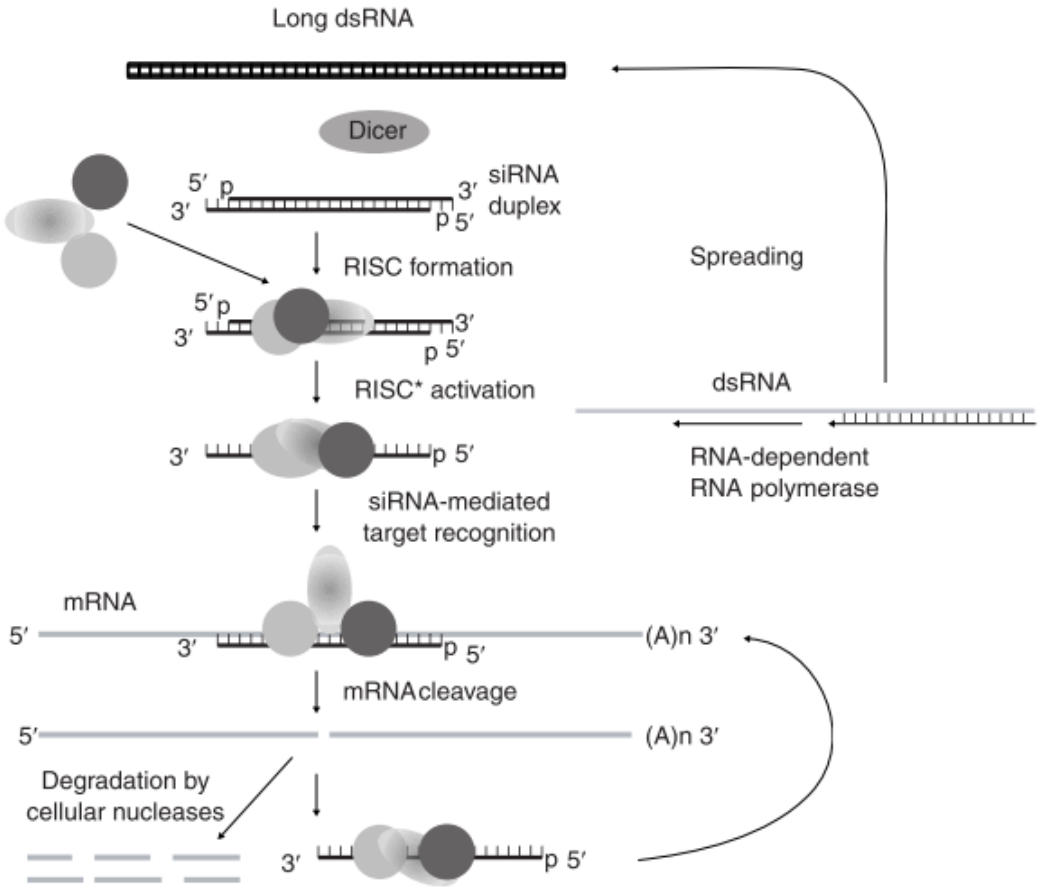


Figure 1 - Representative scheme of RNAi. Double stranded RNA (dsRNA) is processed into short interfering RNA (siRNA) by Dicer enzymes. siRNA molecules formed will attach to a multiprotein complexed known as RISC, which after activation will bind to target mRNA. mRNA is cleaved and degraded by cellular nucleases [12].

The application of dsRNA longer than 30 nucleotides has been associated with the activation of interferon (IFN) response genes, and also to a non-specific inhibition

of protein synthesis, constraining the use of dsRNA in gene therapy [12]–[14]. Only in 2001 a novel solution to this obstacle was found, when studies carried out by Tuschl et al. [11] and Caplen et al. [15] described that successful gene silencing could be achieved by the delivery of siRNAs (21-22 bp long) into mammalian cells [12]–[14].

Until now, two main strategies were tested using the RNAi pathway for genetic therapy purposes, based on either generation or administration of these short dsRNAs. The first approach is based on short hairpin RNAs (shRNAs), single-stranded molecules of 50–70 nucleotides in length, that form stem–loop structures [13]. It is an endogenous approach, since it allows the stable intracellular expression of shRNAs, which are then processed into active siRNAs by the host cell [13]. The control of siRNA levels and the inability to switch off the production of siRNAs are the main disadvantages of using shRNA. The second strategy consists in the exogenous application of synthetic siRNAs. As gene silencing induced by synthetic siRNA is limited by the number of RNA molecules present in the cell, the treatment can be stopped when it's no longer necessary. The use of siRNA for gene silencing has become the prime method for mammalian cell genetic analysis. This method presents potential for therapeutic treatment of a wide variety of diseases [16].

1.3 Molecular targets for gene silencing

Several therapeutic treatments using the RNA interference pathway have been developed, these treatments have different molecular targets. Targets important for gene therapy in cancer are mainly tumour suppressor genes, cell-cycle modulators, and growth factors [17]. Table 1 presents some of the targeted genes for a variety of cancer treatments in animal models [18].

Table 1 - List of common target genes of gene silencing for cancer therapy in a variety of animal models [18].

Animal Models	Targeted Genes
Liver metastasis mouse model	Bcl-2
Prostate cancer xenograft	Raf-1; CD31; Bcl-2
Gastric cancer xenograft	VEGF
Breast cancer xenograft	c-raf; Her-2; RhoA
Ovarian cancer xenograft	EphA2; FAK; ADRB2; IL-8
Lung cancer xenograft	EGFR
Orthotopic glioblastoma	PTN

1.3.1 Epidermal Growth Factor Receptor (EGFR)

One recognized target for gene silencing is the epidermal growth factor receptor (EGFR). This 170-kd glycoprotein consists in an extracellular binding domain, a transmembrane lipophilic region, and an intracellular domain with tyrosine kinase activity. After the binding of a ligand, such as the epidermal growth factor (EGF) or transforming growth factor (TGF-α), EGFR dimerizes and, activates the intrinsic protein tyrosine kinase, leading to a cascade of downstream signalling events that influence the behaviour of epithelial cells and tumours of epithelial cell origin [19], [20]. EGFR is overexpressed in many tumours (colorectal, head and neck, lung, ovarian, breast and renal cancer), and its signalling pathway is involved in cell differentiation, proliferation, migration, development of angiogenesis, and apoptosis inhibition [19], [21]. EGFR is a good target for cancer therapy since it has proven to be involved in the initiation, growth and metastasis of many human tumours. The main strategies for targeting EGFR are based on monoclonal antibodies directed against the extracellular receptor domain, which interferes with ligand binding, small-molecule compounds that target intracellular domain, inhibiting the activation of the tyrosine kinase activity and siRNA molecules which interfere with production of the EGFR protein at the mRNA level [19]. Gene silencing therapeutic approach has been reported to successfully reduce EGFR expression using a variety of vectors to transport siRNA into tumour cells, which makes this approach extremely promising [17], [21]–[23].

2. Vectors for Gene therapy

A successful gene therapy approach depends on the efficient delivery of nucleic acid molecules into cells. Actually, this represents the main problem in gene therapy, since the application of naked nucleic acid molecules is very inefficient due to several obstacles in the delivery process. Due to their small size, naked siRNAs are eliminated by kidney filtration, resulting in a short half-life when applied *in vivo*. Also, the nucleic acid molecules are prone to degradation due to the presence of nucleases in biological fluids and cells [24]. Moreover, nucleic acids are negatively charged due to the phosphodiester present in their backbone, which creates an electrostatic repulsion between nucleic acid molecules and the anionic headgroups of cell membrane phospholipids. This electrostatic repulsion hinders the passive diffusion of nucleic acids into the cells [12]. Therefore, high amounts of naked siRNA are required in order to achieve *in vivo* efficient silencing. Nevertheless, an elevated dose of siRNA is associated with non-specific effects on non-targeted genes, triggering immune responses and anti-angiogenic effects independent of the siRNA sequence [16].

In order to overcome the limitations in the nucleic acid delivery process and to enhance the transfection efficiency, a wide variety of physical and chemical methods have been developed. The use of delivery systems has commonly two main approaches: biological (viral vectors) and chemical (cationic lipids or polymers). The biological approach has been the most common approach, and is based on the use of viral vectors [25], while the chemical approach is based on the use of synthetic non-viral vectors [26].

2.1 Viral vectors *versus* non-viral vectors

Viruses are highly evolved organisms that have developed a very efficient mechanism to internalize their genome into host cells and exploit cellular machinery to facilitate replication [27]. By erasing part or the whole viral coding region, as well as promoting the insertion of siRNA or pDNA into the virus capsid, viruses can be used as vectors for gene therapy. Although the use of viral vectors results in relatively high transfection efficiencies, several drawbacks are associated with this method. A limited loading capacity, the complexity of vector production, and particularly the safety concerns such as the possibility of immunogenic/inflammatory responses and risk of mutations, are important disadvantages associated with this treatment [28], [29].

Actually, clinical trials using viral vectors have already caused the death of a patient when, in 1999, the injection of an adenovirus vector induced systemic inflammatory response syndrome that resulted in intravascular coagulation, acute respiratory disorder and multi organ failure [27].

Non-viral vectors were developed to overcome the safety concerns associated with viral vectors. Non-viral vectors are normally composed of synthetic cationic lipids (lipoplexes) or polymers (polyplexes)[30], although dendrimers[31], chitosan[32], and peptides[33] have also been studied for the delivery of nucleic acids. Unlike viral vectors, these carriers do not trigger specific recognition by the immune system, resulting in lower chance of immune responses. Also they have the potential to transport larger genetic cargos, are much easier to assemble, and may be tuned to enhance specificity [34], [35]. However, the transfection efficiency of non-viral vectors is still relatively low when compared to viral vectors, which is caused by the inability of the vector to surpass the cellular barriers to deliver the genetic cargo.

2.2. Barriers faced by non-viral vectors in gene delivery

In order to obtain successful *in vivo* delivery of nucleic acids using a non-viral vector, it must be able to overcome a variety of extracellular and intracellular barriers (Fig.2).

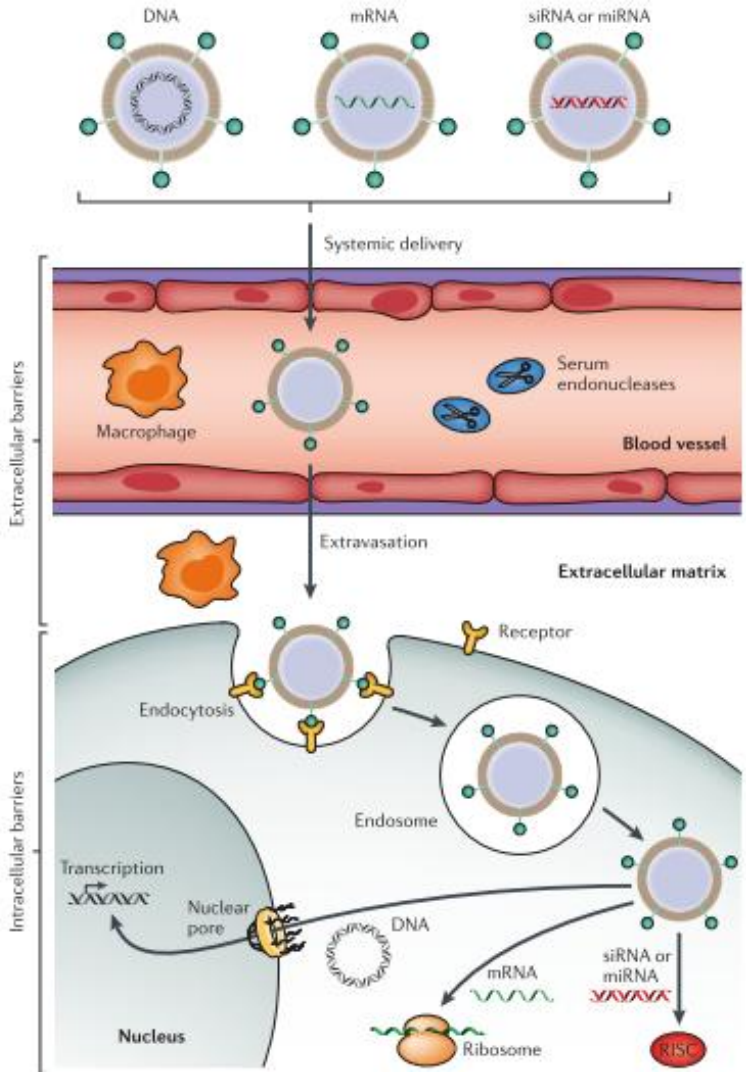


Figure 2 -Extracellular and intracellular barriers to gene therapy. Non-viral vectors such as liposomes were developed to deliver a variety of nucleic acids such as DNA, mRNA, short interfering RNA (siRNA) or microRNA (mRNA) to cells. For efficient delivery, non-viral vectors must be able to overcome extracellular and intracellular barriers. Extracellularly, vectors must be able to prevent degradation of the genetic cargo by serum endonucleases, avoid immune detection, nonspecific interactions and renal clearance from the blood. The vector used must also be able to promote cell entry in target tissues and subsequent endosomal escape. After endosomal escape, DNA must be transported into the nucleus, siRNA and miRNA must bind to the RNA-induced silencing complex (RISC) and miRNA must bind to translational machinery [35].

2.2.1 Extracellular Barriers

As mentioned before, the half-life of nucleic acids in physiological fluids and extracellular space is very short, so the vector selected must be able to protect its cargo in order to improve circulation time. In order to overcome these problems, the vector chosen needs to have colloidal stability, and reduced interaction with blood components such as proteins and cells, which may promote cargo release and particle aggregation. Protein binding can also trigger immune detection, leading to the

clearance of the vector by circulating macrophages before reaching the desired tissues. After evading immune detection and renal clearance the vector needs to extravasate from the bloodstream to the desired tissues and reach the target cells [35].

2.2.2 Intracellular Trafficking

After the vector reaches the desired tissues several obstacles arise, and the ability to promote efficient entry into the cell and induce endosomal escape are critical steps to obtain high transfection efficiencies. The cellular internalization process of non-viral vectors is not yet completely understood, since some studies have shown that the cellular uptake occurs mainly by endocytosis, but others have demonstrated that internalization by fusion with the plasmatic membrane can also occur for certain vectors such as liposomes [29]. The internalization efficiency of the vector is dependent on its size and hydrophilic nature. After cell uptake, the internalized particles become entrapped in the intracellular vesicles (endosomes) that fuse with lysosomes, where particle degradation occurs. Therefore, endosomal escape is an essential step for efficient delivery of the genetic cargo, as well as its dissociation from the vector after endosomal escape [2], [35], [36]. Cationic lipid based vectors (cationic liposomes) may fuse with endosomal membranes, facilitating endosomal escape and cargo release [35]. After dissociation from the vector, DNA molecules have to be further transported to the nucleus and RNA molecules must bind to Argonaute2 (aug2) protein, to form the RNA induced silencing complex (RISC) (siRNA and miRNA), or to the translational machinery (mRNA) [2], [35].

2.3. Liposomes as non-viral vectors

In 1965 Alec Bangham [37] first reported that, when in aqueous system, phospholipids self-assemble into closed bilayer structures forming lipid vesicles. Lipids are amphiphilic molecules, which means they are composed by a hydrophilic polar head group and hydrophobic tails. When in aqueous solution, lipids tend to organize in order to reduce entropically unfavourable interactions between the hydrophobic acyl chains and surrounding aqueous medium. The entropically favourable structure is obtained when the bilayer membrane formed by the lipids curves on itself, forming a closed vesicle, where the hydrophilic headgroups tend to face the aqueous phase [38].

The bilayer structure of liposomes is relatively easy to manipulate and can be formed in a diverse range of morphologies. Vesicle size and number of bilayers are important parameters to determine the ability of liposomes to encapsulate pharmaceutical compounds and their half-life time in circulation [39]. The most common morphologies of the vesicles are unilamellar, multilamellar and multivesicular, depending on the preparation methodology (Fig. 3).

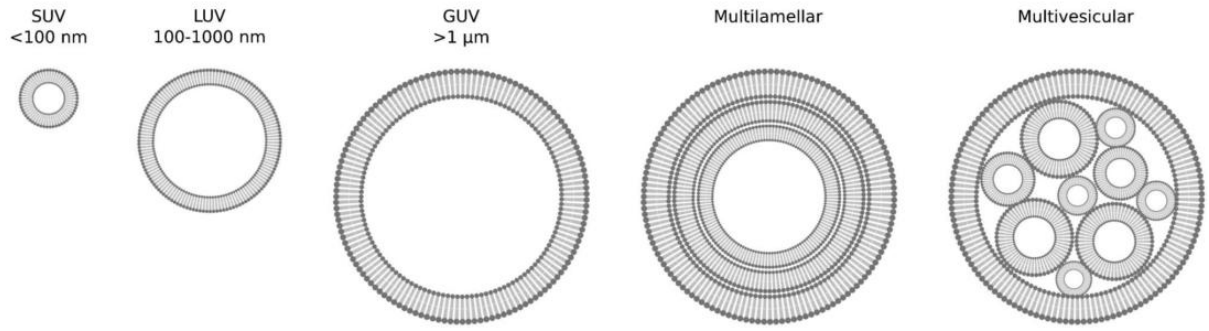


Figure 3 - Common vesicle size and lamellarity. Small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), giant unilamellar vesicles (GUV), Multilamellar vesicles (MLV) and multivesicular vesicles (MVV).

Unilamellar vesicles are composed by a single bilayer membrane and present different size diameters. The size of small unilamellar vesicles (SUVs) ranges from 20 to 100 nm, while large unilamellar vesicles (LUVs) can vary from 100 nm to 1 µm. For sizes bigger than 1 µm, liposomes are described as giant unilamellar vesicles (GUVs). Multilamellar vesicles (MLVs) are formed by multiple concentric bilayers and have a diameter that can vary from 500 nm to over 1 µm. Multivesicular vesicles (MVVs) present roughly the same diameter of MLVs, but are formed by several smaller unilamellar vesicles inside a wider one [38]–[40]. Since larger liposomes are rapidly removed from blood circulation, smaller vesicle diameter are more favourable for drug or acid nucleic delivery [41]. Therefore, different methods for liposome preparation were developed in order to shape the lipid vesicles, and each method will produce different types of liposomes. The most common methods are lipid film hydration, ethanol injection, sonication and extrusion.

In addition to the size and number of lamellae, different liquid-crystalline lipid phases may exist, depending on the lipid molecules used in the system. The type of structure can be predicted by the packing parameter (P) of the lipid. The packing

parameter is the ratio between the area occupied by the hydrophobic region and the area occupied by the hydrophilic region, as defined by Equation 1.

$$\boldsymbol{P = \frac{v}{a\, l_c}} \qquad \textbf{Equation (1)}$$

where (*v)* is the volume of the hydrocarbon, (*a*) is the effective area of the head group, and (l_c) is the length of the lipid tail. As depicted in Fig. 4, different packing parameter values will lead to different lipid structures: lipids with P < 1/3 tend to form spherical micelles; lipids with 1/3 ≤ P < 1/2 form cylindrical micelles; lipids with 1/2 ≤ P < 1 form flexible bilayers or vesicles; when P=1 planar bilayers are formed; and when P > 1 inverted micelles are formed. When P exceeds 1, the area occupied by the hydrocarbon chains is much larger than the area of the head group, and the lipid tends to adopt a bilayer destabilizing structure called inverted micelle or inverted hexagonal phase. These structures seem to improve the transfection efficiency of liposomes [38], [42]. The lipid phase behaviour can also be modulated by changes in several parameters such as hydration, state of ionization (pH and ionic strength) and temperature [41].

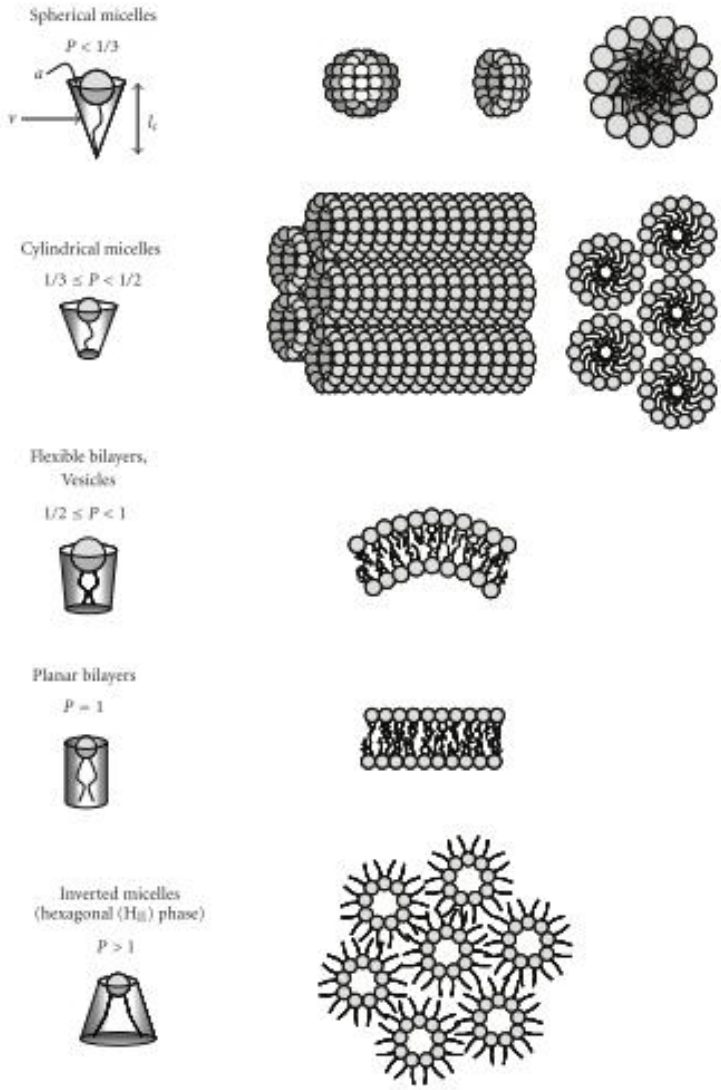


Figure 4 Liposome structures assumed by lipids according to their packing parameter. Lipid structure, when in an aqueous medium, is greatly influenced by the lipid packing parameter. Structures such as spherical micelles, flexible bilayers, vesicles, planar bilayers and inverted micelles can be obtained depending on the ration between hidrophobic and hidrophilic area [38]**.**

2.3.1 Cationic Liposomes

Cationic liposomes are spherical-shaped colloidal structures, formed by the self-assembly of cationic lipid molecules in aqueous solution, composed by one or more concentric lipidic bilayers entrapping an aqueous compartment [39]. Liposomes present several interesting properties such as biocompatibility, biodegradability as well as the ability to entrap and protect either water-insoluble (hidrophobic) pharmaceutical agents into the hydrocarbon chain core of the bilayer, or water soluble molecules (hydrophilic) in the internal water compartment [40]. Due to these

attractive characteristics, cationic liposomes have been extensively studied as non-viral vectors for medical applications and for several other fields such as chemical and biochemical analytics, diagnostics, cosmetics, long lasting immune contraception, food and chemical industry [38], [39].

In the late 80's a study developed by Felgner and colleagues [43] reported, for the first time, the ability of cationic liposomes to efficiently form complexes with DNA and transfect the COS-7 cell line. This study helped to understand the potential of liposomes for gene therapy. Since then several cationic lipids have been used to deliver nucleic acids to the cells [38], [44] (Fig. 5)

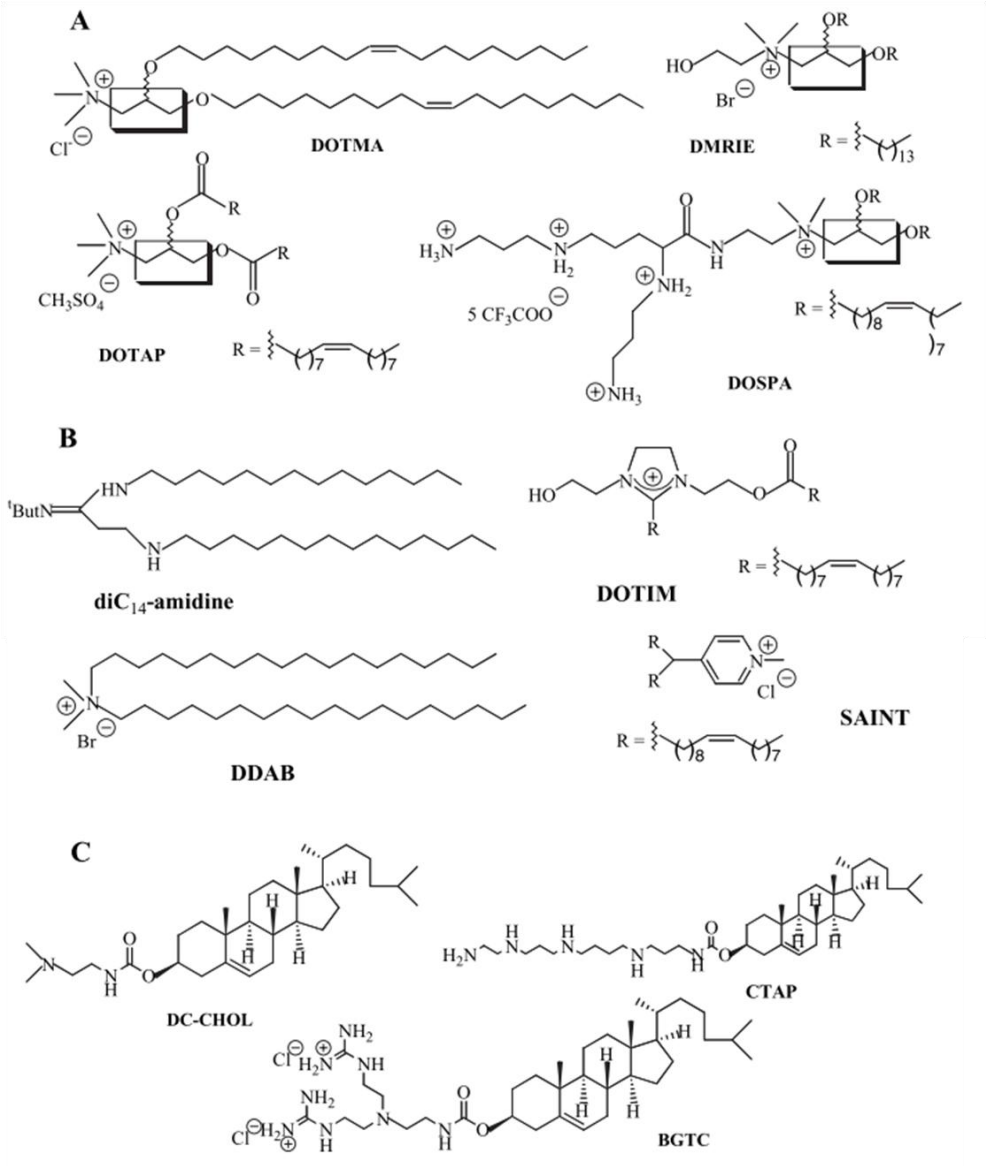


Figure 5 Cationic lipids used in gene therapy. (A) representative structures of glycerol based cationic lipids; (B) cationic lipids not based on glycerol; (C) cholesterol-based cationic lipids.

2.3.2. Optimization of the liposome structure/composition

The use of cationic liposomes for delivery of nucleic acids present some drawbacks, such as fast elimination from blood [40], some cytotoxicity due to their positive charge, and the fact that they may also trigger non-specific interactions with cell components, serum proteins and enzymes [2]. In order to overcome these obstacles, several modifications can be done to improve their properties. The size, charge and surface characteristics of liposomes can easily be altered through the inclusion of specific compounds (*helper* lipids, stimuli-sensitive lipids) in the liposome formulation, or by surface modification.

Different cationic lipid mixtures can be prepared in order to obtain the most favourable structure for gene transfection. For instance *helper* or neutral lipids, such as dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), cholesterol (CHO) and monoolein (MO), are often included in the formulation to improve the characteristics of liposomes. These lipids have the ability to convert the liposome lamellar phase into a non-lamellar phase [42]. The structural organization induced by this lipids is able to promote fusion with endosomal membranes and destabilization of lipoplexes during transfection. Several studies have shown that the presence of a helper lipid improved the transfection efficiency of the liposomes when compared to the cationic lipids alone, as well as decreased the cytotoxicity associated with the high positive charge of the liposomes [38], [45]–[47].

One of the limitations for *in vivo* application of liposomes as non-viral vectors is their circulation half-life, which decreases the possibility of liposomes to reach their site of action. The optimization of liposomes as non-viral vectors has been made not only by changing the liposome composition, but also by the inclusion of molecules at the liposomes surface. A common method to obtain liposomes with increased half-life involves its surface modification with inert and biocompatible polymers, such as polyethylene glycol (PEG). PEG can be used to coat the liposome surface, reducing the peripheral liposome charge, and therefore the binding of proteins. As a consequence, the liposome recognition by opsonins decreases, avoiding liposome clearance, increasing circulation time and reducing toxicity [40], [48]. The specificity of liposomes can also be improved by adding molecules such as folate or immunoglobulins, that will

be covalently coupled to the liposome surface and will allow the targeting to specific cells [41].

2.3.3 Formation of cationic lipoplexes

Cationic liposomes can be used as non-viral vectors for gene therapy due to their ability to easily promote complexation with nucleic acids, forming lipoplexes that are capable of transporting these nucleic acids into cells. The electrostatic interaction between negatively charged phosphate groups of the nucleic acid molecules, and the positively charged liposomes, drives the spontaneous formation of lipoplexes [49]. This interaction will lead to nucleic acid charge neutralization, followed by a disruption of liposomes and intermediate condensation of nucleic acid molecules into lipoplexes. Finally, relaxation and rearrangement into a more ordered states occurs [50]. Efficient complexation is dependent on the charge ratio (CR+/-), which is the ratio between positive charges from liposomes and negative charges from nucleic acids. In order to completely neutralize the negative charges, an excess of cationic lipid is added to the nucleic acids, resulting in positively charged lipoplexes that promote electrostatic interactions with cell membrane. Since siRNA are much smaller than pDNA molecules, the siRNA and pDNA complexation process presents some differences, and siRNA nanocarriers require less negative charges and form smaller sized nanoparticles. However, siRNA lipoplexes are less stable than pDNA lipoplexes, as pDNA promotes a stronger polycation-mediated electrostatic collapse into small compact particles [51]. Due to their different characteristics, siRNA and pDNA transfection mechanisms will be significantly different [52].

2.4. The system DODAB:MO for nucleic acid delivery

In recent years a novel liposome formulation for nucleic acid delivery was proposed, based on the helper lipid monoolein (MO) and cationic lipids from dioctadecyldimethyl family (DODAX) [45], [49], [53]–[59]. Liposomes composed by DODAB/DODAC and MO were extensively studied as pDNA and siRNA carriers, and have proved to be a very promising transfection agent.

2.4.1 DODAB

The long-chained cationic surfactant dioctadecyldimethylammonium bromide (DODAB) is a synthetic lipid that tends to assemble into closed bilayers. DODAB resembles membrane phospholipids since it is composed by two saturated alkyl chains linked to hydrophilic group [60] (Fig. 7A). In an aqueous medium and above the phase transition temperature (T_m= 45 ˚C) [58], [61], [62], DODAB tends to form large unilamellar vesicles [49], [53]. This lipid presents a small critical vesicle concentration (CVC), which allows the formation of vesicles at very low concentrations [45]. When applied in *vivo*, DODAB presents strong rigid lamellar phase due to its elevated T_m that becomes a limiting step for gene delivery since rigid structures do not favour membrane fusion. Therefore, the combination with other lipids has been tested to improve the transfection efficiency of DODAB-based liposomes [58], [61], [62].

2.4.2 Monoolein (MO)

1-monooleoyl-rac-glycerol (MO) is an amphiphilic neutral lipid of natural origin, composed of a single hydrocarbon chain, attached to a glycerol backbone by an ester bond (Fig. 6B). The remaining two hydroxyl groups of the glycerol moiety confer polar characteristics to the polar head of the molecule [63]. A concise summary of phase propensity at different temperatures and water content has been established for MO (Fig.6). This phase diagram shows that, even in excess of water, MO presents two inverted bicontinuous cubic phases, this liquid crystalline phases consisting in a pair of interpenetrating, but non contacting, aqueous channels, separated by a single continuous lipid bilayer [64].

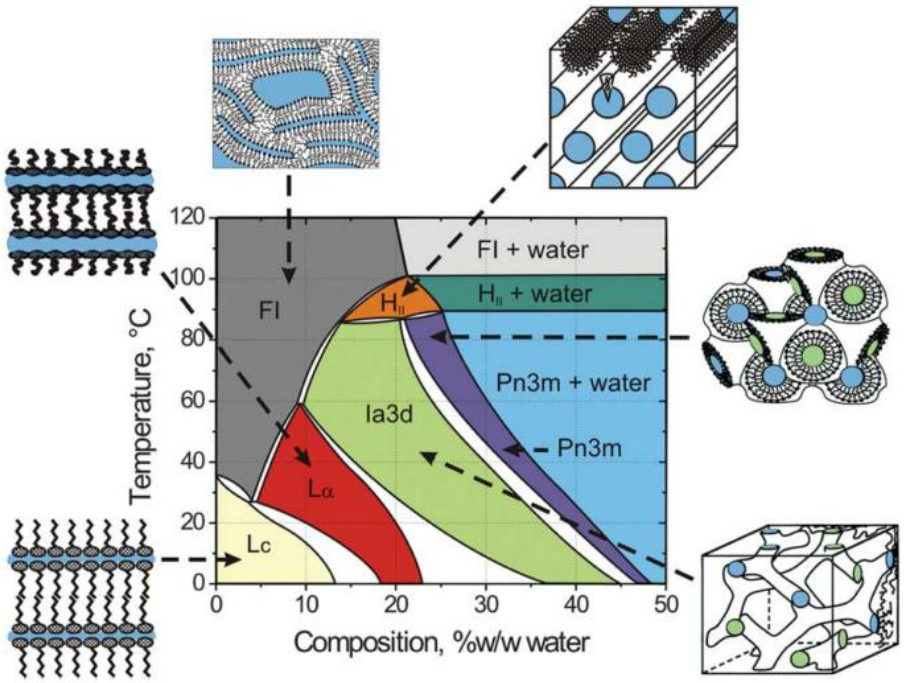


Figure 6 - Temperature-composition phase diagram of monoolein. In small amounts of water monoolein can form a lamellar crystalline phase (Lc) or a similar fluid isotropic phase (FI) depending on the temperature. As the amount of water is increased, a liquid crystalline (Lα), and two inverted bicontinuous cubic phases (Ia3d,Pn3m) are adopted. Schematic representations of the various phases is included, in which colored zones represent water [65].

The phase behaviour of MO presents interesting characteristics. The ability to form non-lamellar phases has been explored in many different fields such as pharmaceutics, cosmetics, agriculture, protein crystallization and drug delivery [63]. Furthermore, due to the richness of MO phase diagram, the addition of this neutral lipid has been proposed in the development of lipoplexes for gene transfection [49].

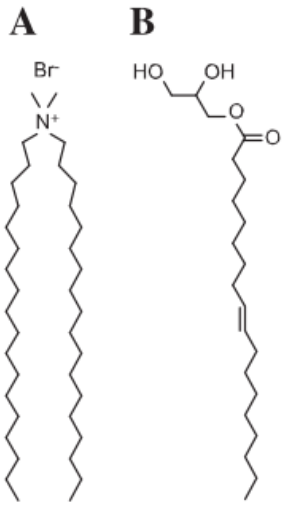


Figure 7 - Molecular structure of DODAB and MO. A - molecular structure of DODAB, B- molecular structure of MO

2.4.3 The mixture DODAB:MO

Nucleic acid complexation with DODAB:MO liposomes has been intensively studied during the past few years [49], [54]–[58] . It was demonstrated that the addition of MO to lipid mixture has a strong influence in liposome/lipoplex morphology and consequent transfection efficiency performance.

The liposomal mixed system composed by MO and DODAB was studied by phase scanning imaging in order to characterize the phase behaviour and aggregate morphology of DODAB:MO liposomes at different molar ratios and temperatures. This study showed that the morphology obtained is strongly dependent on DODAB/MO molar ratio. When the liposomal formulation is of equimolar composition, or when DODAB is in excess, the structures assembled are mostly bilayers; whereas formulations presenting higher amount of MO tend to promote densely packed cubic oriented particles (Fig.8). Additionally, it was found that increasing the temperature of the formulations has a similar influence on the vesicles morphology as the increase of MO concentration [53].

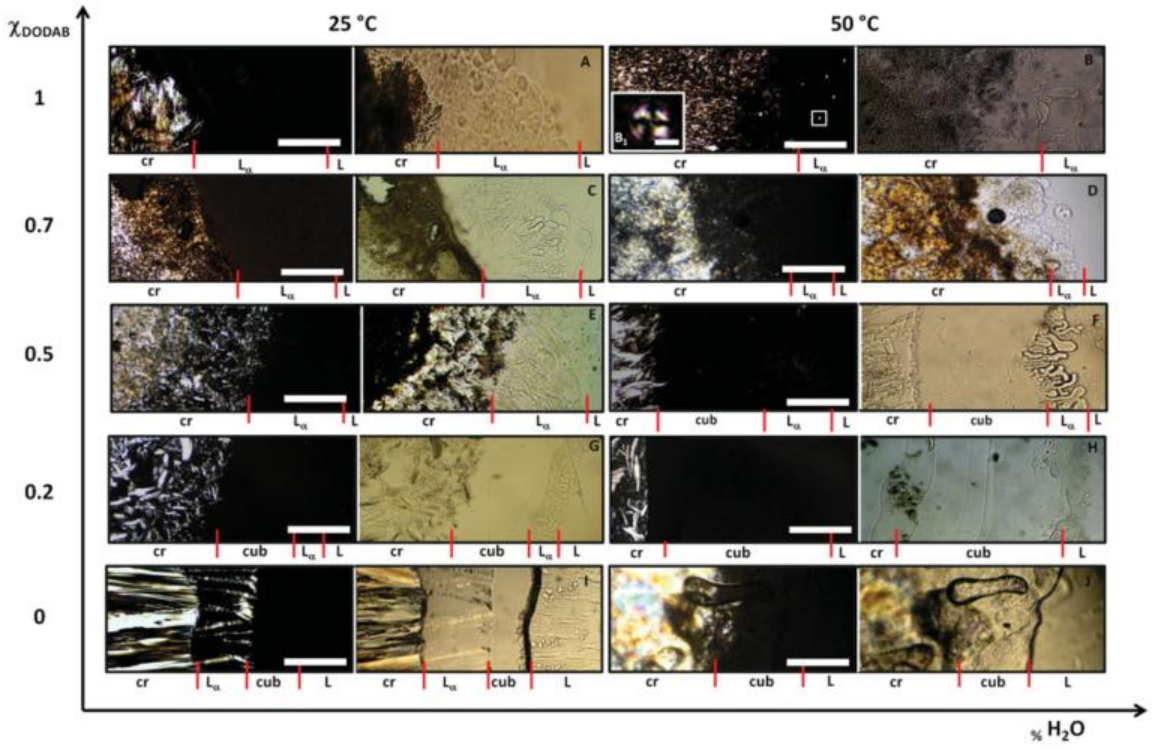


Figure 8 Phase scanning imaging of different DODAB:MO mixtures at 25 ˚C and 50 ˚C. *Lα* is the lamellar liquid crystalline phase, *L* the isotropic phase, *cub* the isotropic cubic phase and *cr*, hydrated crystals [53]

The addition of a *helper* lipid such as MO to DODAB, that presents a very low T_m, can effectively lower the T_m of the final lipid mixture. This has implications on the

liposomal membrane fluidity and lipid organization, which are important parameters influencing nucleic acid encapsulation efficiency and transfection efficiency of the lipoplexes [54]. Additionally, studies regarding the nucleic acids complexation efficiency of DODAB:MO showed that the MO tendency to form inverted non-lamellar structures promotes inter-lamellar attachments (ILAs) and packing defects, resulting in fluidization/destabilization of the lamellar arrangement of DODAB. These structures help in the release of nucleic acids from the lipoplex by membrane fusion, enhancing transfection efficiency [53], [57]. The fluidization promoted by the presence of MO also leads to a high encapsulation efficiency improving lipoplex resistance to biological conditions [29], [45], [49].

Transfection efficiency studies with DODAB:MO at different molar ratios were performed both for siRNA [54] and pDNA [55]–[58]. In both cases, lipoplexes with less MO presented better transfection efficiencies when compared with lipoplexes with higher MO contents. These results show that the use of MO as a *helper* lipid may be finely tuneable in order to improve lipoplexes transfection [66].

2.4.4 Optimization of DODAB:MO for siRNA delivery: inclusion of anionic cargo

pDNA and siRNA molecules present substantial chemical and structural differences, such as molecular weight and molecular topography. These differences may lead to different complex formation when interacting with cationic lipids. Since different complexes are obtained, it is presumable that cell interaction, uptake and distribution will vary, resulting in different intracellular pathways. These differences will have a strong impact on the effectiveness of the gene delivery system.

Recent studies using different liposomal formulations and different cell lines have demonstrated that the addition of non-coding pDNA molecule to siRNA prior to complexation with liposomes results in lipoplexes with enhanced silencing efficiency both *in vitro* [9], [16] and *in vivo* [67], [68]. Similar results have been reported for simultaneous complexation of pDNA and non-coding pDNA in polyplexes [3]. The mechanism behind this effect is still not entirely understood.

Furthermore, no major difference between siRNA lipoplexes, DNA lipoplexes and siRNA/pDNA lipoplexes structures were detected [16]. Also, the administration of these lipoplexes did not translate into significant differences in the amount of transferred siRNA [9]. The differences observed, were associated with the lower

surface of the lipoplexes formed due to the less amount of cationic lipid necessary to efficiently encapsulate siRNA combined with pDNA. This is a benefit, because decreasing the amount of lipid, leads to a lower cytotoxic response and less off-target effects triggered by these lipoplexes [16]. Nevertheless, even though no relevant structural changes were detected, the confocal microscopy studies performed were able to confirm that the internalization pathways may differ for siRNA/pDNA lipoplexes, siRNA lipoplexes and pDNA lipoplexes, since the cellular localization was different for each lipoplex formulation [9]. It is important to realise that, even in a eukaryotic non-coding version, pDNA is not an inert molecule, and it might still lead to the undesirable expression of molecules. Moreover, studies have shown that when applied in high doses to culture cells, pDNA containing siRNA lipoplexes can be highly toxic [10]. Therefore, several anionic polymers have been studied to replace pDNA as the anionic cargo for simultaneous complexation with siRNA, and improve this way the safety profile of the system by reducing immunogenicity [10].

Addition of poly-glutamate to siRNA prior to lipoplex formation instead of pDNA has been recently reported [10], [69], [70]. Poly(L-glutamic acid) (PG) is a biodegradable, non-toxic synthetic polymer, composed by naturally occurring l-glutamic acid molecules linked together through amide bonds. The free γ-carboxyl group in each unit of l-glutamic acid is negatively charged at a neutral pH, and enables drug attachment. PG is widely applied in the fields of drug delivery, tissue engineering, and biomedical materials [71], [72].

Reports have shown that the inclusion of this polymer instead of pDNA also lead to the formation of lipoplexes with colloidal stability and improved gene silencing efficiency [70]. When siRNA/pDNA/cationic lipid and PG/pDNA/cationic lipid complexes where compared, lipoplexes containing pDNA promoted higher transfection efficiency than lipoplexes containing PG. However, a high cytotoxicity was also associated with the first systems. The substitution of pDNA by PG has lead to a significant decrease in cellular toxicity at higher siRNA doses, while maintaining some improvement in gene silencing efficiency. Thus, the addition of polymers such as poly-glutamate appears to have better potential than plasmid DNA for the development of a safe and effective siRNA delivery system [10], [69].

3. Objective

Since the discovery of RNAi as a valuable tool for gene therapy, a lot of effort has been put into developing suitable carriers for siRNA delivery into cells, as naked therapeutic siRNA molecules show very poor biodistribution and pharmacokinetics in the body. The use of vectors based on cationic liposomes emerged as a promising delivery method, due to their relatively high transfection efficiency and low toxicity and immunogenicity, compared to their viral counterparts. A variety of modifications can still be made in liposomes structure in order to enhance their transfection efficiency.

In recent studies, our group has developed DODAB:MO liposome formulations capable of effectively encapsulate and deliver siRNA into cells, resulting in relatively high silencing efficiency. The main objective of this thesis was to develop and characterize novel systems for therapeutic siRNA delivery based on the addition of anionic cargo (pDNA or PG) to the previously tested DODAB:MO (2:1)/siRNA lipoplexes. The aim is to study the differences in encapsulation, cellular uptake, cytotoxicity and silencing efficiency between the previously established system and the newly developed formulations. Moreover, the differences between the anionic cargo (pDNA, PG with low mol. wt. and PG with high mol. wt.) were to be assessed, to understand which one would be more suitable for co-encapsulation with siRNA.

II - Materials and Methods

i - Materials

Dioctadecyldimethylammonium bromide (DODAB) was purchased from Tokyo Kasei (Tokyo, Japan). 1-monooleoyl-rac-glycerol (MO), Poly-L-glutamic acid sodium salt (PG) (3000-15000 and 15000-50000 mol wt), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Thiazolyl Blue Tetrazolium Bromide (MTT), Dimethyl sulfoxide solution (DMSO), GenEluteTM Plasmid Miniprep Kit, Puromycin dihydrochloride and Hoechst Stain solution were obtained from Sigma-Aldrich (St. Louis (Mo.), USA). 293T and MDA-MB-468 cell lines were purchased from American Type Culture Collection (ATCC) (Manassas (Va.), USA) and 293T/GFP-puro cell line from Cell Biolabs (San Diego (CA), USA). Dulbecco's modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine, Penicillin/Streptomycin and Trypsine-EDTA were purchased from Biochrom (Berlin, Germany). The iScript™ Reverse Transcription Supermix for qPCR and the iTaq™ Universal SYBR® Green Supermix kits were obtained from BIO-RAD (Hercules (CA), USA). SV Total RNA Isolation System kit was obtained from Promega (Madison (WS), USA), Quant-iT™ RiboGreen® RNA Assay Kit and Lipofectamine® RNAiMAX Transfection Reagent from Life Technologies (Eugene (OR), USA) and Hank's Balanced Salt Solution (HBSS) from Lonza (Basel, Switzerland). Absolute ethanol was purchased from Merck Milipore (Berlin, Germany), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-7-nitrobenzofurazan (NBD-PE) (λexc = 465 nm; λem = 535 nm) from Avanti Polar Lipids (Alabaster, AL, USA), and pGL4.20 plasmid DNA was kindly given by Cristina Carvalho, Ph.D. student. siRNA targeting the enhanced green fluorescent protein (siEGFP) (5'CAAGCUGACCCUGAAGUUdTdT3'), a siRNA negative control (sicontrol) (5'UGCGCUACGAUCGACGAUdTdT3'), siRNA targeting the EGFR protein (siEGFR) (5'UGAGCUUGUUACUCGUGC3') and a scrambled anti-BCR-ABL sequence (siSCRAMB) (5'GUCUCAAGUUUUCGGGAAGdTdT3') were purchased from Integrated DNA Technologies (Iowa, USA). The siRNA were diluted in DEPC-treated water and stored at -20 °C until used.

ii - Methods

1. Preparation of DODAB:MO liposomes

Stock solutions of dioctadecyldimethylammonium bromide (DODAB) and 1-monooleoyl-rac-glycerol (MO) were prepared in ethanol, at 20 mM. The liposome formulation was prepared by mixing the appropriate volume of DODAB and MO in order to obtain a molar ratio of 2:1 (mol:mol), after which one of the methods described below was performed to obtain the liposomes.

1.1 Methods for liposome preparation

Various methods for the preparation of liposomes were tested, and the produced liposome suspension evaluated by Dynamic Light Scattering (DLS) in order to understand which method produced liposomes with more suitable characteristics for transfection in mammalian cells. Sonication, extrusion and ethanol injection methods where used to shape the liposomes.

1.1.1 Sonication

Sonication is a commonly used method for the preparation of liposomes. The acoustic sound produced by the sonicator induces pressure on lipid suspensions, breaking up the larger, multilamellar vesicles into smaller ones, that may be unilamellar or multilamellar [73]. The size of the particle can be shaped by controlling the time of exposure, as well as the amplitude (A) applied. This method has the advantage of being less time-consuming than other methods, but the liposome size obtained after sonication are not as reproducible as in methods like extrusion [74].

For the preparation of DODAB:MO (2:1) by the sonication method, appropriate volumes of DODAB and MO were added to 5 mL of HEPES buffer (25 mM, pH 7.4), to form liposomes at a concentration of 3 mM. Afterwards, the liposome formulation was subjected to sonication in a Qsonica Misomix S-4000 Sonicator, with the ultrasonic frequency fixed at 20 kHz. Sonication was performed by ultrasonic bath at different amplitudes (60, 70, 80 and 90 % A) and time of exposure (5,10 and 20 min).

1.1.2 Extrusion

Extrusion is one of the most common methods used for controlling the size of liposome suspensions. The lipid suspension is forced through a polycarbonate membrane with a well defined pore size, resulting in monodisperse unilamellar liposomes with diameter similar to the pore size [75]. The primary advantages of this method are the formation of a monodisperse population of liposomes and reproducibility [74]. The retention of lipid by the membrane filter is the main disadvantage, as well as the time consumed in the process.

For the preparation of liposomes by extrusion, suitable amounts of DODAB and MO (20 μM stock solution in ethanol) were added to a round bottom flask, together with 3 mL of ethanol, to obtain a homogeneous film. The solvent was then evaporated in a rotary evaporator at 60 ˚C, under vacuum, for 15 min. After evaporation, the lipid film formed was hydrated with 5 mL HEPES buffer (25 mM, pH7.4). The rotation process was maintained for 15 min at 60 ˚C, and a 3 mM suspension of DODAB MO (2:1) liposomes was obtained. After the lipid film hydration, the liposome preparation was subject to a process of extrusion, in order to obtain liposomes with acceptable size characteristics. Using a Northern Lipids Lipex Extruder with polycarbonate filters (Whatman, USA), the liposome preparation was forced to pass through a 400 nm pore sized filter once, and four times through a 100 nm pore sized filter. The process was conducted at 60 ˚C, the temperature above the phase transition temperature of the lipids, which facilitates the passage through the filters and diminishes the lipid retention.

1.1.3 Ethanol Injection

Ethanol injection is a fast and simple process for the preparation of liposomes. The process is based on the injection of an ethanol solution of lipids, with a thin needle or pipette tip, into an aqueous solution, while stirring. The lipids instantaneously self-assemble into liposomes. The size and shape of the liposomes depend on several parameters and can be tuned to form the more suitable liposomes for a certain application. Parameters such as temperature, type of lipid, lipid concentration, type of buffer, and even the stirring speed, can greatly influence the size and shape of the produced liposomes. This method has the disadvantage of producing more

polydisperse liposome suspensions, as well as less reproducible results than the extrusion method [76][77].

For the production of DODAB:MO (2:1) liposomes by the ethanol injection method, DODAB and MO were mixed at the appropriate ratio, and immersed into a bath above the lipid transition phase temperature (60 ˚C). The lipids were then quickly pipetted drop by drop into 2.5 mL HEPES buffer (25 mM, pH 7.4), under strong vortex for 15 s, alternating with 25 s in the a water bath at 60 °C, in order to maintain the temperature. During the process, the organic solvent evaporates due to the high temperature, resulting in an aqueous solution of DODAB:MO (2:1) with a final concentration of 3 mM.

2. Plasmid DNA

2.1 pGL4.20

The pGL4.20 [luc2/Puro] vector represented in Fig. 9 is responsible for the encoding of luciferase reporter gene luc2 (*Photinus pyralis*), a synthetic reporter gene, which has been codon optimized for mammalian expression. It also contains a mammalian selectable marker for puromycin resistance. Since this vector does not contain a promoter, it contains multiple cloning regions to allow the cloning of a promoter. Thus, it can be used as a non-coding plasmid DNA (pDNA), which makes it suitable for this work.

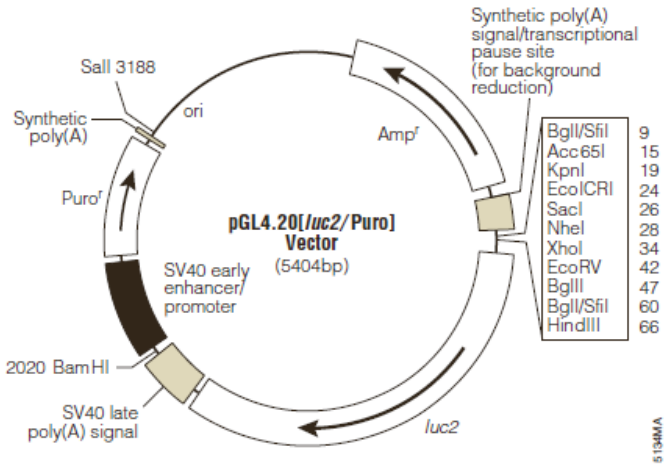


Figure 9 - Schematic representation of pGL4.20 [luc2/Puro] vector

2.2 Transformation of competent cells

Escherichia coli Xl1-Blue competent cells were transformed by the heat shock transformation method [78]. Briefly, a sample of the plasmid pGL4.20 was added to 200 µL competent cells, which were then kept on ice for 30 min. Afterwards, the cells were put in a water bath at 42 ˚C for 30 seconds and then transferred to ice for 10 min. This variation of temperature leads to a heat shock that will enhance the membrane permeability of the competent cells, allowing a more efficient internalization of the plasmid. 800 µL of SOC Medium (2 % tryptone peptone, 0.5 % yeast extract, 2.5 mM KCl, 10 mM NaCl , 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose) were then added to the cells, which were incubated for 1 h one hour at 37 ˚C and 200 rpm. After incubation, the cells were centrifuged at maximum rotation per minute for a few seconds. The pellet obtained was re-suspended in 50 µL supernatant and then spread in a petri dish with LB Medium (1 % tryptone peptone, 0.5 % yeast extract, 1 % NaCl, 2 % agar) with 100 µg/µL ampicillin. The petri dish was left for incubation overnight at 37 ˚C, along with a petri dish with the same competent cells without the transformation process, as a control. The resistance acquired by the transformed cells allowed them to form colonies, while the non-transformed cells showed no formation of colonies due to their susceptibility to ampicillin. One of the colonies was then selected and transferred into approximately 200 mL LB medium, and grown overnight at 37 ˚C and 200 rpm.

2.3 Purification of plasmid DNA

After the amplification of the plasmid DNA (pDNA) by the transformed bacterial cells, the plasmid must be isolated. The isolation and purification was made using the GenEluteTM Plasmid Miniprep Kit (Sigma-Aldrich, USA), according to the manufacturer's protocol. The isolated pDNA was re-suspended in ultra-pure water and analysed in the NanoDrop ND 1000 Spectrophotometer, to determine the nucleic acids concentration and confirm the purity of the sample, by measuring the absorbance ratio at 260/280 nm.

3. Preparation of DODAB:MO (2:1) lipoplexes

Different types of lipoplexes were prepared in this work for the study of their physical characteristics and determine possible advantages in processes such as cellular internalization, cytotoxic effects and silencing efficiency. All the lipoplexes were formed with the same liposome formulation - DODAB:MO (2:1), with siRNA alone, or combinations of siRNA with pDNA, siRNA with poly-L-glutamic acid 1 (PG1, with 3000 to 1500 mol. wt.) and siRNA with poly-L-glutamic acid 2 (PG2 with 1500 to 5000 mol. wt.). The physical characteristics of lipoplexes with pDNA alone and PGA alone were also analysed by dynamic light scattering. A list of the different lipoplexes prepared is presented in Table 2.

Table 2- Different lipoplex conditions prepared throughout this work.

Lipoplexes
DODAB:MO (2:1) + siRNA
DODAB:MO (2:1) + pDNA
DODAB:MO (2:1) + PG1
DODAB:MO (2:1) + PG2
DODAB:MO (2:1) + siRNA and pDNA
DODAB:MO (2:1) + siRNA and PG1
DODAB:MO (2:1) + siRNA and PG2

siRNA and pDNA are composed by nucleotide bases, each containing a negatively charged phosphate group. The L-glutamic acid units in PG are also negatively charged due to a free y-carboxyl group. siRNA, pDNA and both PG polymers where prepared in HEPES buffer (25 mM, pH 7.4), to have the same negative charge concentration. As for all the experiments the concentration of siRNA, pDNA and both PG was maintained in all solutions, lipoplexes containing combinations of siRNA with pDNA, PG1 or PG2 present twice the amount of negative charges when compared to solutions containing only siRNA, pDNA, PG1 or PG2. This means that lipoplexes with siRNA+pDNA, siRNA+PG1 and siRNA+PG2 where prepared with twice the amount of lipid, in order to obtain the same charge ratio (+/-).

The ammonium groups present in DODAB confer a positive charge to this molecule, which will strongly interact with DNA, siRNA and PG due to their negatively

charged molecules. The balance between charges of the lipoplexes formed is given by the charge ratio (+/-):

$$\boldsymbol{Charge\ ratio}\ (+/-) = \frac{[\boldsymbol{ammonium\ groups\ from\ DODAB}]}{[\boldsymbol{Negatively\ charged\ groups}]} \quad \textbf{Equation (2)}$$

For the preparation of the lipoplexes, DODAB:MO (2:1) liposomes prepared by ethanol injection were added to the siRNA, siRNA+pDNA and siRNA+PG1/2 solutions, followed by an incubation period of 20 min, at room temperature (RT), with occasional agitation. Lipoplexes with different charge ratios were obtained.

4. Dynamic Light Scattering (DLS)

4.1 Size measurements

The mean size diameter (z-average) and polydispersity index (PDI) of the lipoplexes prepared were measured by Dynamic Light Scattering (DLS). Particles suspended in a liquid are never stationary, because their collision with solvent molecules leads to a constant movement. This movement is known as Brownian motion. Using a Malvern Zetasizer Nano SZ particle analyzer (Malvern Instruments), the Brownian motion of the nanoparticles can be analysed, as well as the homogeneity of the sample. A laser beam illuminates the sample and analyses the intensity fluctuations in the scattered light due to nanoparticle movement. The speed of the Brownian motion is different for different particle sizes: smaller particles move faster than large particles, which changes the fluctuation intensity. The relationship between the particles speed and diameter is explained by the Stokes-Einstein equation (Equation 3):

$$\boldsymbol{D} = \frac{\boldsymbol{k_B T}}{\boldsymbol{6\pi\eta r}} \quad \textbf{Equation (3)}$$

where (D) is the particle diffusion coefficient, (k_B) Boltzmann constant (1.38 × 10-23 $m^2.kg.s^{-2}.K^{-1}$), (T) is the temperature, (r) the radius of the particle and (η) the medium viscosity [79].

The polydispersity index (PDI) refers to the size distribution of the liposomes, high PDI values (PDI>0.5) indicate that the sample has a very broad size distribution, while low values (PDI<0.5) are associated with monodisperse samples. When samples

present a high polydispersity (PDI>0,5), the Z-average value is not reliable and a distribution analysis data should be used instead, to determine the mean size of the nanoparticles [80].

4.2 ζ-Potential measurements

The ζ-Potential measurements were performed in a Malvern ZetaSizer Nano ZS particle analyzer. Particles in suspension acquire an electrical charge on at their surface that is divided in two layers: the inner layer (stern layer) is formed by ions strongly bond, while the outer layer has ions linked by a weak interaction (Fig.6). In this outer layer, the ions and particles form a stable entity, so when the particle moves, only the ions forming this stable entity move with it. The potential of these ions is known as ζ-potential, and can be determined by applying an electric field to the sample. This will give the charged particles an electrophoretic mobility that can be measured by Laser Dopller Velocimetry (LDV), by pointing a laser beam to the sample and detecting the fluctuations on the scattered light that is proportional to the velocity of the particles. The relation between the electrophoretic motion and the particle's ζ-Potential is obtained by the application of the Henry's equation (Equation 4):

$$\boldsymbol{Ue} = \frac{\mathbf{2}\,\boldsymbol{\varepsilon}\boldsymbol{\zeta}\,\mathbf{f(Ka)}}{\mathbf{3}\boldsymbol{\eta}} \qquad \textbf{Equation (4)}$$

where (*Ue*) is the electrophoretic mobility, (ε) is the dielectric constant, (ζ) the zeta potential, (η) is the viscosity of the medium and *f(Ka)* is the Henry's function, which generally presents two different values, either 1.5 or 1 [80].

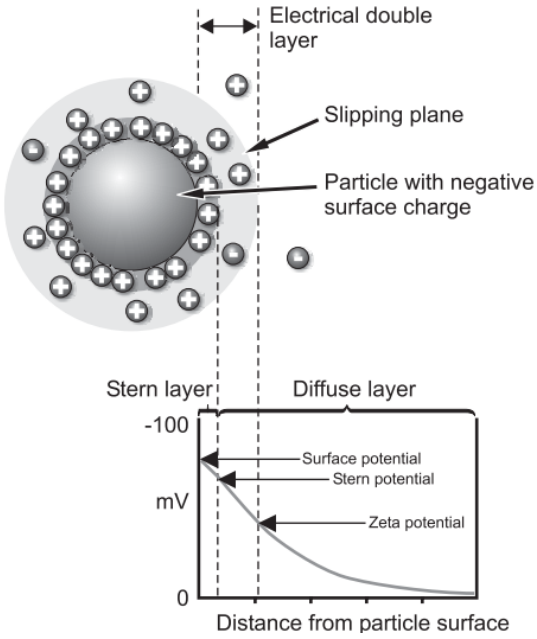


Figure 10 - Representation of the electrical double layer surrounding particles dispersed in a aqueous solution. The boundary that divides the strongly bond inner region (stern Layer) and the diffuse outer region has a potential known as ζ-Potential [80].

4.3 Liposomes and lipoplexes Z-average and ζ-Potential

The mean size and surface charge of the liposomes prepared by the different methods described were analysed by DLS, in order to determine which liposome preparation method promotes more suitable characteristics for siRNA delivery into cells. In order to analyse the mean size, 200 µL of liposomes were pipetted into disposable polystyrene cuvettes, and diluted with 800 µL HEPES buffer (25 mM, pH 7.4) before measurement at 25 ˚C. Z-average, polydispersity index (PDI) and error values were taken in consideration. After the size analysis, 800 µL of the samples were pipetted into universal dip cells (Malvern Instruments) for ζ-Potential determination at 25 ˚C. The Malvern Dispersion Technology Software (DTS) was used for data processing, and the ζ-Potential average and error values were taken into consideration.

5. Dynamics of siRNA encapsulation by DODAB:MO (2:1) liposomes

Lipoplexes described before, were prepared with different charge ratios (+/-) and analysed by DLS and RiboGreen® assay to characterize the dynamics of siRNA complexation rate by DODAB:MO (2:1) liposome formulation.

5.1 Dynamic Light Scattering

Lipoplexes were prepared by adding growing amounts of DODAB:MO (2:1) liposomes to siRNA, pDNA, PG1, PG2, siRNA+pDNA, siRNA+PG1 and siRNA+PG2 solutions, in order to form different charge ratios (+/-). The siRNA used for DLS and ζ-potential measurements was a single stranded scrambled siRNA (siSCRAMB) containing 22 nucleotide bases, at a concentration of 4 µM. After a 20 min incubation period at RT, 200 µL of lipoplexes were diluted with 800 µL HEPES buffer (25 mM, pH 7.4), and the Z-average, PDI and ζ-Potential were measured by DLS, using a Malvern Zetasizer Nano ZS.

5.2 RiboGreen assay

Although the RNA concentration in solution can be determined by absorbance at 260 nm, there are important disadvantages associated with this commonly used technique, like poor sensitivity and interference from contaminating components such as nucleotides, proteins and salts in the RNA solution. To make a more accurate siRNA quantification, the RiboGreen assay, based on an ultra-sensitive fluorescent nucleic acid stain, was used. RiboGreen alone exhibits a poor fluorescence, but when it bounds to nucleic acids, the dye fluoresces with several orders of magnitude greater than the unbound form. This allows for a rapid and simple procedure for measuring RNA concentration in solution [81], [82]. If siRNA is encapsulated within the liposome, RiboGreen will exhibit a low fluorescence signal, since there are no free siRNA molecules to attach to.

In the RiboGreen assay, lipoplexes were prepared by adding increasing amounts of DODAB:MO (2:1) liposomes to siRNA alone, siRNA+pDNA and siRNA+PG, in order to obtain lipoplex solutions with charge ratios (+/-) 1, 2, 3, 5, 10 and 15. The lipoplex dispersions were diluted fivefold with HEPES buffer (25 mM, pH 7.4), and 100 µL of these mixtures were transferred into a 96 black well plate (Corning, USA). A sample containing only siRNA diluted in HEPES buffer (25 mM, pH 7.4) was used as a negative control. Afterwards, 100 µL of a 200-fold diluted RiboGreen® was added to each well. After 5 min incubation in the dark, the fluorescence was assessed on a Fluoroskan Ascent FL (Thermo Scientific) microplate fluorometer and luminometer, with an excitation/emission filter pair of λ_{ex}=485 nm, λ_{em}=538 nm.

6. Cell lines and culture conditions

Highly transfectable derivative of human embryonic kidney (293T cells) and Human breast carcinoma (MDA-MB-468) cell lines where cultured in Dulbecco's minimal essential medium (DMEM), supplemented with 10 % (v/v) heated inactivated fetal bovine serum (FBS), 1 % (v/v) penicillin-streptomycin, 1 % (v/v) L-glutamine and 1 % (v/v) sodium piruvate. 293T/GFP-Puro (siEGP) Cell line was also cultured in the same medium, supplemented with 2 µg/mL puromicyn for selection. The cells were kept in 25 or 75 cm^2 tissue culture flasks, in an incubator with 5 % CO_2 and at 37 ˚C. In order to maintain sub-confluence, all cell lines were subcultured regularly using 0.05 % Trypsin solution. All work with cell lines was performed in sterile conditions in a laminar flux chamber Bio II A (Telstar), to avoid contaminations.

6.1 Lipoplexes cellular uptake

The lipoplexes cellular internalization was determined on the 293T and MDA-MB-468 cell lines. Lipoplexes were prepared at charge ratios (+/-) 5 and 10, with liposomes labelled with NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoylsn-glycero-3-phosphoethanolamine), and sterilized by filtration with a 0.22 µm pore sized filter before addition to the cells. The labelled liposomes (3 mM) were prepared by adding 2 % (mol:mol) NBD-PE to the lipid mixture prior to ethanol injection. NBD-PE is composed of a phospholipid (PE) labelled in the head group by a fluorophore (NBD). NBD-PE will be incorporated in the liposomes and therefore allow internalization measurements in the cells.

293T and MDA-MB-468 cell lines where seeded into 24-multiwell plates (TPP, Switzerland) at a cell density of 1×10^5 cells per well in complete cell culture medium. 293T cells were incubated 24 h and MDA-MB-468 48 h in 5 % CO_2 and 37 ˚C, and then kept at 4 ˚C for 30 min, before lipoplexes were added to synchronize cell's metabolism. Scrambled siRNA (siSCRAMB) was used to prepare the lipoplexes and the final siRNA concentration on each well was set to 25nM. Immediately before the addition of siRNA lipoplexes, siRNA/pDNA lipoplexes and siRNA/PG lipoplexes, the complete cell culture medium was replaced by HBSS (Hank's Balanced Salt Solution), in order to avoid interferences caused by the medium components on the fluorescence measurements. Lipoplexes were incubated for 6 h

6.1.1 Fluorescence Spectrophotometry

After the incubation period, medium was removed and cells washed twice with PBS to remove any non-internalized lipoplexes. PBS was removed, 500 µL Triton X-100 (5 %) was added to the wells to lyse the cells, and 200 µL of each sample was added to a 96 black well plate (Corning, USA). A negative control was done by simply adding triton X-100 (5 %) to cells without lipoplexes. In order to quantify the amount of NBD-PE internalized by the cells, a calibration curve was done by successively diluting the concentration of NBD-PE labelled DODAB:MO in Triton X-100 (5 %). Samples were analysed in a Synergy Mx Multi-Mode Plaque Reader with Gen5TM software (Bio-Tek Instruments, Inc., EUA), with λ_{ex}=480 nm and λ_{em}=530 nm. Uptake experiments were performed in triplicate and. The concentration of the lipid internalized was determined using the calibration curves. The percentage of cellular internalization was determined after subtracting the fluorescence intensity of the negative control, and considering the total amount of lipid added to each well as the total amount of fluorescence.

6.1.2 Fluorescence microscopy

After incubation, HBSS was removed from the wells, and MDA-MB-468 cells were washed twice with PBS. Afterwards, cells were stained with Hoechst Stain solution, a fluorescent DNA stain with λ_{ex}≈350 nm, λ_{em}≈460nm [83]. After 15 minutes incubation, PBS was removed and cells were washed three times with an acidic buffer (0.2M glycine–0.15M NaCl, pH 3.0), to promote the removal of liposomes attached to the cell-surface. Samples were analyzed by fluorescence microscopy in an Olympus IX71 inverted microscope with Cell F software. Bright field micrographs and fluorescence micrographs (FITC filter, λ_{ex}=490 nm, λ_{em}=520 nm and DAPI filter, λ_{ex}=372 nm, λ_{em}=456 nm.) were acquired with 40x objective for each sample.

6.2 Cytotoxicity assay

MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a tetrazolium salt permeable to the cell membrane. Once inside the cells, if they are viable, MTT tetrazolium salt is metabolized by mitochondrial dehydrogenase enzyme activity into insoluble formazan coloured crystals. This crystals absorb light at a wave length of 570 nm, which can be easily assessed by absorption measurements. This way, MTT is a rapid method to assess the cell viability, since cells with a compromised metabolism will not metabolize the salt, and present lower light absorption [84].

Both 293T and MDA-MB-468 cell lines were used to evaluate the liposomes and lipoplexes cytotoxicity. DODAB:MO (2:1) liposomes were freshly prepared by ethanol injection and sterilized by filtration through a 0.22 μm pore sized filter. For 293T cell line, lipoplexes were prepared at charge ratios (+/-) 10, and the siRNA final concentration in the well was 25 nM and 50 nM. Liposomes, siRNA, pDNA and both PG molecules were also tested individually in this cell line to evaluate their toxicity. For MDA-MD-468 cell line, lipoplexes were prepared only with 25 nM siRNA, at charge ratio (+/-) 5 and 10.

Cells were seeded into 24 well plates (TPP, Switzarland) at a cell density of 1×10^5cells per well, and incubated for approximately 24 h at 5 % CO_2 and 37˚C. After the incubation period, the cell culture medium was replaced by 400 μL fresh medium. Then, 100 μL of the different lipoplex suspensions were added to the wells, in duplicates. A cell viability control was done by adding 500 mL of fresh medium to the cells, while a cell death control was done with 30 % (v:v) DMSO.

The lipoplexes were incubated for 48 h, after which 50 μL of MTT (5 mg/mL) was added to each well and left to incubate for 1 h at 5 % CO_2 and 37˚C, for formation of the formazan crystals. The medium was then removed and 500 μL of a DMSO/Ethanol [1:1 (v/v)] solution was added to each well to dissolve the crystals. 200 μL of each condition was transferred into a 96 well plate (Nunc, Thermo scientific, USA), and 200 μL of DMSO/Ethanol [1:1 (v/v)] was used as blank. The absorbance measurements were done in a SpectraMax Plus 384 absorbance Plate Reader (Molecular Devices), with the SOFT Max Pro software.

6.3 Gene silencing assays

Different methods were used to analyse the lipoplex gene silencing efficiency in the two cell lines. 293T cells stably expressing the EGFP protein (293TeGFP) were used to evaluate DODAB:MO (2:1) silencing efficiency by the decrease in the EGFP fluorescence signal, using fluorescence microscopy. DODAB:MO (2:1) silencing efficiency on MDA-MB-468 cell line was analysed by knocking down the epidermal growth factor receptor (EGFR) expression, using quantitative Real Time PCR (qPCR).

6.3.1 Fluorescence microscopy

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is commonly used as a marker for gene expression and protein localization in a variety of organisms. This protein absorbs light with an excitation maximum of 395 nm, and fluoresces at an emission maxima of 508 nm, exhibiting bright green fluorescence [85]. In this work we used a siRNA anti EGFP (siEGFP) to evaluate the transfection efficiency of DODAB:MO (2:1) lipoplexes on 293TeGFP cells, by knocking down the EGFP expression. The decrease in green fluorescence was observed by fluorescence microscopy and measured using the Image J software.

293TeGFP cells were seeded into 24 well plates (TPP, Switzerland), at a cell density of 1×10^5cells per well, and left over-night at 5 % CO_2 and 37˚C for adhesion. Liposomes were freshly prepared as described above and filtered through a 0.22 μm filter for sterilization. siRNA, siRNA+pDNA, siRNA+PG1 and siRNA+PG2 lipoplexes were prepared at charge ratios (+/-) 5 and 10, and the siRNA final concentration in the wells was set to 25 nM and 50 nM. After 20 min incubation, the cell culture medium was replaced by 400 μl of fresh medium, and 100 μL of each lipoplex formulation was added to the wells, in duplicates. Lipofectamine, a common lipofection reagent with relatively high transfection efficiency, was used as a positive control, whilst non encapsulated siRNA was added to the wells as a negative control.

After a 48 h incubation period, cells were washed with PBS and analyzed by fluorescence microscopy in an Olympus IX71 inverted microscope with the Cell F software. Bright field micrographs and fluorescence micrographs (FITC filter, λ_{ex}=490 nm, λ_{em}=520 nm) were acquired with 10x objective for each sample, in duplicate. The exposure time was maintained in all fluorescence photographs taken.

The Image J software image processing program was used to count the number of cells in bright field micrographs, and to determine the fluorescence intensity on FITC micrographs. Cell count was performed through the enhancement of the contrast between cells and extracellular medium, achieved by removing the background and adjusting image threshold, and by using Analyze Particles function, which counts the number of cell nuclei with sizes within a range of pre-established pixels. To quantify the fluorescence intensity, background fluorescence was removed and the fluorescence micrographs converted to multi-channel composite images in order to

split the channels. RGB pictures are composes of red, green and blue channels, and by splitting the channels, the red and blue interference can be removed. Fluorescence intensity was quantified using the function histogram. Finally, for each condition, the fluorescence intensity was divided by the number of cells counted on the corresponding bright field micrographs, which gives the ratio of total fluorescence intensity per number of cells.

6.3.2 Reverse transcription and quantitative real time PCR (qPCR)

For qPCR, MDA-MB-468 cells were seeded into 12 well plates (TPP, switzerland) at a cell density of 2×10^5 cells per well, and left approximately 48 h in an atmosphere of 5 % CO_2 and 37 ˚C. DODAB:MO (2:1) Liposomes were prepared as described above, filtered through a 0.22 µm pore sized filter for sterilization, and added to siRNA, siRNA+pDNA, siRNA+PG1 and siRNA+PG2 to form lipoplexes at C.R. (+/-) 5. siRNA anti EGFR protein (siEGFR) was used, and lipoplexes prepared in order to have a final siRNA concentration of 50 nM in the wells. After 20 min incubation for the formation of lipoplexes, cell culture medium was replaced by 900 µl fresh medium, and 100 µl of each lipoplex formulation was added to the wells. Cells without lipoplexes were used as a negative control, to have the normal expression of EGFR, while, Lipofectamine was used as a positive control. After a 24 h incubation period, EGFR down regulation was analyzed by qPCR, using GADPH as the reporter gene.

6.3.2.1 RNA Isolation

PCR is often used for the quantification of miRNAs and other regulatory RNAs, cellular mRNA and mRNA splice variants [86]. In order to analyse changes in gene expression by real time polymerase chain reaction (RT-PCR), first it is necessary to extract the mRNA from the cells and convert it to complementary DNA (cDNA) by reverse transcriptase (RT). Since the mRNA obtained after extraction is extremely sensitive and prone to degradation, it is important to work under tightly controlled and well defined conditions to avoid mRNA degradation. RNA purity and integrity must also be analysed before reverse transcription, since impurities in RNA sample may lead to the inhibition of PCR reaction or to inaccuracies in gene expression evaluation. RNA quality can be assessed by various absorbance measurements such as NanoDrop [87].

RNA isolation was performed using a SV Total RNA Isolation kit (Promega, USA) according to manufacturer's instructions. Briefly, DMEM medium was removed, cells were washed with PBS ice cold and collected in RNA Lysis Buffer into sterile tubes, to which RNA Dilution Buffer was added. After a heating step (70 ˚C for 3 min), the tubes were centrifuged (10 min at 12000 rpm) and the cleared lysate transferred into fresh tubes. 95 % ethanol was added, the mixture is transferred into a Spin Basket Assembly and centrifuged for 1 min at 12000 rpm. The eluates were discarded and the DNase incubation mix (Yellow Core Buffer solution, $MnCL_2$ at 0.09 M and DNase I) was added to the membranes of the spin basket. After 15 min at RT, DNase Stop Solution was added to the membranes and a centrifugation of 1 min at 12000 rpm performed. RNA Wash Solution was used to wash the membranes several times and, finally, RNA was eluted with Nuclease-Free Water. Isolated RNAs were quantified with the Nanodrop ND-1000 spectrophotometer. Absorbance ratios of 260/280 and 260/230 were determined to check the purity of the RNA samples

6.3.2.2 Reverse Transcription

For reverse transcription a mixture containing dNTPs, random primers and RNase H must be prepared and added to the extracted RNA, so that the enzyme reverse transcriptase RNase H+ will promote the conversion of RNA to cDNA. The amount of siRNA must be equal for every sample in order to obtain reliable results.

Reverse transcription was performed using the iScript™ Reverse Transcription Supermix for qPCR kit (BioRad, USA). This supermix contains all the necessary components for reverse transcription (reverse transcriptase, RNase inhibitor, dNTPs, oligo(dT), random primers, buffer, $MgCl_2$ and stabilizers), except the RNA template. The reaction was performed according to manufacturer's instructions, and the RNA concentration normalized to 1 µg for all the samples, using nuclease-free water to adjust the final reaction volume to 20 µl. The complete mixture was incubated in a T100 thermal cycler (Bio-Rad, USA) and the following protocol established: 5 min at 25 °C to allow the binding of the primers, followed by 60 min at 42 °C to promote optimal conditions for reverse transcription, and finally 5 min at 85 °C to inactivate the reverse transcriptase.

6.3.2.3 qPCR

Quantitative Real-time PCR (qPCR) is a widely used technique to analyse and quantify differences in gene expression levels between samples. qPCR is performed on single or double-stranded DNA templates. For qPCR to occur, a mixture must be prepared containing: two oligonucleotide primers that attach to the DNA sequence and promote amplification; the four deoxynucleotide triphosphates dNTPs; DNA polymerase, which extends the primers by incorporating the dNTPs; magnesium ions; and a dye or dye-labelled probe that allows the miniaturization of the amplification and quantification of its products in real time. Asymmetric cyanine dyes such as SYBR Green I and BEBO are often used as reporters for qPCR [88]. The method is based on repeated heating and cooling cycles, where high temperature is applied in order to promote the separation of the DNA double strand, and temperature is lowered to allow the binding of the primers to the template. The last step involves the application of high temperatures (72 ˚C) which are ideal for polymerase function [86][88][89]. In qPCR experiments, various reference genes such as glyceraldehyde-3-phosphate dehydrogenase (GADPH), β-actin or rRNA are used as an internal standards, which are assumed to have constant expression between experimental conditions. This way, experimental data can be normalized to the reference gene in each sample [90]. A series of guidelines known as MIQE guidelines were written to establish par1ameters that should be met to publish acceptable results from qPCR experiments [91].

Real-Time PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, USA). For each condition, 1 µl of EGFR primer forward at 10nM and EGFR primer reverse were added to the iTaq Universal SYBR Green Supermix containing the antibody-mediated hot-start iTaq polymerase, dNTPS, $MgCl_2$, SYBR Green I dye, enhancers, and stabilizers. After the prepared mixture was loaded into a 96-well PCR plates (iCycler iQ, Bio-Rad), 1 µl cDNA template of each condition was added to each well, and the final reaction volume normalized with DNase free H_2O. The mixture was prepared on ice, to avoid sample degradation and mixture components were carefully pipetted, to avoid contaminations that would alter the results. The same protocol was followed using primers for the endogenous reference gene GADPH (forward primer 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse primer 5'-

TGTAGACCATGTAGTTGAGGTCA-3'), for normalization of the cDNA expression in each condition.

The qPCR reaction was performed in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), by applying one cycle of 5 min at 95 °C, followed by 40 cycles of PCR at 95 °C × 15 s and 60 °C × 30 s. A melting curve (1 cycle of 95 °C × 60 s and 55 °C × 60 s, followed by an increase in temperature from 55 to 95 °C, with 0.5 °C increments in each step) was made immediately after the reaction, to demonstrate the specificity of the amplification. No template controls were evaluated for each target gene. Quantification cycle (Cq) values were generated automatically by the Bio-Rad CFX Manager 2.0 Software, and the relative gene expression values were determined according to Equation 4 [92]:

$$\boldsymbol{ratio} = \frac{E_{target}^{\Delta Cq_{target}}}{E_{ref}^{\Delta Cq_{ref}}} \quad \textbf{Equation (5)}$$

where E_{target} is the real-time PCR efficiency of the EGFR transcript; E_{ref} is the real-time PCR efficiency of the GADPH reference gene transcript; ΔCq_{target} = $Cq_{control}$ - Cq_{sample} of the target gene transcript; and ΔCq_{ref} = $Cq_{control}$ – Cq_{sample} of the reference gene transcript. After a calibration curve was done for both genes, PCR efficiencies were calculated according to $E = 10^{(-1/slope)}$, and found to be between 94 % and 112 %, with $R^2 > 0.99$.

7. Statistical analysis

Prior to data analysis all assumptions were met testing for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test).

To investigate the influence of different lipoplexes on GFP silencing of 293T/GFP-Puro cell line, a three-level nested design ANOVA was conducted: composition: DODAB:MO (2:1) liposomes and siRNA-related systems; charge ratio (+/-): 0, 5 and 10; concentration: 25 nM and 50 nM.

ANOVA model [two factors: composition (five levels: siRNA lipoplexes, siRNA coupled with small mol weight poly-glutamate lipoplexes, siRNA coupled with large mol weight poly-glutamate lipoplexes, siRNA coupled with pDNA lipoplexes, and death control) and concentration (two levels: 25 nM and 50 nM)] was conducted to determine differences on metabolic activity of 293T cells, pre-exposed to the different

lipoplexes. *In vitro* cytotoxicity (MTT assay) of the lipoplexes single components was further investigated for this cell line, following the same statistical analysis: ANOVA model [two factors: type (seven levels: DNA, small mol weight poly-glutamate, large mol weight poly-glutamate, siRNA, siRNA liposomes, liposomes and death control) and concentration (two levels: 25 nM and 50 nM)]. The lipoplexes interference on metabolic activity of MDA-MB-468 human breast carcinoma cells was also investigated following, a two-level nested design ANOVA: composition: DODAB:MO (2:1) liposomes and siRNA lipoplexes, and charge ratio (+/-): 0, 5 and 10. To investigate differences on cellular metabolic activity among human cells of different origin, a two-level nested design ANOVA was considered: cell line: 293T cells and MDA-MB-468, and condition (25 nM; C.R. (+/-) 10): DODAB:MO (2:1) liposomes and siRNA lipoplexes. All data were tested for differences among replicates of different-time performed experiments using a MANOVA design.

Post hoc comparisons were conducted using Student-Newman-Keuls. A *P* value of 0.05 was used for significance testing. Analyses were performed in STATISTICA (StatSoft v.7, US).

III - Results and Discussion

In this study liposomes prepared by three different methods were analysed by DLS in order to select the most suitable method to develop a new siRNA delivery system based on DODAB:MO (2:1) liposomes. After choosing liposome preparation method, DODAB:MO (2:1) lipoplexes encapsulating siRNA, and co-encapsulating siRNA+pDNA, siRNA+PG1 and siRNA+PG2 were formed. Different parameters such as Z-average, *ζ*-potential, siRNA encapsulation efficiency, cellular uptake, cytotoxicity and silencing efficiency were assessed for all lipoplex formulations, in order to understand if the addition the anionic cargo (pDNA or PG) to the lipoplexes could promote substantial differences in such parameters.

1. Liposome Preparation Method

DODAB:MO (2:1) liposomes were prepared by three different methods: ethanol injection, sonication and extrusion after lipid film hydration. Liposomes obtained for each method were analysed by Dynamic and Electrophoretic Light Scattering (DLS) to understand how these methods affect their physicochemical properties, such as size and surface charge.

1.1 Sonication

Sonication by ultrasonic bath was the first method tested, using different amplitudes and times of exposure. The size and polidispersity index (PDI) of the liposomes obtained were measured by DLS (Table 3).

Table 3 - Liposome mean size diameter (nm) and PDI values obtained after different amplitudes (A) and times of exposure to sonication.

A (%)	Time (min)	Size (nm)	PDI
60	5	268.5	0.498
	10	101.4	0.472
	20	94.23	0.434
70	5	91.14	0.266
	10	81.72	0.282
	20	78.14	0.242
80	5	126.9	0.432
	10	83.69	0.366
	20	62.24	0.262
90	5	118.6	0.289
	10	79.11	0.289
	20	70.43	0.233

Ultrasonic bath sonication leads to the formation of liposomes with a hydrodynamic diameter ranging from 70 to 270 nm. The results presented in Table 1 show that increasing the amplitude and time of exposure during sonication resulted in smaller liposomes and lower PDI values. Despite the particle size obtained being suitable for application in gene therapy, the high PDI values obtained (0.25 to 0.5 for most liposome formulations) are not ideal. In addition to the high PDI value, liposomes prepared by this method presented very low stability, since only a few hours after preparation, it was possible to see the increase of turbidity in the samples, possibly due to aggregation, reorganization into multilamellar structures and precipitation of the liposomes. DLS measurements showed that smaller sized liposomes have aggregated into larger ones (data not shown), and this aggregation behavior can be linked to the inability of smaller liposomes to promote electrostatic repulsion with surrounding liposomes. Another hypothesis is that sonication method may promote hydrophobic defects in the liposomes which might be responsible for the aggregation observed [61]. Even though the mean size diameter of liposomes obtained by sonication was considered suitable for encapsulation and delivery of nucleic acids, this preparation method presents several disadvantages, such as the lack of reproducibility between batches, the lack of homogeneity between liposomes and the particle's aggregation behavior observed. Due to these disadvantages, no further studies were made with liposomes prepared by sonication.

1.2 Extrusion and Ethanol injection

After excluding the sonication method, another two methods were tested, lipid film hydration followed by extrusion and ethanol injection. The mean size diameter and ζ-potential of liposomes obtained by these methods were analyzed by Dynamic and Electrophoretic Light Scattering (Fig.11A, B).

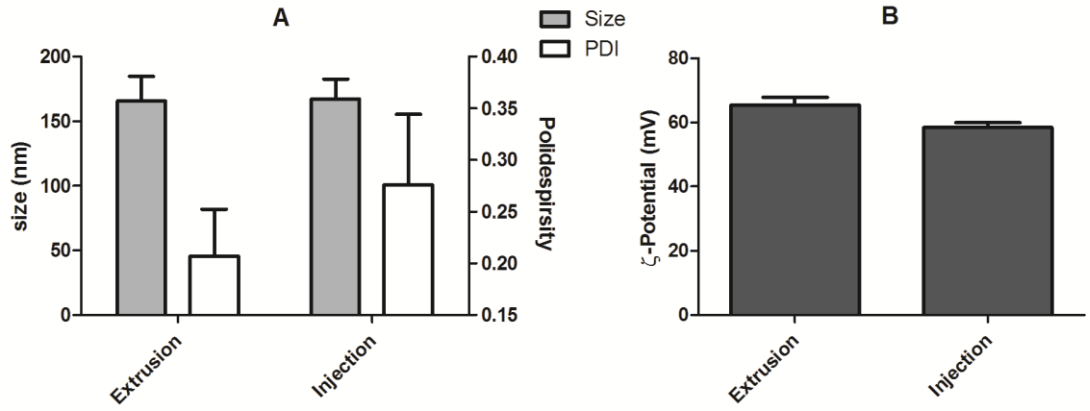


Figure 11 - DLS measurements of liposomes prepared by extrusion and injection methods. **(**A) shows the Z-average (nm) and polidispersity (PDI) of liposomes prepared by extrusion and injection and (B) shows the *ζ*-potential values (mV) for both methods.

Fig. 11A shows the mean size (nm) and PDI of DODAB:MO (2:1) liposomes prepared by ethanol injection and lipid film hydration followed by extrusion. No significant size differences can be observed in liposomes obtained by either method: both liposomes present relatively small hydrodynamic diameter (around 160 nm). Liposomes prepared by extrusion showed lower PDI (≈0.2), suggesting that this preparation method promotes a more uniform size distribution compared with ethanol injection method, which presents a relatively high PDI (≈0.28) value. Liposomes prepared by ethanol injection present similar physicochemical characteristics as observed in previous work from our group [93]. As for extrusion, liposome sizes and mainly PDI values obtained were slightly higher than the ones previously reported for the same DODA:MO (2:1) formulation [54]. These differences might be associated with technical problems.

The superficial charge of the particles obtained is an important physicochemical property. Fig. 11B presents the *ζ*-potential of liposomes prepared by both methods. As expected, liposomes present a highly positive surface charge due to the presence of the cationic lipid DODAB. Liposomes prepared by extrusion present a slightly higher value of *ζ*-potential (≈65.3 mV) than liposomes prepared by ethanol injection (≈58.4 mV). This additional superficial charge can be the result of conformational changes in the liposomes during extrusion, with MO relocating to the interior of the liposomes. The high *ζ*-potential values can improve the particle's ability to encapsulate nucleic acids and promote cell entry through electrostatic attraction. However, positively

charged particles also present higher cytotoxicity and are easily cleared when applied *in vivo*, due to the interaction of serum proteins which might trigger the immune system [35], [94], [95].

Liposomes prepared by both methods present roughly the same size and ζ-potential, yet, extrusion is a more reproducible method to obtain stable and homogenous liposome formulations with appropriate and adjustable sizes. However, retention of lipid in the polyester filters during extrusion is a main disadvantage of this method. The retention of lipid can greatly influence DODAB:MO ratios as well as the concentration of liposomes in solution. These alterations, if not measured, interfere with all the following experiments. Therefore, ethanol injection was the chosen method to proceed with the rest of the experiments of this thesis.

2. Dynamics of nucleic acid encapsulation

One of the main objectives of this work is to study how the addition of pDNA or of PG molecules to siRNA lipoplexes influences the final structure of lipoplexes. DODAB:MO (2:1) liposomes encapsulation rate of siRNA, with either pDNA, PG1 or PG2, was studied by DLS and Ribogreen assay.

2.1 Dynamic Light Scattering

Lipoplexes with different C.R. (+/-) (1, 2, 3, 4, 5 and 10) were prepared for each condition, and Z-average and ζ-potential measurements were performed in order to characterize the different lipoplexes formed and to understand if the addition of anionic charges (pDNA and PG) to the mixture influences the complexation efficiency of DODAB:MO (2:1) liposomes.

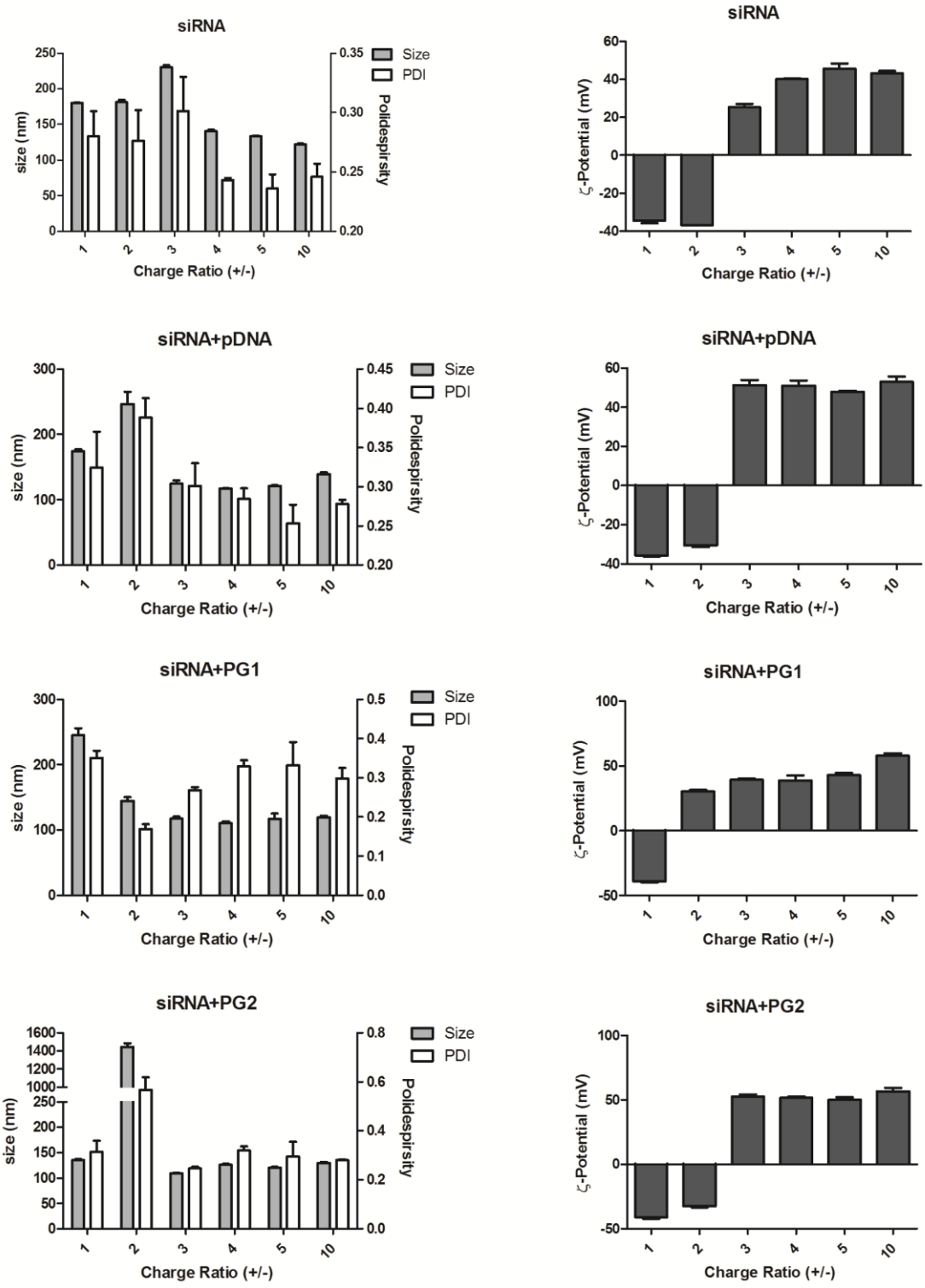


Figure 12 - DLS measurements of different lipoplexes prepared at increasing C.R. (+/-). Z-average, polidispersity and ζ-potential measurements of siRNA lipoplexes, siRNA+pDNA lipoplexes, siRNA+PG1 and siRNA+PG2 lipoplexes, prepared with 4μM siRNA in HEPES buffer, 25mM, pH 7.4..

Fig. 12 shows the size measurements of lipoplexes prepared wih siRNA alone showed an increse in lipoplex mean size diameter and PDI up to C.R. (+/-) 3, and a

subsequent reduction of both parameters for higher C.R. (+/-). When pDNA or PG2 are added to the mixture, the increase in mean size diameter and PDI occurs at C.R. (+/-) ≤ 2. For higher C.R.s (+/-), lipoplex mean diameter and size distribution is reduced and maintained. For lipoplexes with PG1 and siRNA, the mean size diameter reaches its peak at C.R. (+/-) 1 and is reduced until C.R. (+/-) 3. For higher charge ratios, no significant alteration is observed. The high PDI of siRNA+PG1 lipoplexes at C.R. (+/-) 4, 5 and 10 can be related to a less homogenous DODAB:MO (2:1) liposome batch.

ζ-potential measurements of siRNA, siRNA+pDNA and siRNA+PG2 lipoplexes showed negative values (≈-35 mV) for C.R. (+/-) 1 and 2. For higher C.R.s (+/-), *ζ*-potential becomes positive and stabilizes around +50 mV. siRNA+PG1 lipoplexes *ζ*-potential measurements only present negative values for C.R. (+/-) 1.

The addition of DODAB:MO (2:1) liposomes to the nucleic acid solutions promotes an eletrostatic attraction between the positively charged ammonium groups of DODAB, and the negatively charged groups of either nucleic acids or polymers. This attraction will promote the formation of aggregates, responsible for the increase in mean size observed for lipoplexes with low C.R. (+/-). *ζ*-potential measurements for the same C.R. (+/-) present negative values, which implies the presence of either free siRNA, pDNA and PG, or adsorbtion of these molecules to the surface of the aggregates. With the increase of C.R. (+/-), the excess of liposomes promotes a neutralization of the negative charges from the nucleic acid/polymer backbone. This neutralization leads to the reorganization of the aggregates, promoting the insertion of siRNA inside the liposome vesicles [54]. This reorganization step is responsible for the maximum mean size diameter and PDI observed. This reorganization also promotes the encapsulation of the negatively charged nucleic acids or polymers, resulting in the increase of *ζ*-potential values. Further increase in the charge ratio results in smaller mean size diameters and highly positive *ζ*-potential values. DLS measurements also showed slight differences between physicochemical properties of lipoplexes prepared with PG1 and PG2. This differences are probably caused by the mean size of PG molecules. PG2 has a higher mol. wt. and, therefore, resembles pDNA molecules, which might explain the similarities observed between these systems. siRNA+PG1 lipoplexes, due to the smaller molecule size of PG1, seems to promote

quicker compaction of siRNA, slightly reducing the amount of lipid necessary to promote the neutralization of the negative charges.

All lipoplex formulations prepared at C.R. (+/-) > 4 present roughly the same small size (≈120 nm) and highly positive surface charge (≈+51 mV). The ζ-potential values are similar to the ones obtained for liposomes (≈+58.4 mV) suggesting that most of the nucleic acid or polymer molecules in the solution were efficiently encapsulated. However, small changes can be observed in the encapsulation rate for C.R. (+/-) < 4. SiRNA+pDNA, siRNA+PG1 and siRNA+PG2 lipoplexes reach stable values of ζ-potential and Z-average at C.R. (+/-) 3 while siRNA lipoplexes only reach stability at C.R. (+/-) 4. Taken together, these results show that all formulations promote siRNA encapsulation, and the addition of pDNA or PG molecules can reduce the amount of lipid necessary to efficiently encapsulate siRNA. This might be the result of an increased eletrostactic interaction between negative and positive charges due to the addition of anionic cargo to the siRNA suspension.

2.2 RiboGreen assay

siRNA encapsulation efficiency of each lipoplex formulation was determined using the RiboGreen assay. When this probe intercalates with nucleic acids, its fluorescence intensity is enhanced, allowing the quantification of nucleic acids in solution. Lipoplexes for each condition were prepared at increasing C.R. (+/-) and siRNA encapsulation efficiency was determined.

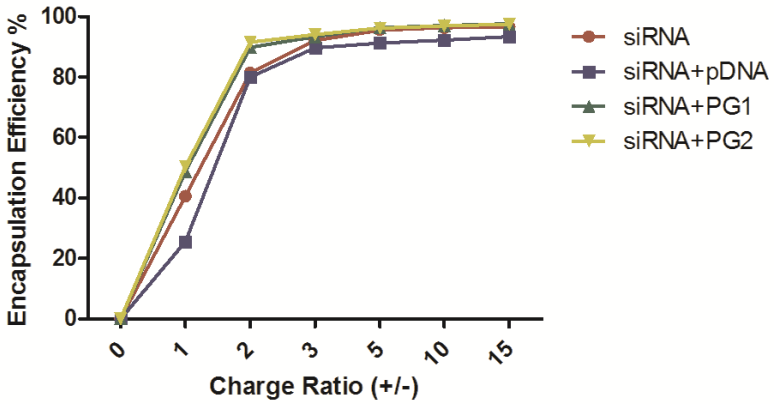


Figure 13 - siRNA encapsulation efficiency for each lipoplex formulation prepared at increasing charge ratios (+/-)

The RiboGreen assay (Fig. 13) shows that even though at C.R. (+/-) > 5 all lipoplex formulations promote efficient siRNA encapsulation (≈95 %), the rate of encapsulation is slightly different for the different lipoplexes. In accordance to DLS measurements, lipoplexes containing PG present slightly higher encapsulation efficiency, and reach maximum encapsulation at lower C.R. (+/-), suggesting that the additional anionic charges provided by the polymer enhance siRNA compaction inside DODAB:MO (2:1) liposomal formulation. The differences on PG molecular size do not seem to promote any differences in the liposomes encapsulation efficiency. Contrarily to what DLS measurements showed, siRNA+pDNA lipoplexes present a lower encapsulation efficiency than siRNA lipoplexes, for all C.R. (+/-). This lower encapsulation efficiency may be due to the binding of RiboGreen to both free siRNA and pDNA, enhancing fluorescence signal intensity.

Overall, DLS and fluorescence measurements allowed to understand that DODAB:MO (2:1) liposomes efficiently encapsulate siRNA at C.R. (+/-) 5 or higher, and that the addition of anionic cargos to lipoplexes can slightly improve the encapsulation efficiency by reducing the amount of lipid necessary. siRNA encapsulation efficiency was slightly higher for C.R. (+/-) 15, however, the excess of lipid can result in high cytotoxicity. Therefore, only lipoplexes prepared at C.R. (+/-) 5 and 10 were tested *in vitro* for cellular uptake, cytotoxicity and silencing efficiency.

3. Cellular uptake

Lipoplexes cellular internalization was evaluated by adding liposomes and lipoplexes, labeled with the fluorescent NBD-PE probe, to 293T and MDA-MB-468 cell lines. Two different C.R. (+/-), 5 and 10, were used for a final siRNA concentration of 25 nM. Internalization in 293T cell line was analyzed by fluorescence measurements, after 6 h incubation with liposomes and lipoplexes. For MDA-MB-468, apart from the fluorescence measurements, also fluorescence microscopy was used to observe the cellular uptake of the different lipoplexes. Three independent experiments were performed for fluorescence measurements in both cell lines.

3.1 Fluorescence measurements for evaluation of cell uptake

To determine the cellular uptake of liposomes and lipoplexes in 293T and MDA-MB-468 cell lines, calibration curves of the fluorescence emission spectrum area in function of the lipid concentration of all systems were constructed. This way it was possible to estimate the labeled lipid concentration internalized by the cells. Data was normalized for lipid concentration present in lipoplexes or liposomes used.

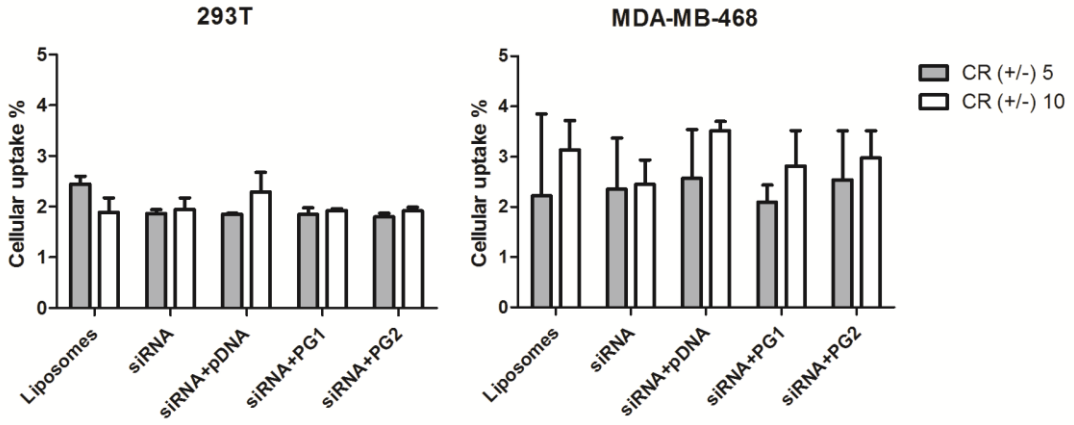


Figure 14 - 293T and MDA-MD-468 cellular association with NBD-labeled liposomes and lipoplexes after 6 h incubation. Liposomes and siRNA, siRNA+pDNA, siRNA+PG, siRNA+PG2 lipoplexes were prepared at CR (+/-) 5 and 10, incubated at a final siRNA concentration of 25 nM. Results were normalized in function of maximum lipid concentration added to cells.

Fluorescence measurements in 293T cell lines (Fig. 14) showed a very small lipoplex internalization percentage (≈2 %), and no significant difference between conditions or C.R (+/-) were detected. Liposomes and lipoplexes internalization in MDA-MB-468 cells (Fig. 14) was equally low, although some differences in internalization efficiency were observed for different C.R.s: at C.R. (+/-) 5 internalization efficiency was approximately 2 % for every condition, while the liposomes and lipoplexes at C.R. (+/-) 10 resulted in a slightly higher internalization efficiencies (≈3 %). Even though poor internalization efficiency was observed, it is possible to understand that no significant differences were observed due to the addition of an anionic cargo to lipoplexes, suggesting that cellular internalization is not affected by the addition of these compounds. The low uptake efficiency observed might be linked with a loss of cells while washing the wells with PBS, inefficient cell lysis promoted by Triton-X, or quenching of the fluorescence by compaction or by dilution of the fluorescent lipid in the cellular membrane [16].

In order to confirm if these low internalization percentages obtained by fluorescence measurements were accurate, MDA-MB-468 cell lines internalization efficiency was also analyzed by fluorescence microscopy.

3.2 Fluorescence Microscopy

MDA-MB-468 cells were treated with NBD-PE labeled liposomes and lipoplexes prepared at C.R. (+/-) 10. After a 6 h incubation period, cells were subjected to acidic wash to remove non internalized particles [96] and incubated with Hoechst for nucleus staining. Bright field and fluorescence (DAPI and FITC) micrographs were obtained for each test condition. Fig. 15 shows the merging of bright field, FITC and DAPI micrographs.

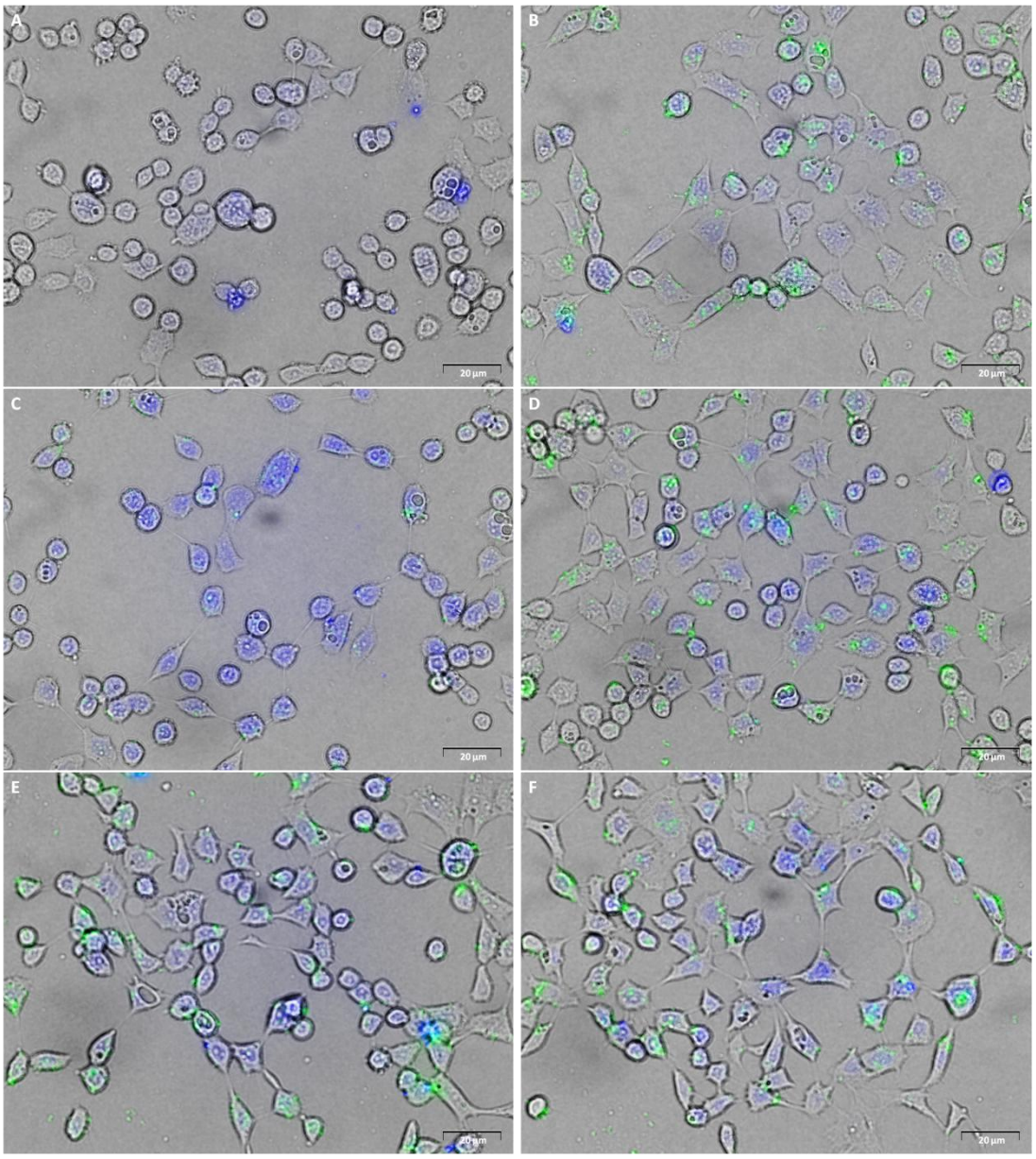


Figure 15 - Merged bright-field and fluorescence micrographs of internalized NBD-labeled liposomes and lipoplexes by MDA-MB-468 cells. Liposomes/lipoplexes were incubated with 293T cell line at charge ratio (+/-) 10 and final siRNA concentration of 25 nM for 6 h. Untreated cells (A), liposomes (B), siRNA lipoplexes (C), siRNA+pDNA lipoplexes (D), siRNA+PG1 lipoplexes (E) and siRNA+PG2 lipoplexes (F). Objective 40x was used. Scale bar represents 20 µm.

The green fluorescence observed by fluorescence microscopy represents the binding and/or internalization of NBD-labeled liposomes and lipoplexes by the cells, while blue fluorescence is the result of Hoechst binding to DNA, labeling the cell nucleus. Merged micrographs allow the visualization of internalization of liposomes and lipoplexes co-encapsulating siRNA with pDNA or PG. Although low internalization is seen for siRNA lipoplexes, this lipoplex formulation presents half the amount of lipid

than the other formulations, explaining the low internalization efficiency observed. In sum, by observing these micrographs, it becomes evident that systems interact with the cells, and are probably internalized. These micrographs suggest that a higher internalization efficiency has occurred when compared to the one reported by fluorescence measurements, reinforcing the need to optimize this method.

To better understand the cellular uptake of the systems, additional methodologies should be used, such as flow cytometry, which would allow for better quantification of cellular association, and confocal microscopy, a powerful imaging technique that would allow a better observation of lipid nanoparticles internalization.

4. Cytotoxicity assay

One of the most important parameters to evaluate the biomedical applicability of liposome formulations is the toxicity levels induced by these systems when interacting with cells. Therefore, the cytotoxicity induced by liposomes and each lipoplex formulation was evaluated in 293T and MDA-MB-468 cell lines. MTT assay was used to determine the cell's metabolic activity after 48 h incubation with lipoplexes. The reduction in cell metabolic activity can be used to understand the level of cytotoxicity induced by lipoplexes.

4.1 293T cell line

Cytotoxicity induced in 293T cell lines was evaluated using lipoplexes prepared at C.R. (+/-) 10, at two different siRNA concentrations, 25 and 50 nM. The cytotoxicity of siRNA, pDNA, PG1, PG2 and liposomes was also evaluated, for the same concentrations as the ones used for the preparation of the complexes. Since siRNA concentration is maintained, lipoplexes containing siRNA alone will present half the amount of lipid as the other lipoplex formulations. The results were presented as the mean values of three independent experiments.

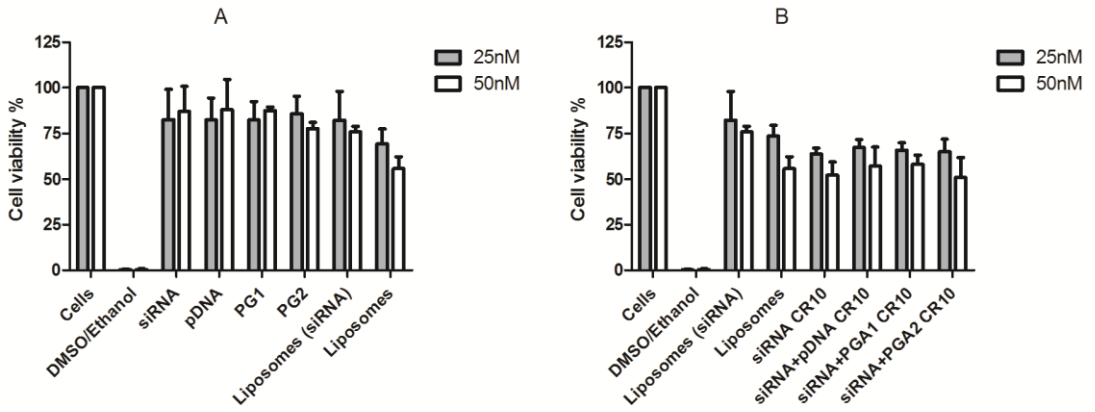


Figure 16 - Cell viability evaluation by MTT assay of individual lipoplex components (A) and different liposome and lipoplex formulations (B). Lipoplexes were prepared at charge ratio (+/-) 10 with 25 and 50 nM of siRNA, and incubated for 48 h with the 293T cell line. Liposomes (siRNA) and Liposomes were repeated in (B) to allow for better comparison between lipoplex systems and the liposomes used to form them.

The results of metabolic activity for the single compounds of the lipoplexes (Fig. 16 A) show that siRNA, pDNA, PG1, and PG2 alone promote a very little decay in cell viability for both 25 nM and 50 nM concentrations. Interestingly, the liposome concentration needed for the preparation of siRNA lipoplexes (Liposomes/siRNA), also did not promote a significant decrease in cell viability for neither concentration. However, the liposome concentration used for co-encapsulation of siRNA with pDNA or PG molecules, induced a significant decrease in metabolic activity for both concentrations. This was expected, since co-encapsulation requires twice the amount of lipid, which is the main responsible for the decrease in cell viability. When analyzing the percentage of metabolic activity induced by the lipoplex single components, significant statistical differences were observed among groups ($F(6,154)=4.307$, $P<0.05$), where liposomes used for co-encapsulation induced higher cytotoxicity, a tendency significantly different from all the other conditions (SNK, data not shown).

Fig. 16B shows the decrease in cell viability induced by each lipoplex and liposomal formulation for 25 and 50 nM. It is possible to observe that, all lipoplex formulation promote roughly the same cell viability (65 and 55 for 25 and 50 nM, respectively). siRNA+pDNA, siRNA+PG1 and siRNA+PG2 lipoplexes cytotoxicity levels resemble the ones obtained for liposomes, suggesting that most of the cell viability reduction is caused by liposome concentration, and that the addition of anionic cargo to lipoplexes does not influence cytotoxicity. Even though only half the amount of liposomes was used to encapsulate siRNA alone, the same level of cytotoxicity was

observed for this lipoplex formulation. Contrarily to what happens for the other lipoplexes, encapsulation of siRNA alone appears to promote liposome cytotoxicity. Statistical analysis allowed us to confirm that an increase in siRNA concentration caused a significantly decrease in 293T cellular metabolic activity ($F(1,154)=6.432$, $P<0.05$), and that no statistical differences were observed for *in vitro* cytotoxicity of the different lipoplexes (see Table 4). Also, no differences among replicates or different experiences were found in any case (MANOVA, data not shown).

Table 4 - Factorial ANOVA results on cellular metabolic activity of 293T cells after incubation with different lipid-siRNA based nanoparticles for 48 h.

Source of variation	**DF**	***MS***	***F***	***P***
cellular metabolic activity				
Composition	4	14870.482	68.435	<0.05
Concentration	1	5385.627	24.785	<0.05
composition*concentration	4	425.292	1.957	0.106
Error	110	5.832		

4.2 MDA-MB-468

MDA-MB-468 metabolic activity 48 h after administration of each lipoplex formulation at C.R. (+/-) 5 and 10, was measured for a final siRNA concentration of 25 nM. Lipoplexes were not prepared with 50 nM siRNA since it was expectable that these formulations would induce high levels of cytotoxicity. As in the previous experiment, siRNA concentration was maintained, therefore for the formation of siRNA lipoplexes half the amount of liposomes used in the other lipoplexes was required. The measurements presented were obtained in two independent experiments.

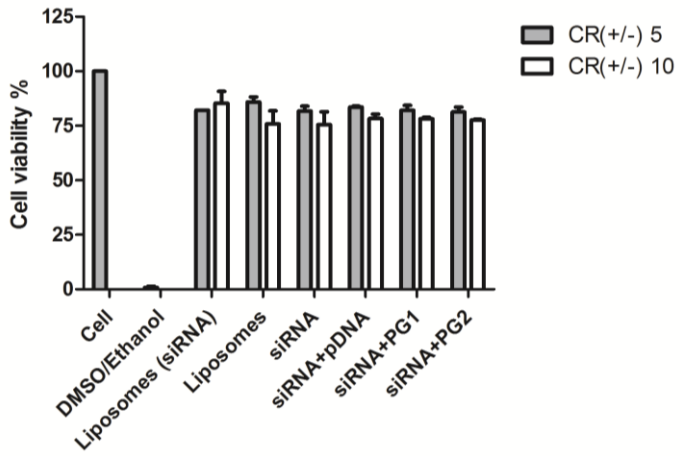


Figure 17 - **MDA-MB-468 cell viability, evaluated by MTT assay, after incubation with different liposome and lipoplex formulations for 48 h.** Lipoplexes were prepared at charge ratio (+/-) 5 and 10, with 25 nM final siRNA concentration.

No significant differences in cell viability can be observed between each of the lipoplexes and liposome formulations prepared at C.R. (+/-) 5 (≈ 82%). The fact that all lipoplexes cause roughly the same cytotoxicity as the liposomes used to prepare them (Fig. 17), reinforces the idea that liposomes are the major parameter affecting cell metabolism. When lipoplexes were prepared at C.R. (+/-) 10, the increase in liposome concentration lead to slightly lower values of cell viability for every condition, except for siRNA liposomes. Cells treated with liposomes at the same concentration used to encapsulate siRNA plus an anionic cargo present the same cell viability values as lipoplexes (≈ 76 %) prepared at C.R. (+/-) 10, once again suggesting that most of the reduction in cell viability is caused by the lipid present in the lipoplexes. In accordance to what was observed with the 293T cell line, even though liposome concentration used to form siRNA lipoplexes presents no significant reduction in cell viability, siRNA lipoplexes application results in significant cell metabolism decrease, suggesting that the structural differences promoted by the presence of siRNA alone, result in more cytotoxic systems. The nested ANOVA results on metabolic activity of MDA-MB-468 breast carcinoma line, exposed to the different lipoplex and liposome formulations, confirmed that a statistically significant increase in the cytotoxicity effect of siRNA lipoplex incubation ($F(6,75)=7.889$, $P<0.01$) occurred. Also, an increase in the charge ratio caused a significantly decrease in cellular metabolic activity of this line (one-way ANOVA, $F(2,85)=1846.800$, $P<0.01$).

Both cell lines were treated with lipoplexes prepared at C.R. (+/-) 10 for a final siRNA concentration of 25 nM, therefore, cytotoxicity levels induced by these conditions can be compared between cell lines. When comparing the metabolic activity between the cells exposed to the different lipoplexes and liposome formulations, significant statistical differences were observed among groups ($F(12,118)=83.527$, $P<0.01$). The metabolic activity percentages of 293T cells were found to be lower than those of MDA-MB-468 breast carcinoma cell line (Fig. 18). The highest metabolic activity value was obtained for cells exposed to siRNA liposomes, a tendency that for 293T cell line, but not for MDA-MB-468 breast carcinoma line, is significantly different from all the other conditions (SNK, data not shown).

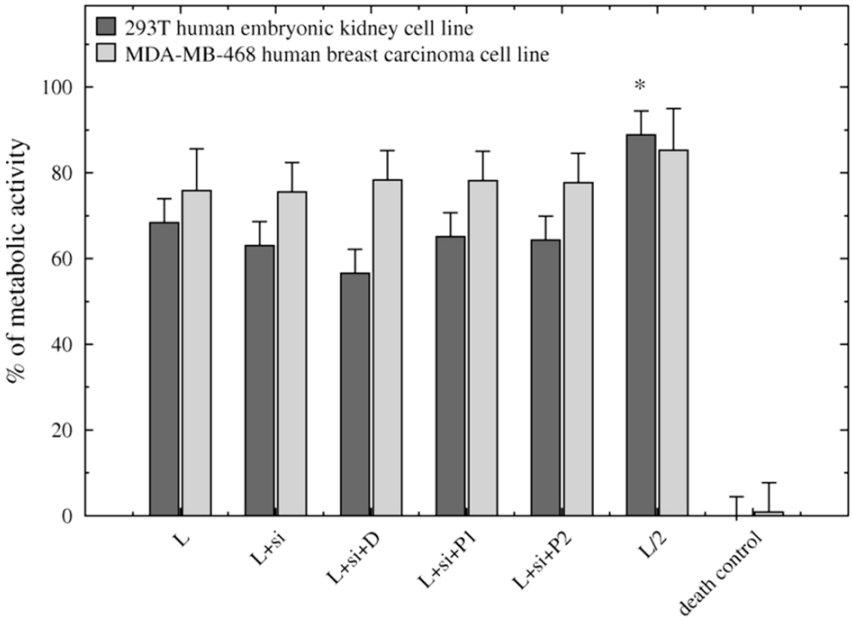


Figure 18 Metabolic activity (MTT assay) of 293T and MDA-MB-468 cell lines exposed to liposomes and lipoplexes for 48 h. Lipoplexes were prepared at charge ratio C.R. (+/-) 10 with 25 nM final siRNA concentration. (*) indicate significant differences among treatments ($P<0.05$, nested design ANOVA).

Taken together, these results show that after a 48 h incubation period with liposomes and lipoplexes, 293T cells presented lower metabolic activity than MDA-MB-468 cells for C.R. (+/-) 10, and 25 nM final siRNA concentration. For both cell lines the same tendency is maintained, with lipoplexes co-encapsulating siRNA with pDNA, PG1 and PG2 promoting the same levels of cytotoxicity as siRNA lipoplexes, even though twice the amount of lipid was used to prepare the first formulations. No significant differences were observed between siRNA+PG lipoplexes and siRNA+pDNA lipoplexes

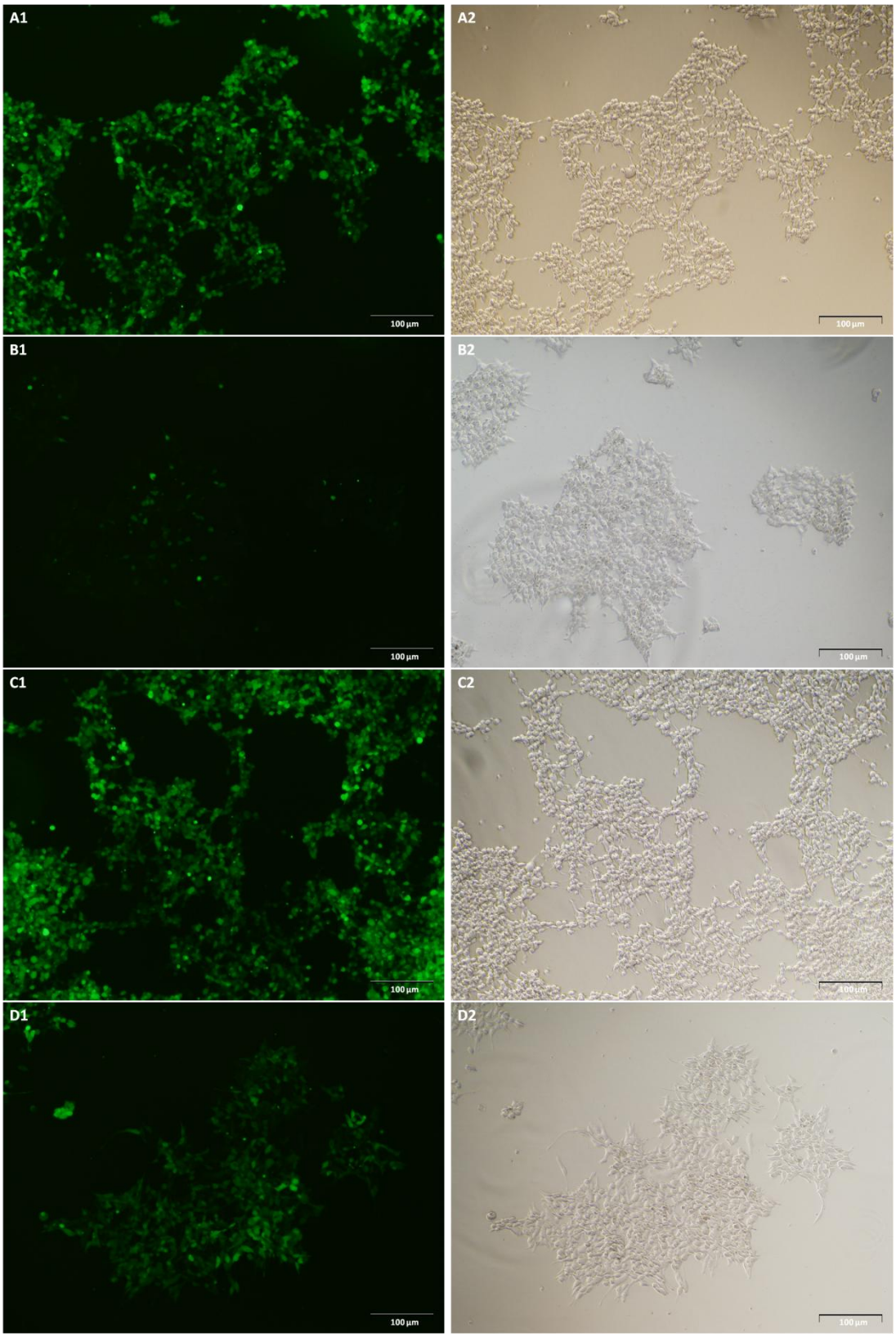
for these conditions. However, a previous study has reported that when the concentration is increased, siRNA lipoplexes with pDNA promote higher cytotoxicity when compared to siRNA lipoplexes with PG molecules, suggesting that in terms of cytotoxicity, addition of PG molecules to lipoplexes is preferable to the addition of pDNA [10], [69]. Also, the difference in PG molecular size does not seem to affect the cytotoxicity induced by the lipoplexes.

5. Silencing efficiency

Silencing efficiency of siRNA lipoplexes was evaluated for two different cell lines, 293T/GFP-puro and MDA-MB-468. A different method was used to study the silencing efficiency of lipoplexes in each cell line. 293T/GFP-puro EGFP gene knockdown was assessed by fluorescence microscopy while EGFR gene silencing was measured in MDA-MB 468 cell line by qPCR.

5.1 Fluorescence Microscopy

293T/GFP-puro cells were treated with the different lipoplex formulations at C.R. (+/-) 5 and 10, and two different siRNA concentrations, 25 and 50 nM. However, cell measurements for CR (+/-) 10 and 50 nM were not performed since significant cytotoxicity was observed by microscopy. Fig. 19 shows representative brightfield and fluorescence micrographs of cells treated with the different lipoplex formulations.


A1
A2
100 µm
B1
B2
100 µm
C1
C2
100 µm
D1
D2
100 µm

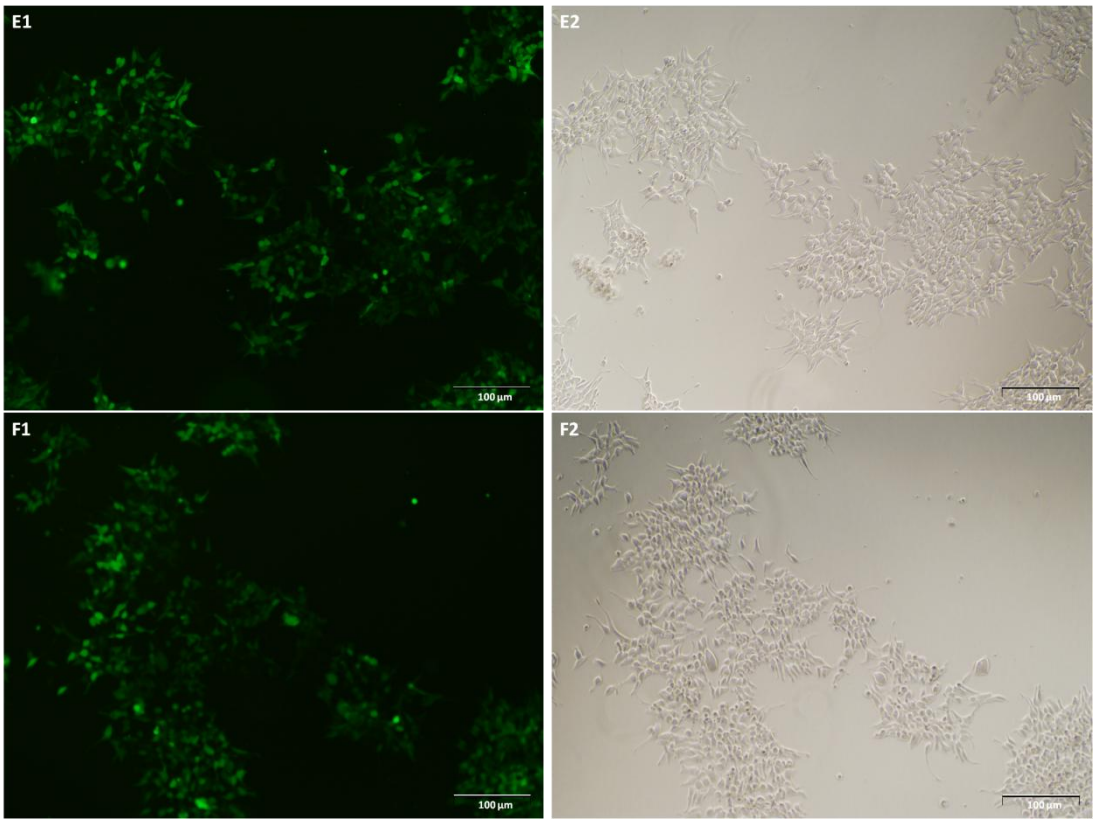


Figure 19 - Fluorescence (1) and bright-field (2) micrographs of 293T/GFP-puro cells. cells were treated with liposomes and lipoplexes at charge ratio (+/-) 10 and siRNA concentration of 25nM for 48 h. Untreated cells (A), lipofectamine (B), siRNA lipoplexes (C), siRNA+pDNA lipoplexes (D), siRNA+PG1 lipoplexes (E) and siRNA+PG2 lipoplexes (F). Objective 10x was used. Scale bar represents 100 µm.

As expected, when compared to the control cells (A), it is visible that lipofectamine treatment (B) results in accentuated decrease in fluorescence intensity, which is the result of the EGFP protein silencing by siEGFP. A smaller decrease can be observed for lipoplexes where siRNA is co-encapsulated with pDNA or PG (D, E and F), whereas for siRNA lipoplexes (C) no significant decrease in fluorescence intensity can be observed. Fluorescence intensity values were measured using Image J software, and the results were statistically analyzed (Fig. 20).

The nested design ANOVA results on EGFP silencing percentage of 293T/GFP-Puro cells showed a significant interaction among the selected factors. For lipoplex formulations, significant statistical differences were observed among groups. siRNA+pDNA, siRNA+PG1 and siRNA+PG2 lipoplexes induced a higher percentage of EGFP silencing than siRNA lipoplexes ($F(5,168)=13.815$, $P<0.05$). No significant differences were observed between C.R. (+/-) and siRNA concentrations.

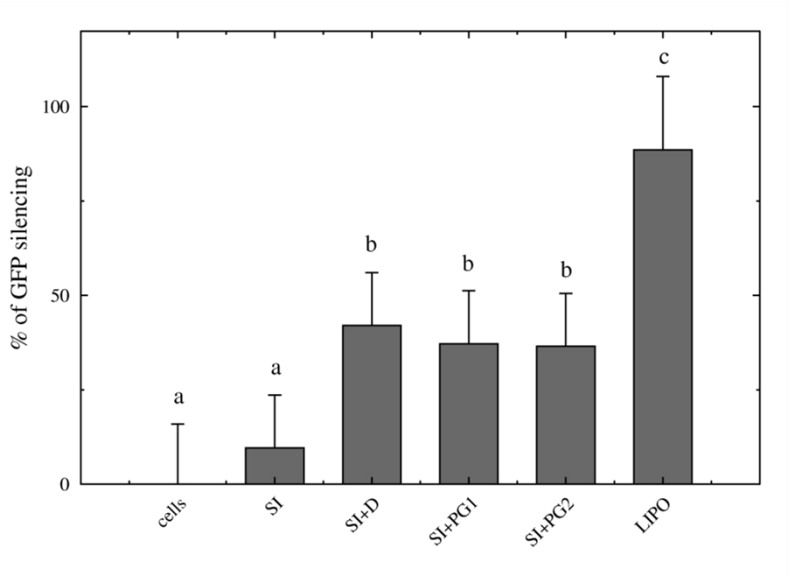


Figure 20 - EGFP silencing on 293T/GFP-Puro cell line, incubated with the different lipoplex formulations, for 48 h. Different letters indicate significant differences among treatments (P<0.05, nested design ANOVA). Untreated cells (cells), siRNA lipoplexes (SI), siRNA+pDNA lipoplexes (SI+D), siRNA+PG1 lipoplexes (SI+PG1), siRNA+PG2 (SI+PG2) and lipofectamine (LIPO).

The results on Fig. 20 show that siRNA lipoplexes did not promote a significant decrease in fluorescence intensity when compared to the non-treated cells. However, when pDNA, PG1 and PG2 were included in the lipoplex formulation, it was possible to observe a significant decrease in fluorescence intensity, even though the same amount of siRNA was used for each formulation. This suggests that less siRNA is needed to achieve EGFP silencing when pDNA or PG molecules are added to the formulation. One possible explanation is that the addition of the anionic cargo may lead to the formation of different lipoplex structures when compared to lipoplexes encapsulating siRNA alone. Addition of anionic cargo may result in less stable lipoplexes, which will facilitate endosomal escape and cargo release, resulting in higher siRNA delivery, and thus promoting higher levels of gene silencing for the same siRNA concentration. Also, the presence of pDNA or PG molecules could help the dissociation of the lipoplexes by interaction/competition with cell surface proteoglycans [16]. Since internalization efficiency studies showed that very little number of siRNA was successfully internalized by cells, it is also possible to observe that a small amount of siRNA is needed inside the cell to promote significant reduction in gene expression.

5.2 qPCR

Lipoplex ability to silence EGFR expression was tested in MDA-MB-468 cells. To avoid the significant toxicity observed in previous experiments with C.R. (+/-) 10, lipoplexes were prepared at C.R. (+/-) 5, at a final siRNA concentration of 50 nM per well. Two independent experiments were performed for the PCR assay.

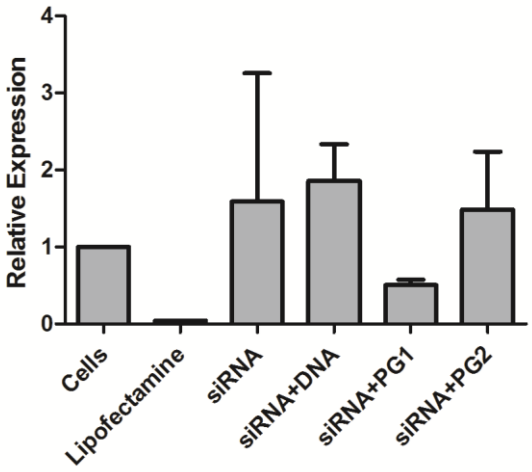


Figure 21 - EGFR silencing on MDA-MB-468 cell line as determined by qPCR, after treatment with the different lipoplexes prepared at charge ratio (+/-) 5 and final siRNA concentration of 50 nM for 24 h.

The results observed in Fig. 21 show that cells treated with lipofectamine presented the highest levels of EGFR gene silencing. siRNA+PG1 lipoplexes also promoted significant reduction in EGFR expression, while siRNA, siRNA+pDNA and siRNA+PG2 lipoplexes promoted overexpression of the gene. This overexpression might be associated with the formation of primer dimers (PD), leading to competition for PCR reagents, which may interfere with accurate gene expression quantification. Another hypothesis is that maybe GADPH was not the most suitable endogenous gene for this experiment. Studies have reported that GADPH is not the most adequate gene to quantify cellular normal expression due to its relatively large expression error [90]. This experiment was just a preliminary test, since only one time-point was analyzed. Besides, only two individual PCR experiments were performed without replicas, therefore, results with high standard deviation are unreliable. Additional experiments, with a different reference gene, should be performed in order to better evaluate the silencing efficiency of the systems. Also, protein expression studies such as western-blot assays would help to further understand the silencing efficiency of these systems.

Nevertheless, results for siRNA+PG1 lipoplexes show some level of EGFR gene silencing for both experiments performed, which can be a good indication of the silencing potential of these systems.

IV - Conclusion and future work

In this work, liposomes obtained by sonication, ethanol injection and extrusion were analyzed by DLS. We were able the see that different methods of liposome preparation result in liposomes with distinct characteristics. Liposomes prepared by sonication can be tuned to present suitable sizes for siRNA delivery, however, the liposome suspensions obtained were very unstable. As for ethanol injection and extrusion, even though both methods resulted in liposomes with approximately the same relatively small hydrodynamic diameter (≈ 160 nm), PDI values of liposomes obtained by extrusion were lower. From ELS measurements we have understood that extrusion promotes liposomes with more positively charged surface (≈ 65 mV) when compared to liposomes obtained by ethanol injection (≈ 58 mV). Even though extrusion is a more reproducible method, the time consumed in the process and the retention of lipid in the membranes are important disadvantages for these technique. These studies led us to conclude that ethanol injection was the more suitable preparation method for our purposes.

DLS measurements of siRNA, siRNA+pDNA, siRNA+PG1 and siRNA+PG2 lipoplexes, showed that the addition of pDNA, PG1 and PG2 promote the compaction of siRNA at lower C.R. (+/-). However, at C.R. (+/-) > 4, all lipoplex formulations present a similar mean diameter (≈120 nm) and ζ-potential (≈+50 mV), suggesting that the addition of an anionic cargo does not significantly affect the final siRNA complexation efficiency, with the different lipoplexes progressing towards structures with similar mean sizes and ζ-potential. In what encapsulation efficiency was concerned, lipoplexes prepared with PG1 or PG2 seem to promote a slight increase in siRNA encapsulation for lower C.R. (+/-). As discussed before, the same behavior was not observed for siRNA+pDNA lipoplexes possibly due to the unspecific binding of RiboGreen to pDNA molecules. Therefore, no distinctions can be made between the different anionic cargos, as for C.R. (+/-) > 5 all lipoplexes present high encapsulation efficiency (≈95 %). These results are in accordance with DLS measurements, a fact that further attests that the presence of the different anionic components did not had a significant outcome in the siRNA encapsulation/complexation process.

Concerning lipoplexes cellular uptake, fluorescence measurements have shown poor internalization for all systems in both 293T (≈2 %) and MDA-MD-468 (≈3 %) cell

lines. However, fluorescence microscopy analysis seems to suggest a more efficient cellular uptake, mainly in systems where siRNA was co-encapsulated with PG and pDNA.

Metabolic activity of 293T cells after administration of the lipoplex compounds individually showed that, for the tested concentrations (25 and 50 nM of siRNA), the only compound that significantly reduced metabolic activity were the liposomes used to co-encapsulate siRNA with either pDNA or PG. This reduction in metabolic activity is similar to the effects obtained with all lipoplex formulations. Interestingly, lipoplexes co-encapsulating siRNA and pDNA or PG presented the same cytotoxicity levels as siRNA lipoplexes, even though these systems were prepared with twice the amount of lipid. The same tendency was observed for the MDA-MB-468 cell line, reinforcing the idea that addition of anionic cargo does not enhance the cytotoxicity of the systems.

Finally, regarding gene silencing efficiency, the fluorescence microscopy assay has shown that no significant differences were observed between different lipoplex C.R. (+/-) or concentrations. More importantly, lipoplexes with pDNA, PG1 and PG2, clearly show an enhanced silencing efficiency of EGFP expression when compared to siRNA lipoplexes. The qPCR assay showed that siRNA lipoplexes prepared with PG1 might be a promising system to promote knockdown of EGFR expression. However, as discussed before, the results obtained are preliminary, since more experiments should be performed at different time points and using a more suitable reporter gene.

In sum, the different lipoplexes prepared in this work were highly efficient in the encapsulation of siRNA, did not promote a significant cytotoxicity to 293T and MDA-MB-468 cells, and showed promising results in gene silencing. Moreover, the co-encapsulation with the anionic compounds show some improvements on the siRNA/DODAB/MO base system.

Several studies can be developed in the future in order to better understand the influence of an anionic cargo in siRNA lipoplexes. Methods such as calorimetry or cryo-TEM should be performed in order to understand the lipoplexes structural differences. The stability in physiologic fluids, such as serum, and membrane fusion assays might also be valuable methods to infer if the additional anionic components can enhance the system's ability to deliver siRNA. As referred before, flow cytometry, and confocal microscopy would allow for better assessment of the systems

internalization in cells. Additional qPCR and western-blot assays should be performed at different time-points to conclusively report the gene silencing ability of the systems.

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