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Development of Dioctadecyldimethylammonium Bromide/Monoolein Liposomes for Gene Delivery

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Additional information is available at the end of the chapter

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1. Introduction

The electrostatic repulsion between the phosphodiester anionic charges of nucleic acids (NA) and the negatively-charged headgroups of cell membrane phospholipids hinders naked NA to permeate the plasma membrane [1, 2]. Additionally, nucleases present in the cells and in biological fluids enzymatically degrade NA, limiting their biofunctionality. Although many alternative methods have been developed to deliver NA to cells, factors such as versatility [3, 4], applicability [5, 6] and efficiency [7, 8] have discouraged their disseminated use in gene therapy.

Given these limitations, the intracellular delivery of genetic material can only be achieved through the use of physical, biological or chemical methods that promote gene insertion into cells. Physical methods have generally low *in vivo* applicability and include direct injection of NA into organs of live animals, micro projectile biolistics with a gene-propelling gun, cell sonication using an ultrasonic transducer and cell electroporation by exposure to an electric field. Biological methods rely on attenuated or inactivated versions of adenoviruses, lentiviruses and retroviruses, whose deactivated components can be used as gene vectors to obtain relatively high *in vivo* transfection efficiencies (transfection mediated by viral vectors) [9, 10]. Viral vectors pose nevertheless important safety, toxicity and immunogenicity issues, which greatly limit their use in humans. Chemical methods are based on the use of chemical adjuvants with relatively high levels of biocompatibility, such as synthetic polymers (polyfection) [11, 12] and cationic liposomes (lipofection) [13-15]. These molecules self-assemble in highly organized structures capable of complexing the genetic material and later releasing it inside the cells. Whereas viruses generally impose issues of mutagenicity and immunogenicity [16, 17], polymers such as polyethylenimine (PEI) are known to be highly cytotoxic [18, 19]. By exclusion of alternatives, cationic liposomes have emerged as

the carriers of excellence for intracellular delivery of nucleic acids due to their high versatility [20], reduced cytotoxicity [21], and high transfection efficiency [22].

Cationic liposomes are spherical vesicles composed of one or more cationic lipid or phospholipid bilayers [23, 24]. They include both cationic and neutral surfactants in their composition and may differ in size [25], lamellarity [26] or charge [27]. The cationic amphiphiles (which are mainly of synthetic nature) share two common features: the net cationic charge on the hydrophilic headgroup, and the hydrophobic tail that anchors the molecule to the liposome lipid bilayer [28]. The chemical structure of the cationic lipids varies markedly and each molecule can have a single (monovalent surfactant) or multiple cationic charges (multivalent surfactant) [29]. The neutral *helper* lipid also plays an important role in lipoplex fate by promoting the formation of inverted non-lamellar structures. These structures facilitate lipoplex fusion with the cell membrane and the subsequent release of the genetic material in the cytoplasm [30]. In addition, the presence of *helper* lipid reduces the amount of cationic lipid required for NA condensation, which reflects itself on a reduction of the toxic effects towards the cells, by decreasing the number of positively-charged headgroups in the lipoplex formulation [31, 32].

The driving force for lipoplex formation is the electrostatic interaction between the net positive charge of the cationic liposomes and the negatively charged DNA at an optimal ratio (+/-). This fact also enables the resulting complex to adsorb to the negatively charged cell surface [33-36]. After adsorption, cellular uptake of the complexed DNA facilitates intracellular DNA delivery and subsequent transgene expression [37]. In the case of DODAB/MO formulations, in which DODAB acts as a monovalent cationic surfactant and MO as *helper* lipid, the inclusion of MO leads to a dual-lipoplex phase diagram with lamellar structures prevalent at DODAB molar fractions above 0.5 and inverted bicontinuous cubic mesophases below 0.5 [38].

The high structural dependence of the system on MO content and temperature [39, 40] could reveal itself useful for optimizing lipoplex resistance against deleterious interactions with biological fluids and cell components, while remaining biocompatible and efficient as delivery agent. The presence of MO in these formulations also reduces the net positive charge necessary for successful NA complexation, thus reducing transfection-associated cytotoxicity [41]. In summary, a multidisciplinary approach to lipofection vectors will lead to the development of formulations with the most appropriate characteristics. Careful design of liposomal composition is essential for overcoming biological barriers, in order to achieve optimal transfection efficiency *in vitro* and *in vivo*.

2. Cationic lipid-mediated gene transfection

2.1. DODAB:MO liposomes

When assessing the potential of a new lipofection reagent, it is fundamental to study the physicochemical properties of the base liposomal formulation, to better adjust lipoplex

morphology (lipoplex size, charge ratio (+/-), fluidity and structure) for optimal transfection conditions. In this way, the behaviour of the lipofection reagent (resistance to extracellular components, cytotoxicity) *in vitro* and *in vivo* is more predictable [42-44].

MO was first proposed as *helper* lipid for non-viral transfection [45, 46] in a new liposomal formulation also including synthetic surfactant Dioctadecyldimethylammonium Bromide (DODAB) [41]. DODAB is a bilayer-forming cationic lipid that tends to form large unilamellar vesicles (LUV's) in excess water [47, 48]. It features a hydrophobic moiety consisting of a double acyl chain (C18:0) attached to a quaternary ammonium headgroup (one single positive charge per molecule) [49, 50]. DODAB's phase behaviour has been extensively studied [51, 52], thus its physicochemical characteristics can be easily controlled, making it straightforward to design DODAB-based formulations with specific molecular structures. DODAB's main limitation is the relatively high gel-to-liquid crystalline phase transition temperature ($T_M = 45^\circ\text{C}$) [53-56], superior to the human physiological temperature ($T_M = 37^\circ\text{C}$), meaning that DODAB's bilayers display a strong rigidity at normal body temperature which greatly limits its use as a delivery agent. This limitation can be counteracted by including a co-lipid with a lower T_M value, such as DOPE [57, 58], cholesterol [59, 60] or MO [40], which will lower the T_M of the lipid mixture.

The use of MO in liposomal formulations brings other advantages apart from the fluidization of DODAB's membranes. MO is a natural-occurring neutral surfactant that has the particularity of forming two inverted bicontinuous cubic phases (Q_{II}^D and Q_{II}^G) in excess water [61, 62]. It possesses a single unsaturated acyl chain (C18:1) attached to a glycerol headgroup [63]. Its tendency to form inverted bicontinuous cubic phases has been explored in the past for different applications such as protein crystallization [64, 65] or matrix for gel electrophoresis [66], and justifies the structural richness of the liposomal system formed with DODAB [40].

The aggregation behaviour of concentrated DODAB/MO mixtures has been studied through different techniques including phase scan imaging (Fig. 1) that reveals a two-region phase diagram consisting of either DODAB or MO enriched zones [40]. If $X_{\text{DODAB}} \geq 0.5$, bilayer-based structures dominate (Fig. 1A', 1B') and their size and fluidity depend on the molar composition of the mixture, with DODAB gel phase appearing as hydrated crystals [40]. When $X_{\text{DODAB}} < 0.5$, aggregates are dominated by densely packed cubic-oriented particles, visible as a cubic isotropic phase (Q) associated with high MO contents (Fig. 1D', 1E') [40].

This dual phase behaviour of DODAB/MO lipid mixtures confers a structural complexity to the system that extends itself to lipoplex organization, which can be fine-tuned to suit the biological application. Additionally, results show that MO has a similar effect on aggregate morphology than an increase in temperature, which can be modulated to produce formulations more suitable for gene transfection [39, 40].

2.2. Role of MO as helper lipid in pDNA/DODAB/MO lipoplexes

The incubation of nucleic acids with DODAB/MO mixtures or other cationic vesicle formulation leads to the formation of lipoplexes [67, 68]. The electrostatic interaction between opposite charges is the key factor that determines the adsorption of the cationic vesicles to the DNA molecules, a transient state that ends when a critical cationic vesicle concentration is reached. This leads to the disruption of the lipid vesicles which allows the formation of highly organized structures where the DNA molecules are tightly condensed between adjacent bilayers – the so-called lipoplexes [69-71]. The excess of cationic lipid is required for lipoplex binding to the cell surface but any subsequent addition of cationic lipid to the complex does not enhance DNA delivery and only increases toxicity in the exposed cells [72].

Lipoplexes such as the pDNA/DODAB/MO system can be directly visualized by techniques such as cryo-TEM imaging (Fig. 2), which also gives information on the structural properties of the system (size, compactation, organization) [38]. Cryo-TEM imaging reveals that pDNA/DODAB/MO lipoplexes present the same dual phase diagram as obtained for DODAB/MO lipid mixtures [38]. pDNA/DODAB/MO lipoplexes at $X_{\text{DODAB}} > 0.5$ (Fig. 2A) exhibit a multilamellar structure consisting of stacked alternating lipid bilayers and pDNA monolayers. The analysis using Fast Fourier Transforms (FFT) corroborates this observation, by denoting a mono-orientated organization pattern at repeating distances of about 5 nm (Fig. 2A'', 2A''' and 2A''') [38].

In contrast, pDNA/DODAB/MO lipoplexes at $X_{\text{DODAB}} \leq 0.5$ (Fig. 2B) show high-curvature zones where lipid bilayers intercross each other with pDNA monolayers stacked between them. These high-curvature zones have been interpreted as MO-rich domains that alternate with DODAB-rich domains presenting multilamellar organization. The FFT diagrams show that these MO-rich domains possess a distinct structural organization with bi-orientated patterns in angles of 90° between them, consistent with the existence of cubic inverted bicontinuous mesophases (Fig. 2B'', 2B''' and 2B''') [38].

The DODAB/MO aggregate organization influences the final structural properties of the resulting pDNA/DODAB/MO lipoplexes, with MO content having a dramatic effect on how DNA is condensed and protected within the membrane.

By definition, a *helper* lipid (also termed as co-lipid or simply adjuvant) is any neutral surfactant not directly contributing to NA condensation or to targeting of the cell membrane by the lipoplex. *Helper* lipids enhance transfection efficiency by forming non-lamellar structures that intervene in several steps of the transfection process [73, 74]. These non-lamellar structures influence transfection efficiency in at least two ways: i) lipoplex-cell membrane fusion promoted by the fusogenic character of the *helper*; ii) improved endosomal escape of NA due to the disruption of the endosomal membrane by these structures prior to endosome/lysosome fusion, which would lead to NA degradation [75].

Dioleoylphosphatidylethanolamine (DOPE) is the most established *helper* used in non-viral vectors and is known to enhance transfection mediated by different cationic liposomal formulations [76-79]. DOPE stimulates the formation of inverted hexagonal structures (H_{II} –

Fig 3A) which represent a major structural variation from the classic multilamellar sandwich model of lipoplex organization (L_{α}^C – Fig. 3B) [80]. However, its application in gene therapy has been strongly limited because of the strong cytotoxicity associated with it [81, 82].

Figure 2. Cryo-TEM imaging of pDNA/DODAB:MO lipoplexes at C.R. (+/-) 4.0 (1mM total lipid). Panels A and B represent two different DODAB:MO molar fractions (2:1 and 1:1, respectively) from which have been selected two distinct zones A' and B'. The corresponding FFT diagrams A'' and B'' are shown after the appliance of "Mask" tool in the Digital Micrograph™ (GATAN) software (A''' and B'''). The inverse FFT diagrams of the previous images allow the emergence of distinct structural patterns: mono-orientated organization consistent with the existence of lamellar structures for high DODAB contents (A''', 2:1) and 90° bi-orientated organization associated with inverted bicontinuous cubic mesophases for high MO contents (B''', 1:1). Magnification: 50 000x. Adapted from [38].

This evidence has motivated the search for new *helpers* with higher levels of biocompatibility while maintaining the same efficiency as DOPE. Cholesterol is one of such molecules due to its ability to modify bilayer fluidity [83]. Inclusion of cholesterol results in the formation of complexes that are more stable but less efficient *in vitro* compared to DOPE-containing lipoplexes. In contrast, addition of cholesterol results in more efficient complexes for *in vivo* application [84, 85].

MO is another promising alternative to common *helper* lipids, as it seems to combine positive aspects of both DOPE and cholesterol: tendency to promote inverted non-lamellar structures similarly to DOPE (although different from the common inverted hexagonal structures – Fig. 3C) and the fluidizing effect of cholesterol, which increases the fusogenicity of the lipoplexes.

Figure 3. Different types of pDNA/cationic lipid structural organizations: A - inverted non-lamellar hexagonal structure characteristic of cationic vesicles containing DOPE at $X_{\text{DOPE}} \geq 0.5$; B - lamellar structural characteristic of cationic vesicles containing $X_{\text{Helper}} \leq 0.5$; and C - inverted bicontinuous cubic structure characteristic of cationic vesicles containing MO at $X_{\text{MO}} \geq 0.5$. Double-tailed surfactant with grey-headgroup represents cationic lipid, double-tailed surfactant with white-headgroup represents DOPE and single-tailed surfactant with white-headgroup represents MO. Grey-coloured regions represent cationic lipid rich-domains and white-coloured regions represent MO or DOPE rich-domains.

The fluidizing effect of MO contributes favourably to the complexation efficiency of DNA, quickening lipoplex formation [41]. At the same time, the formed inverted bicontinuous cubic mesophases improve the resistance of aggregates to extracellular component

destabilization, thereby potentially enhancing transfection efficiency [38]. MO-based aggregates induce a relatively low cytotoxicity level, which further reinforces its use as a new *helper* lipid in this type of non-viral systems. *In vivo* evaluation of MO-based lipoplexes shall confirm the potential for this neutral surfactant to replace classic *helpers* in lipofection formulations, although some promising results have already been obtained for other cationic lipid formulations that also form inverted bicontinuous cubic structures [86-88]. Ethylphosphatidylcholines are a family of positively charged membrane lipid derivatives that promote the formation of Q_{II}^G and Q_{II}^P structures, having been linked to high levels of transfection efficiency with low cytotoxicity in several animal cell lines [89, 90], consubstantiating MO's potential role in gene delivery.

2.3. Recent progress in gene delivery with cationic lipids

The quest for the perfect cationic liposome formulation has been based on empirical testing of novel surfactant molecules that had never been previously used for NA delivery [91-93]. The only goals for candidate molecules are the attainment of high transfection efficiency with low cytotoxicity [94, 95].

After the first generation of cationic lipids based on double-chain surfactants with plain ammonium headgroups (DODAB, DOTAP, DOTMA or DMRIE) [96, 97], soon came cationic lipids with poly-ammonium and multivalent functional radicals (DOGS, DOSPA). The latter exhibited higher transfection efficiencies but also higher cytotoxicity due to the immunogenicity of the cationic ammonium headgroups [98, 99]. This negative effect was balanced with the appearance of *helpers* (DOPE) [74] and natural lipid-derivatives such as cholesterol [100] or glycerol [101], although sometimes compromising transfection efficiency. Gemini-dimeric surfactants also presented promising potential but with significant toxicological consequences [102-106].

Polyethylene glycol (PEG)-based lipids emerged as interesting hydrophilic polymer-based surfactants that could provide steric stability to cationic liposomes, increasing lipoplex lifetime in the bloodstream and also decreasing the toxic effects observed *in vitro* and *in vivo* [107-109]. The polymeric counterpart of the PEG-based surfactants (variable both in chain length and branching) forms a protective surface coating that inhibits the adhesion of plasma components which could promote NA release and particle aggregation [109]. This protective effect is enhanced by including up to 5-10% of PEG in the liposomal formulation, with no visible effects on lipoplex structure [110]. PEG addition reduces net electric charge and increases hydration of the liposome surface, decreasing immunogenicity and cytotoxicity elicited by the particles. Nevertheless, at high concentrations, these polymers are known to be toxic and of difficult clearance from the organism. Therefore, when developing PEGylated particles, one must weigh advantages and disadvantages of including PEG, especially when aiming for long-term therapeutic administration [111].

Inclusion of pH-sensitive molecules in the formulations has been shown to improve transgene expression by favouring DNA release from the endosomal compartment. Examples of pH-sensitive molecules used in non-viral gene delivery include polyhistidine,

dioleoyldimethylammonium propane (DODAP) or cholesteryl hemisuccinate (CHEMS) [112, 113].

Another major breakthrough with impact in gene therapy was the possibility of specific cell targeting by liposomes. Amphiphiles with hydrophilic headgroups could be chemically linked to molecules such as folate, transferrin or the epidermal growth factor that potentiate specific delivery to cancer cells, markedly increasing the therapeutic benefits achieved with lipoplexes, with little secondary effects [114-118].

More recently, cationic lipids with amino acid headgroup (serine, alanine) [119, 120] and sugar-based cationic lipids (D-galactose) have appeared as promising families of cationic surfactants [121, 122]. Small molecular weight peptides (glutamate, cysteine) augment the hydrophilicity of the lipoplex surface, as with small surface sugars (galactose, mannose) that additionally allow targetability of the lipoplexes.

3. Lipoplex interaction with extracellular milieu

3.1. Resistance to components of biological fluids

An effective delivery system must confer stability to complexed NA in physiological conditions [123, 124]. Systemic delivery of NA requires a stealth carrier that protects NA from indiscriminate interaction with complement and coagulation pathways that lead to rapid removal from blood circulation of the lipoplexes by opsonization [125-127]. pDNA/DODAB/MO lipoplexes were therefore tested regarding their sensitivity when simulating their interaction with the body (temperature, salt, exposure to serum, nucleases and membrane lipases), to be validated for systemic applications [128].

Fig. 4 shows the variation of free pDNA fraction after incubation of pDNA/DODAB:MO lipoplexes (2:1, 1:1 and 1:2) with different constituents of the plasma. Increasing the temperature from 25°C to physiological temperature (37°C) leads to a reduced but visible release of pDNA from the lipoplex, more evident for lower MO contents. The gel phase of DODAB ($X_{\text{DODAB}} > 0.5$) is clearly more disturbed by incubation at higher temperature than the liquid-crystalline phase of DODAB/MO lipid mixtures ($X_{\text{DODAB}} \leq 0.5$). This tendency is maintained upon NaCl addition at physiological concentration (150mM), showing the protective role of MO upon the electrostatic imbalance provoked by salt addition.

DODAB/MO formulations with varying MO content behave very differently when exposed to serum (Fig. 4). Serum may strongly interfere with lipoplexes, both *in vitro* and *in vivo*, causing lipoplex-protein aggregation that lead to degradation of the genetic material and possibly clogging the blood vessels in intravenous application [129]. pDNA/DODAB/MO lipoplexes release up to 30% of the initially complexed pDNA when incubated for 30min with bovine serum albumin, the major constituent of bovine serum, particularly in the case of formulations with low MO content. MO contributes, in fact, to a better resistance of pDNA/DODAB/MO to extracellular components, eventually related to the inverted bicontinuous cubic structures present that reduce the exposure of DNA molecules to the

plasma constituents. In fact, the results suggest a direct correlation between lipoplex stability and MO content.

Some authors have managed to transiently overcome this inhibitory effect of serum on lipofection by increasing the charge ratio (+/-) of cationic liposome to DNA [130, 131]. Significantly enhanced gene transfer has also been achieved by pre-incubating the delivery system with serum proteins prior to NA complexation [132, 133].

Figure 4. Resistance of pDNA/DODAB:Monoolein lipoplexes to components of biological fluids. Variation on the percentage of free pDNA upon incubation with DODAB:Monoolein liposomes (2:1, 1:1, and 1:2) at CR (+/-) 2.0, and subsequently exposed to a temperature increase from 25°C to 37°C in the presence of NaCl salt (150mM) and BSA (0.5g/L) at incubation times of 30 min. The values were calculated through spectral decomposition of ethidium bromide steady-state fluorescence, as described elsewhere [38].

3.2. Lipoplex adhesion to the cell surface

The adsorption and uptake of lipoplexes may be affected by the presence of proteoglycans at the plasma cell membrane surface. It is therefore important to study how lipoplexes interact with these extracellular matrix components during cell transfection. Association of lipoplexes with negative polyelectrolytes free in solution might also be useful to evaluate eventual loss of pDNA at the cell surface [134].

Proteoglycans (membrane receptors consisting of a protein core and one or more anionic glycosaminoglycan chains including heparin, dermatan and chondroitin sulphates) were identified as the mediating agents for cationic liposome/DNA cellular uptake both *in vitro* and *in vivo* [135]. Lipoplex/proteoglycan interaction is suggested to depend upon three major aspects: the ionic strength, the effect of *helper* lipids and of the glycosaminoglycan structure [134, 135].

When the lipoplexes interact with heparin and heparin sulphate, the negative charge of the polyelectrolytes determines NA release from the lipoplex through the same type of cooperative process that is responsible for lipoplex formation [136-138].

On subjecting pDNA/DODAB:MO (2:1 and 1:1) lipoplexes to increasing amounts of heparin (HEP), the improved resistance and stability of the lipoplexes obtained with increasing amounts of MO could be confirmed (Fig. 5). The fact that the system with higher MO content ($X_{\text{DODAB}} = 0.5$) shows enhanced resistance to heparin relatively to pDNA/DODAB:MO (2:1) lipoplexes suggests that pDNA dissociation is mainly dependent on structural properties (Fig. 2) rather than physicochemical properties of the lipoplexes.

4. Modulation of cell behaviour by lipoplexes

4.1. Cytotoxicity

In addition to the efficiency of MO based lipoplexes, patient tolerability is determinant for therapeutic application of these systems. *In vitro* toxicity tests are a useful, time and cost-effective first approach in the validation process of a therapeutic agent. The adverse effects of liposomes on cells can be identified through different assays that look at particular aspects of cell behavior, such as metabolism, proliferation or cell membrane integrity. To determine if liposomal formulations will be well tolerated by all cells it will contact with, it is important to test cytotoxicity using different cell types. The cell lines should be selected: i) to evaluate how target cells will react; ii) to predict eventual toxicity for the heart and liver, by using cardiomyocytes and hepatocytes, respectively; iii) to screen if the liposomes can be applied to all types of cells. In the case of DODAB:MO liposomes, four different mammalian cell lines (HEK 293, BJ5ta, L929 and C2C12) were exposed for two days to increasing concentrations of these systems, after which different analytical methods were applied (Figs. 6 to 8).

The cell lines presented here are routinely used for toxicity studies and are commercially available. The human Embryonic Kidney (HEK) 293 cell line was originally derived from

human embryonic kidney cells grown in tissue culture, from which 293T cell line is derived. BJ5ta cells are normal human foreskin fibroblasts immortalized with telomerase. Murine cell lines L929 and C2C12 are fibroblasts and myoblasts, respectively.

Figure 5. Resistance of pDNA/DODAB:Monoolein lipoplexes to model proteoglycans. Variation on the percentage of free pDNA upon incubation of pDNA/DODAB:Monoolein lipoplexes (2:1, 1:1, and 1:2) at CRs (+/-) 2.0/4.0 with increasing amounts of heparin (HEP) at incubation times of 30 min. The values were calculated through spectral decomposition of ethidium bromide steady-state fluorescence, as described elsewhere [38]. Adapted from [38].

Another aspect to be taken into account is the possibility that the liposomes and lipoplexes may differently affect parameters such as metabolism, cell membrane structure and chemistry, cell proliferation and mobility. For a comprehensive study, a minimum of three different methodologies, monitoring at least two of these parameters, should be used. From our own results, it was observable that the metabolism of L929 and C2C12 cells was more pronouncedly affected by the contact with DODAB:MO liposomes compared to the other

cell lines, especially with a lipid concentration ≥ 20 $\mu\text{g/ml}$ (Fig. 6). At these higher concentrations, DODAB:MO (1:1) induced lower levels of cytotoxicity in all the cell types, which probably reflects the higher content of MO and concomitant lower content of cationic lipid. Interestingly, the cell membrane integrity assay did not reveal such obvious, concentration-dependent variations in cytotoxicity (Fig. 7). The results obtained with the proliferation test (Fig. 8) were quite concordant with those from the metabolic assay (Fig. 6), indicating again the L929 and C2C12 cells as more sensitive, while BJ5ta proliferation was clearly increased when incubated with up to 20 $\mu\text{g/ml}$ lipid (Fig. 8).

Lipoplexes prepared from these liposomal formulations, at concentrations typically used in transfection experiments, constantly leading to slightly lower viability rates compared to the base DODAB:MO liposomes (data not shown).

The fact that MO-based aggregates cause reduced levels of cytotoxicity for concentrations typically used on transfection assays, reinforcing the use of MO as a new *helper* lipid in this type of non-viral systems. Even if there is general agreement in the reduced toxicity of liposomes as non-viral vectors, these results emphasize the need for accurate liposome/lipoplex evaluation to better assess human risk prior to using them as lipofection vectors.

Figure 6. Evaluation of the cytotoxicity (metabolic assay) in four different mammalian cell lines (BJ5-ta, L929, 293 and C2C12) induced by varying concentrations of DODAB:MO-based liposomes after 48 h of incubation. C_DMSO: cells incubated with 30 % DMSO; C_Cells: cells alone. The mean (+/-) SD was obtained from two independent experiments. MTT assay can be used to estimate cell viability, specifically as marker of the cell metabolic capacity. The soluble tetrazolium MTT is reduced by metabolically active cells, thus the developed purple color proportional to the number of viable cells.

Figure 7. Evaluation of the cytotoxicity (cell membrane integrity) in four different mammalian cell lines (BJ5-ta, L929, 293 and C2C12) induced by varying concentrations of DODAB:MO-based liposomes after 48 h of incubation. C_DMSO: cells incubated with 30 % DMSO; C_Cells: cells alone. The mean (+/-) SD was obtained from two independent experiments. The LDH assay is used to estimate cell viability, as the intracellular enzyme LDH is released into the extracellular medium when cell membranes are damaged.

Figure 8. Evaluation of the cytotoxicity (proliferation) in four different mammalian cell lines (BJ5-ta, L929, 293 and C2C12) of varying concentrations of DODAB:MO-based liposomes after 48 h of incubation. C_DMSO: cells incubated with 30 % DMSO; C_Cells: cells alone. The mean (+/-) SD was obtained from two independent experiments. Sulforhodamine B (SRB) is considered a proliferation assay, used for cell density determination, based on the determination of the cellular protein content.

4.2. Cellular uptake and intracellular trafficking

In spite of extensive efforts to unravel the *in vitro/in vivo* mechanisms of internalization of lipoplexes, doubts remain as to whether the topology of lipoplexes facilitates the entry of DNA by fusion with the plasma membrane or with endosomal vesicles. Other studies have indicated that endocytosis is possibly the preferred mechanism of lipoplex internalization by cells [139, 140]. After the formation of the endocytic vesicle containing the lipoplexes, the internal pH of the endosomes decreases to about 5.5 [141]. The endosomes then fuse with the lysosomes, in which the condensed NA component may be hydrolysed by lysosomal enzymes [142, 143]. Endosomal release of the NA should occur, avoiding the lysosomal lytic pathway, leading to successful transfection.

Different mechanisms for complex internalization have been proposed, in particular for lipoplexes and polyplexes. Endocytosis at the plasma membrane may be clathrin-dependent or -independent. Clathrin-independent mechanisms include fusion of lipoplexes with the plasma membrane, phagocytosis, macropinocytosis and caveolae-mediated uptake [144]. *In vitro* cell culture systems provide the opportunity to experimentally address how lipoplexes interact with the plasma membrane. Although it is widely accepted that endocytosis is the most important route for lipoplex entry, different endocytic pathways may be used in parallel. The most likely explanation is that different cell types prefer a particular mechanism but use more than one. Therefore, optimization remains largely dependent of trial and error.

Intracellular trafficking of lipoplexes can be followed by co-localization studies of labeled particle components and dyes, or antibodies that recognize cell organelles or molecules playing a role in the process (e.g. clathrin coating endocytic pits in the plasma membrane) [139, 145] (Fig. 9). Cell lines harboring mutations in some of these molecules may also be used to evaluate their importance for the internalization process of specific formulations. The use of inhibitors of endocytosis has also been widely used but has two major limitations: the significant toxicity induced by the inhibitors themselves and the evidence corroborating that internalization can be simultaneously mediated by different pathways.

The endosomal escape is thought to be the major limitation for efficient gene transfection [146]. A number of strategies have been explored to enhance NA endosomal release. For example, the incorporation of a non-lamellar forming lipid such as DOPE that disrupts the endosome membrane or inclusion of a pH-dependent molecule that senses the acidification in the endosome compartment leading to disruption of its membrane [147].

Modulation of the endosomal escape during lipoplex intracellular trafficking was replicated by exposing pDNA/DODAB:MO (2:1, 1:1 and 1:2) lipoplexes to acidic conditions in the presence of increasing amounts of hydrochloric acid (pH ranging from 7.5 to 2.5) (Fig. 10). The percentage of released DNA steadily increased upon milieu acidification from pH 7.4 to 4.5, which is the pH range typical in the endosome. This trend correlates negatively with the MO content in the formulation, suggesting that MO's inverted bicontinuous cubic structures may protect more efficiently the lipoplex structure in this environment. More stringent acidification of the environment (pH 4.5 to pH 2.5) inverts the release tendency, which can be related to degradation of naked pDNA in solution.

Figure 9. Visualization of cellular uptake of DODAB:MO lipoplexes by HEK 293T cells. Liposomes are labelled with Bodipy-PE (green) and endo-lisosomes with dextran (red). Co-localization (yellow) indicates sites of active endocytosis of lipoplexes. DODAB:MO (2:1) (+/-) 4.0, 1 μ g pDNA/well, amplification 200x.

Lipoplex charge ratio (+/-) also affects the intensity of pDNA release. Using the same DODAB:MO base formulation, increasing charge ratio (+/-) seems to prevent pDNA release from the lipoplex. This effect was already visible in the destabilization of pDNA/DODAB/MO lipoplexes by plasma constituents such as serum and salt, and probably reflects more efficient pDNA condensation in presence of excess cationic lipid.

Increasing ammonium/phosphate ratio carries the risk of increased cytotoxicity. One possible solution may be using increasing amounts of MO in lipoplex formulation for better protection of pDNA integrity without imposing major toxic effects to the target cell.

Non-viral vectors, although less toxic than viral vectors, may still elicit a strong, nonspecific immune response. Toxicity frequently results from characteristics of the encapsulating polymer or lipid such as the length, saturation, or branching of the polymer. Efforts to reduce the toxicity of nonviral vectors have largely resulted in attempts to make the vectors more biodegradable and biocompatible. Many of the aforementioned systems (i.e. triggered release with disulfides, PEG copolymers) incorporated more biologically active components, thereby reducing the elicited immune response. For example, the incorporation in liposomes of molecules known to suppress the production of the cytokine tumor necrosis factor (TNF- α), as compared to lipoplex alone, succeeded in maintain its levels low while achieving comparable levels of transgene expression [148]. Another method explored by Tan [149] significantly reduced toxicity through the sequential injection of liposome and later of DNA, as opposed to using formed lipoplexes. With this approach, cytokine levels (IL-12, TNF- α) were reduced by greater than 80% compared to lipoplex delivery [149]. Thus, significant advances have been made towards decreasing the toxicity of these non-viral vectors.

Interestingly, DODAB:MO based liposomes and lipoplexes were found to induce production of low levels of TNF- α by macrophages, comparable or lower than DOTMA/DOPE and DOTMA/cholesterol lipoplexes (data not shown) [150].

Figure 10. Resistance of pDNA/DODAB:Monoolein lipoplexes to pH decrease (modulation of endosomal escape). Variation on the percentage of free pDNA upon incubation of pDNA/DODAB:Monoolein lipoplexes (2:1, 1:1, and 1:2) at CRs (+/-) 2.0/4.0 with increasing amounts of hydrochloric acid at incubation times of 30 min. The values were calculated through spectral decomposition of ethidium bromide steady-state fluorescence, as described elsewhere [38].

4.3. Transfection efficiency

Transfection efficiency of plasmid DNA can be directly evaluated by detecting the protein encoded by the reporter gene. Examples of reporter genes are: green fluorescent protein (GFP) and similar, detectable by techniques as microscopy or flow cytometry; β -galactosidase, whose activity can be evaluated by a colorimetric assay; luciferase, whose

activity can be measured with a luminometer, after a substrate is converted into a luminescent form by luciferase. In Figure 11 is depicted an experiment that allows to identify the effect on transfection efficiency of varying the content of MO in the liposomal formulations, lipid:DNA charge ratio in the lipoplexes and also the quantity of pDNA added to the cells, as pDNA dosage is known to affect transfection efficiency. It can be observed that the incorporation of MO in the liposomes resulted in a transfection efficiency improvement when compared to the cationic lipid DODAB alone. When using 1 μg DNA/well, the transfection levels of pDNA/DODAB:MO systems are of the same order of magnitude as Lipofectamine™ LTX. For a lower MO content (pDNA/DODAB:MO (2:1) formulation), a dose effect response (0.5 μg and 1 μg of pDNA) was observed. For higher MO content (pDNA/DODAB:MO (1:1) formulation), the transfection efficiencies remained constant at both CRs. This result strengthens the role of MO as *helper* lipid in the transfection agent.

Figure 11. Transfection efficiency of HEK 293T cells by MO-based lipoplexes. Transfected pDNA encoded the β -galactosidase gene whose activity was evaluated by a colorimetric assay after 48 h of incubation. Lipoplexes prepared at charge ratio (+/-) 4.0 or 2.0, 0.5 or 1.0 μg pDNA/well. Controls: cells incubated with free pDNA; cells transfected using Lipofectamine® as lipofection agent. The mean (+/-) SD was obtained from three independent experiments. Adapted from [38].

5. Conclusions

The identification of the most important formulation parameters and how they influence macromolecule delivery and bioactivity will give direction towards the development of novel therapeutic solutions. The morphology and structure of the lipoplex is influenced by the surrounding environment and the chemical nature of its constituents. Physicochemical properties of the systems define the course of most events when lipoplex interact with the body, tissues and cells. The effectiveness of vector internalization, its intracellular trafficking and successful transgene expression in target cells, is directly dependent on the *helper* lipid features, net charge of the lipoplex and the degree of NA compactation within the complex. Different target cells may impose specific challenges to transfection and many inherent factors are unknown. The advent of controlled cell targeting for improved specificity holds great promise for application of these formulations in nanomedicine.

A good lipofection system must protect NA from deleterious interaction with biological fluids and cell components, while remaining biocompatible and efficient as delivery agent. In summary, with this work we intend to demonstrate that MO can be used safely and efficiently as *helper* lipid in the preparation of non-viral vectors for transfection. The presence of this natural lipid in the formulations reduces the net positive charge necessary for successful NA complexation, thus decreasing transfection associated cytotoxicity.

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6. References

- [1] Patil, S.D., D.G. Rhodes, and D.J. Burgess, *DNA-Based Therapeutics and DNA Delivery Systems - A Comprehensive Review*. American Association of Pharmaceutical Scientists Journal, 2005. 7: p. E61-E77.

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- [2] Levin, Y. and J.J. Arenzon, *Kinetics of Charge Inversion*. Journal of Physics A: Mathematical and General, 2003. 36: p. 5857-5863.
- [3] Pitt, W.G., G.A. Hussein, and B.J. Staples, *Ultrasonic Drug Delivery - A General Review*. Expert Opinion on Drug Delivery, 2004. 1(37-56).
- [4] Miller, D.L. and C. Dou, *Induction of Apoptosis in Sonoporation and Ultrasonic Gene Transfer*. Ultrasound in Medicine and Biology 2009. 35: p. 144-154.
- [5] Rebersek, M. and D. Miklavcic, *Advantages and Disadvantages of Different Concepts of Electroporation Pulse Generation*. Automatika, 2011. 52: p. 12-19.
- [6] Gehl, J., *Electroporation Theory and Methods, Perspectives for Drug Delivery, Gene Therapy and Research*. Acta Physiologica Scandinavica, 2003. 177: p. 437-447.
- [7] André, F.M., et al., *Variability of Naked DNA Expression After Direct Local Injection - The Influence of the Injection Speed*. Gene Therapy, 2006. 13: p. 1619-1627.
- [8] Herweijer, H. and J.A. Wolff, *Progress and Prospects - Naked DNA Gene Transfer and Therapy*. Gene Therapy, 2003. 10: p. 453-458.
- [9] Machida, C.A., *Viral Vectors for Gene Therapy - Methods and Protocols*. 1st ed, ed. H. Press. 2003, New Jersey (U.S.A). 606.
- [10] Heiser, W.C., *Gene Delivery to Mammalian Cells - Viral Gene Transfer Techniques*. 1st ed, ed. H. Press. 2004, New Jersey (U.S.A). 584.
- [11] Arima, H., *Polyfection and Nonviral Gene Transfer Method - Design of Novel Nonviral Vector using Cyclodextrin*. Journal of the Pharmaceutical Society of Japan, 2004. 124: p. 451-464.
- [12] Eliyahu, H., Y. Barenholz, and A.J. Domb, *Polymers for DNA Delivery*. Molecules, 2005. 10: p. 34-64.
- [13] Felgner, P.L., et al., *Lipofection Procedure - A Highly, Efficient, Lipid-mediated DNA-transfection Procedure*. Proceedings of the National Academy of Sciences U.S.A., 1987. 84: p. 7413-7417.
- [14] Pampinella, F., et al., *Analysis of Differential Lipofection Efficiency in Primary and Established Myoblasts*. Molecular Therapy, 2002. 5: p. 161-169.
- [15] Felgner, P.L., et al., *Nomenclature for Synthetic Gene Delivery Systems*. Human Gene Therapy, 1997. 8: p. 511-512.
- [16] Douglas, K.L., *Toward Development of Artificial Viruses for Gene Therapy A Comparative Evaluation of Viral and Non-Viral Transfection*. Biotechnology Progress, 2008. 24: p. 871-883.
- [17] Stone, D., et al., *Viral Vectors for Gene Delivery and Gene Therapy Within the Endocrine System*. Journal of Endocrinology, 2000. 164: p. 103-118.
- [18] Florea, B.I., et al., *Transfection Efficiency and Toxicity of Polyethylenimine in Differentiated Calu-3 and Nondifferentiated COS-1 Cell Cultures*. American Association of Pharmaceutical Scientists Journal, 2002. 4: p. 1-11.
- [19] Lee, M., *Apoptosis Induced by PolyethylenimineDNA Complex in Polymer Mediated Gene Delivery*. Bulletin of the Korean Chemical Society, 2007. 28: p. 95-98.
- [20] Egilmez, N.K., Y. Iwanuma, and R.B. Bankert, *Evaluation And Optimization Of Different Cationic Liposome Formulations*. Biochemical and Biophysical Research Communications, 1996. 221: p. 169-173.

- [21] Choi, W., et al., *Low Toxicity Of Cationic Lipid-based Emulsion For Gene Transfer*. Biomaterials, 2004. 25: p. 5893-5903.
- [22] Yingyongnarongkul, B., et al., *High Transfection Efficiency and Low Toxicity Cationic Lipids with Aminoglycerol-Diamine Conjugate*. Bioorganic & Medicinal Chemistry, 2009. 17: p. 176-188.
- [23] Lasic, D.D. and Y. Barenholz, *Nonmedical Applications of Liposomes - From Gene Delivery and Diagnostics to Ecology*. 1st ed, ed. C. Press. 1996, Boca Raton (USA).
- [24] Lasic, D.D., *Liposomes*. Science & Medicine, 1996. 3: p. 34-43.
- [25] Gonçalves, E., R.J. Debs, and T.D. Heath, *The Effect of Liposome Size on the Final Lipid-DNA Ratio of Cationic Lipoplexes*. Biophysical Journal, 2004. 86: p. 1554-1563.
- [26] Zuidam, N.J., et al., *Lamellarity of Cationic Liposomes and Mode of Preparation of Lipoplexes Affect Transfection Efficiency*. Biochimica et Biophysica Acta, 1999. 1419: p. 207-220.
- [27] Claessens, M.M.A.E., et al., *Charged Lipid Vesicles Effects of Salts on Bending Rigidity, Stability, and Size*. Biophysical Journal, 2004. 87: p. 3882-3893.
- [28] Labas, R., et al., *Nature as a Source of Inspiration for Cationic Lipid Synthesis*. Genetica, 2010. 138: p. 153-168.
- [29] Ahmad, A., et al., *New Multivalent Cationic Lipids Reveal Bell Curve for Transfection Efficiency Versus Membrane Charge Density Lipid-DNA Complexes for Gene Delivery*. Journal of Gene Medicine, 2005. 7: p. 739-748.
- [30] Zuhorn, I.S., et al., *Phase Behavior Of Cationic Amphiphiles And Their Mixtures With Helper Lipids*. Biophysical Journal, 2002. 83: p. 2096-2108.
- [31] Lasic, D.D. and D. Papahadjopoulos, *Medical Applications of Liposomes*. 1st ed, ed. Elsevier. 1998, Amsterdam (Netherlands). 795.
- [32] Madeira, C., et al., *Liposome Complexation Efficiency Monitored by FRET - Effect of Charge Ratio, Helper Lipid and Plasmid Size*. European Biophysical Journal, 2007. 36: p. 609-620.
- [33] Zelphati, O., et al., *Stable and Monodisperse Lipoplex Formulations for Gene Delivery*. Gene Therapy, 1998. 5: p. 1272-1282.
- [34] Gershon, H., et al., *Mode of Formation and Structural Features of DNA-Cationic Liposome Complexes Used for Transfection*. Biochemistry, 1993. 32: p. 7143-7151.
- [35] Akao, T., et al., *Conformational Change in DNA Induced by Cationic Bilayer Membranes*. Federation of European Biochemical Societies Letters, 1996. 391: p. 215-218.
- [36] Dan, N., *Formation of Ordered Domains in Membrane-Bound DNA*. Biophysical Journal, 1996. 71: p. 1267-1272.
- [37] Cruz, M.T.G., et al., *Kinetic Analysis of the Initial Steps Involved in Lipoplex-Cell Interactions*. Biochimica et Biophysica Acta, 2001. 1510: p. 136-151.
- [38] Silva, J.P.N., et al., *DODAB: Monoolein-based Lipoplexes as Non-viral Vectors for Transfection of Mammalian Cells*. Biochimica et Biophysica Acta, 2011. 1808: p. 2440-2449.
- [39] Silva, J.P.N., P.J.G. Coutinho, and M.E.C.D.R. Oliveira, *Characterization of Mixed DODAB-Monoolein Aggregates Using Nile Red as a Solvatochromic and Anisotropy Fluorescent Probe*. Journal of Photochemistry and Photobiology A: Chemistry, 2009. 203: p. 32-39.

- [40] Oliveira, I.M.S.C., et al., *Aggregation Behavior of Aqueous Dioctadecyldimethylammonium Bromide/Monoolein Mixtures: A Multitechnique Investigation on the Influence of Composition and Temperature*. Journal of Colloid and Interface Science, 2011. 374: p. 206-217.
- [41] Silva, J.P.N., P.J.G. Coutinho, and M.E.C.D.R. Oliveira, *Characterization of Monoolein-Based Lipoplexes Using Fluorescence Spectroscopy*. Journal of Fluorescence, 2008. 18: p. 555-562.
- [42] Kearns, M.D., A.M. Donkor, and M. Savva, *Structure-Transfection Activity Studies of Novel Cationic Cholesterol-Based Amphiphiles*. Molecular Pharmaceutics, 2008. 5: p. 128-139.
- [43] Tarahovsky, Y.S., R. Koynova, and R.C. MacDonald, *DNA Release from Lipoplexes by Anionic Lipids Correlation with Lipid Mesomorphism, Interfacial Curvature, and Membrane Fusion*. Biophysical Journal, 2004. 87: p. 1054-1064.
- [44] Koynova, R., et al., *Lipoplex Formulation of Superior Efficacy Exhibits High Surface Activity and Fusogenicity, and Readily Releases DNA*. Biochimica et Biophysica Acta, 2007. 1768: p. 375-386.
- [45] Real-Oliveira, M.E.C.D., et al., *Aplicação da Monooleína como Novo Lípido Adjuvante em Lipofecção*, in Portuguese Patent n. PT104158, I.N.d.P. Industrial, Editor. 2011. p. 1-27.
- [46] Real-Oliveira, M.E.C.D., et al., *Use of Monoolein as a New Auxiliary Lipid in Lipofection*, in International Patent n. WO2010/020935 A2, W.I.P. Organization, Editor. 2010. p. 1-27.
- [47] Tsuruta, L.R., M.M. Lessa, and A.M. Carmona-Ribeiro, *Interactions Between Dioctadecyldimethylammonium Chloride or Bromide Bilayers in Water*. Langmuir, 1995. 11: p. 2938-2943.
- [48] Nascimento, D.B., et al., *Counterion Effects on Properties of Cationic Vesicles*. Langmuir, 1998. 14: p. 7387-7391.
- [49] Okuyama, K., et al., *Molecular and Crystal Structure of the Lipid-Model Amphiphile Dioctadecyldimethylammonium Bromide Monohydrate*. Bulletin of the Chemical Society of Japan, 1988. 61: p. 1485-1490.
- [50] Feitosa, E. and W. Brown, *Fragment and Vesicle Structures in Sonicated Dispersions of Dioctadecyldimethylammonium Bromide*. Langmuir, 1997. 13: p. 4810-4816.
- [51] Schulz, P.C., et al., *Phase Behaviour of the Dioctadecylammonium Bromide-Water System*. Journal of Thermal Analysis, 1998. 51: p. 49-62.
- [52] Feitosa, E., P.C.A. Barreleiro, and G. Olofsson, *Phase Transition in Dioctadecyldimethylammonium Bromide and Chloride Vesicles Prepared by Different Methods*. Chemistry and Physics of Lipids, 2000. 105: p. 201-213.
- [53] Benatti, C.R., et al., *Structural and Thermal Characterization of Dioctadecyldimethylammonium Bromide Dispersions by Spin Labels*. Chemistry and Physics of Lipids, 2001. 111: p. 93-104.
- [54] Proverbio, Z.E., P.C. Schulz, and J.E. Puig, *Aggregation of the Aqueous Dodecyltrimethylammonium Bromide - Didodecyldimethylammonium Bromide System at Low Concentration*. Colloid and Polymer Science, 2002. 280: p. 1045-1052.
- [55] Feitosa, E., et al., *Cationic Liposomes in Mixed Didodecyldimethylammonium Bromide and Dioctadecyldimethylammonium Bromide Aqueous Dispersions Studied by Differential*

- Scanning Calorimetry, Nile Red Fluorescence and Turbidity*. Langmuir, 2006. 22: p. 3579-3585.
- [56] Feitosa, E., G. Karlsson, and K. Edwards, *Unilamellar Vesicles Obtained by Simply Mixing Dioctadecyldimethylammonium Chloride and Bromide with Water*. Chemistry and Physics of Lipids, 2006. 140: p. 66-74.
- [57] Rodriguez-Pulido, A., et al., *A Theoretical and Experimental Approach to the Compaction Process of DNA by Dioctadecyldimethylammonium Bromide-Zwitterionic Mixed Liposomes*. Journal of Physical Chemistry B, 2009. 113: p. 15648-15661.
- [58] Mel'nikov, Y.S., S.M. Mel'nikova, and J.E. Lofroth, *Physico-chemical Aspects of the Interaction between DNA and Oppositely Charged Mixed Liposomes*. Biophysical Chemistry, 1999. 81: p. 125-141.
- [59] Hungerford, G., et al., *Domain Formation in DODAB-Cholesterol Mixed Systems Monitored Via Nile Red Anisotropy*. Journal of Fluorescence, 2005. 15: p. 835-840.
- [60] Hungerford, G., et al., *Interaction of DODAB with Neutral Phospholipids and Cholesterol Studied Using Fluorescence Anisotropy*. Journal of Photochemistry and Photobiology A: Chemistry, 2006. 181: p. 99-105.
- [61] Briggs, J., H. Chung, and M. Caffrey, *The Temperature-Composition Phase Diagram and Mesophase Structure Characterization of the Monoolein-Water System*. Journal de Physique II, 1996. 6: p. 723-751.
- [62] Czeslik, C., et al., *Temperature- and Pressure-Dependent Phase Behavior of Monoacylglycerides Monoolein and Monoelaidin*. Biophysical Journal, 1995. 68: p. 1423-1429.
- [63] Vacklin, H., et al., *The Bending Elasticity of 1-Monoolein upon Relief of Packing Stress*. Langmuir, 2000. 16: p. 4741-4748.
- [64] Caffrey, M., *A Lipid's Eye View of Membrane Protein Crystallization in Mesophases*. Current Opinion in Structural Biology, 2000. 10: p. 486-497.
- [65] Caffrey, M., *Membrane Protein Crystallization*. Journal of Structural Biology, 2003. 142: p. 108-132.
- [66] Carlsson, N., et al., *Bicontinuous Cubic Phase of Monoolein and Water as Medium for Electrophoresis of Both Membrane-bound Probes and DNA*. Langmuir, 2006. 22: p. 4408-4414.
- [67] Dias, R. and B. Lindman, *DNA Interactions with Polymers and Surfactants*. 1st ed, ed. J.W.S. Incorporated. 2008, Hoboken (USA). 425.
- [68] Bielke, W. and C. Erbacher, *Nucleic Acid Transfection*. 1st ed, ed. S. Verlag. 2010, Berlin (Germany). 316.
- [69] Hofland, H.E.J., L. Shepard, and S.M. Sullivan, *Formation of Stable Cationic Lipid/DNA Complexes*. Proceedings of the National Academy of Sciences U.S.A., 1996. 93: p. 7305-7309.
- [70] Oberle, V., et al., *Lipoplex Formation under Equilibrium Conditions Reveals a Three-Step Mechanism*. Biophysical Journal, 2000. 79: p. 1447-1454.
- [71] Barreleiro, P.C.A., R.P. May, and B. Lindman, *Mechanism of Formation of DNA-Cationic Vesicle Complexes*. Faraday Discussions, 2002. 122: p. 191-201.

- [72] Masotti, A., et al., *Comparison of Different Commercially Available Cationic Liposome-DNA Lipoplexes Parameters Influencing Toxicity and Transfection Efficiency*. Colloids and Surfaces B: Biointerfaces, 2009. 68: p. 136-144.
- [73] Hui, S.W., et al., *The Role of Helper Lipids In Cationic Liposome-Mediated Gene Transfer*. Biophysical Journal, 1996. 71: p. 590-599.
- [74] Farhood, H., N. Serbina, and L. Huang, *The Role of Dioleoyl Phosphatidylethanolamine in Cationic Liposome Mediated Transfer*. Biochimica et Biophysica Acta, 1995. 1235: p. 289-295.
- [75] Hirsch-Lerner, D., et al., *Effect of Helper "Lipid" on Lipoplex Electrostatics*. Biochimica et Biophysica Acta, 2005. 1714: p. 71-84.
- [76] Ciani, L., et al., *DOTAP-DOPE and DC-Chol-DOPE Lipoplexes for Gene Delivery Zeta Potential Measurements and Electron Spin Resonance Spectra*. Biochimica et Biophysica Acta, 2004. 1664: p. 70-79.
- [77] Esposito, C., et al., *The Analysis of Serum Effects on Structure, Size and Toxicity of DDAB-DOPE and DC-Chol-DOPE Lipoplexes Contributes to Explain their Different Transfection Efficiency*. Colloids and Surfaces B: Biointerfaces, 2006. 53: p. 187-192.
- [78] Maitani, Y., et al., *Cationic Liposome (DC-Chol-DOPE 1-2) and a Modified Ethanol Injection Method to Prepare Liposomes, Increased Gene Expression*. International Journal of Pharmaceutics, 2007. 342: p. 33-39.
- [79] Rodriguez-Pulido, A., et al., *A Physicochemical Characterization of the Interaction between DC-Chol/DOPE Cationic Liposomes and DNA*. Journal of Physical Chemistry B, 2008. 112: p. 12555-12565.
- [80] Smisterová, J., et al., *Molecular Shape of the Cationic Lipid Controls the Structure of Cationic Lipid-DOPE-DNA Complexes and the Efficiency of Gene Delivery*. Journal of Biological Chemistry, 2001. 276: p. 47615-47622.
- [81] Yuan, X.B., *Non-Viral Gene Therapy*. 1st ed, ed. Intech. 2011, Rijeka (Croatia). 707.
- [82] Kiefer, K., J. Clement, and P. Garidel, *Transfection Efficiency and Cytotoxicity of Non-viral Gene Transfer Reagents in Human Smooth Muscle and Endothelial Cells*. Pharmaceutical Research, 2004. 21: p. 1009-1017.
- [83] Tenchov, B.G., R.C. MacDonald, and D.P. Siegel, *Cubic Phases in Phosphatidylcholine-Cholesterol Mixtures - Cholesterol as Membrane Fusogen*. Biophysical Journal, 2006. 91: p. 2508-2516.
- [84] Huang, L., M. Hung, and E. Wagner, *Nonviral Vectors for Gene Therapy - Part I (1st Edition)*. 1st ed, ed. E.A. Press. 1999, California (U.S.A.). 442.
- [85] Huang, L., M. Hung, and E. Wagner, *Nonviral Vectors for Gene Therapy - Part I (2nd Edition)*. 2nd ed, ed. E.A. Press. 2005, California (U.S.A.). 377.
- [86] Koynova, R., L. Wang, and R.C. MacDonald, *Cationic Phospholipids Forming Cubic Phases - Lipoplex Structure and Transfection Efficiency*. Molecular Pharmaceutics, 2008. 5: p. 739-744.
- [87] Koynova, R., L. Wang, and R.C. MacDonald, *An Intracellular Lamellar-Nonlamellar Phase Transition Rationalizes the Superior Performance of Some Cationic Lipid Transfection Agents*. Proceedings of the National Academy of Sciences U.S.A., 2006. 103: p. 14373-14378.

- [88] Koynova, R. and R.C. MacDonald, *Mixtures of Cationic Lipid O-Ethylphosphatidylcholine with Membrane Lipids and DNA*. Biophysical Journal, 2003. 85: p. 2449-2465.
- [89] Koynova, R., *Lipid Phases Eye View to Lipofection - Cationic Phosphatidylcholine Derivatives as Efficient DNA Carriers for Gene Delivery*. Lipid Insights, 2008. 2: p. 41-59.
- [90] Tenchov, B.G., et al., *Modulation of a Membrane Lipid Lamellar-Nonlamellar Phase Transition by Cationic Lipids - A Measure for Transfection Efficiency*. Biochimica et Biophysica Acta, 2008. 1778: p. 2405-2412.
- [91] Taira, K., K. Kataoka, and T. Niidome, *Non-viral Gene Therapy - Gene Design and Delivery*. 1st ed, ed. S. Verlag. 2005, Tokyo (Japan). 490.
- [92] Wickstrom, E., *Clinical Trials of Genetic Therapy with Antisense DNA and DNA Vectors*. 1st ed, ed. M.D. Incorporated. 1998, New York (U.S.A). 421.
- [93] Templeton, N.S., *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*. 2nd ed, ed. M.D. Incorporated. 2005, New York (U.S.A.). 896.
- [94] Villiers, M.M., P. Aramwit, and G.S. Kwon, *Nanotechnology in Drug Delivery*. 1st ed, ed. S. Verlag. 2009, Berlin (Germany). 663.
- [95] Lamprecht, A., *Nanotherapeutics - Drug Delivery Concepts in Nanoscience*. 1st ed, ed. P. Stanford. 2009, Danvers (USA). 293.
- [96] Rose, J.K., *Liposomal Transfection of Nucleic Acids into Animal Cells*, in U.S. Patent n. 005279833, U.S.I.P. Organization, Editor. 1994. p. 1-13.
- [97] Ashley, G.W., R.C. MacDonald, and M. Shida, *Cationic Phospholipids for Transfection*, in U.S. Patent n. 005651981, U.S.I.P. Organization, Editor. 1997. p. 1-14.
- [98] Niedzinski, E.J. and M. Bennet, *Multi-Functional Polyamines for Delivery of Biologically-Active Polynucleotides*, in International Patent n. 2003/102150, W.I.P. Organization, Editor. 2003. p. 1-37.
- [99] Barenholz, Y. and D. Simberg, *Sphingolipids Polyakylamine Conjugates for Use in Transfection*, in International Patent n. 2004/110499, W.I.P. Organization, Editor. 2004. p. 1-49.
- [100] Reszka, R., *Cholesterol Derivative for Liposomal Gene Transfer*, in U.S. Patent n. 005888821, U.S.I.P. Organization, Editor. 1999. p. 1-4.
- [101] Mori, H. and N. Nishikawa, *Glycerol Derivatives*, in U.S. Patent n. 005221796, U.S.I.P. Organization, Editor. 1993. p. 1-14.
- [102] Camilleri, P., et al., *A Novel Class of Cationic Gemini Surfactants Showing Efficient In Vitro Gene Transfection Properties*. Chemical Communications, 2000. 31: p. 1253-1254.
- [103] Kirby, A.J., et al., *Gemini Surfactants: New Synthetic Vectors for Gene Transfection*. Angewandte Chemie International Edition, 2003. 42: p. 1448-1457.
- [104] Sekhon, B.S., *Gemini (Dimeric) Surfactants*. Resonance, 2004. 9: p. 42-49.
- [105] Wettig, S.D., R.E. Verrall, and M. Foldvari, *Gemini Surfactants - A New Family of Building Blocks for Non-Viral Gene Delivery Systems*. Current Gene Therapy, 2008. 8: p. 9-23.
- [106] Bombelli, C., et al., *Gemini Surfactant Based Carriers in Gene and Drug Delivery*. Current Medicinal Chemistry, 2009. 16: p. 171-183.
- [107] Palmer, L.R., et al., *Transfection Properties of Stabilized Plasmid-Lipid Particles Containing Cationic PEG Lipids*. Biochimica et Biophysica Acta, 2003. 1611: p. 204-216.

- [108] Buyens, K., et al., *Elucidating the Encapsulation of Short Interfering RNA in PEGylated Cationic Liposomes*. *Langmuir*, 2009. 25: p. 4886-4891.
- [109] Kim, J.Y., et al., *The Use of PEGylated Liposomes to Prolong Circulation Lifetimes of Tissue Plasminogen Activator*. *Biomaterials*, 2009. 30: p. 5751-5756.
- [110] Harvie, P., F.M.P. Wong, and M.B. Bally, *Use of Poly(ethylene glycol)-Lipid Conjugates to Regulate the Surface Attributes and Transfection Activity of Lipid-DNA Particles*. *Journal of Pharmaceutical Sciences*, 2000. 89: p. 652-663.
- [111] Veronese, F.M., *PEGylated Protein Drugs: Basic Science and Clinical Applications (Milestones in Drug Therapy)*. 1st ed, ed. B. Basel. 2009, Basel (Switzerland). 297.
- [112] Sakaguchi, N., et al., *The Correlation Between Fusion Capability and Transfection Activity in Hybrid Complexes of Lipoplexes and pH-Sensitive Liposomes*. *Biomaterials*, 2008. 29: p. 4029-4036.
- [113] Wasungu, L., et al., *Transfection Mediated by pH-Sensitive Sugar-Based Gemini Surfactants; Potential for In Vivo Gene Therapy Applications*. *Journal of Molecular Medicine*, 2006. 84: p. 774-784.
- [114] Chang, E.H., L. Xu, and K. Pirollo, *Targeted Liposome Gene Delivery*, in *U.S. Patent n. 006749863*, U.S.I.P. Organization, Editor. 2004. p. 1-25.
- [115] Gorman, C.M. and M. McClarrinon, *Cationic Lipid-DNA Complexes for Gene Targeting*, in *U.S. Patent n. 005830878*, U.S.I.P. Organization, Editor. 1998. p. 1-24.
- [116] Hattori, Y. and Y. Maitani, *Folate-Linked Lipid-Based Nanoparticle for Targeted Gene Delivery*. *Current Drug Delivery*, 2005. 2: p. 243-252.
- [117] Petrak, K., *Essential Properties of Drug-Targeting Delivery Systems*. *Drug Discovery Today*, 2005. 10: p. 1667-1673.
- [118] Russ, V. and E. Wagner, *Cell and Tissue Targeting of Nucleic Acids for Cancer Gene Therapy*. *Pharmaceutical Research*, 2007. 24: p. 1047-1057.
- [119] Yang, P., et al., *Enhanced Gene Expression in Epithelial Cells Transfected With Aminoacid-Substituted Gemini Nanoparticles*. *European Journal of Pharmaceutics and Biopharmaceutics*, 2010. 75: p. 311-320.
- [120] Rosa, M., et al., *DNA Pre-Condensation with an Amino Acid-based Cationic Amphiphile - A Viable Approach for Liposome-Based Gene Delivery*. *Molecular Membrane Biology*, 2008. 25: p. 23-34.
- [121] Letrou-Bonneval, E., et al., *Galactosylated Multimodular Lipoplexes for Specific Gene Transfer into Primary Hepatocytes*. *Journal of Gene Medicine*, 2008. 10: p. 1198-1210.
- [122] Higuchi, Y., et al., *Effect of the Particle Size of Galactosylated Lipoplex on Hepatocyte-Selective Gene Transfection after Intraportal Administration*. *Biological and Pharmaceutical Bulletin*, 2006. 29: p. 1521-1523.
- [123] Audouy, S. and D. Hoekstra, *Cationic Lipid-Mediated Transfection In Vitro and In Vivo (Review)*. *Molecular Membrane Biology*, 2001. 18: p. 129-143.
- [124] Bihan, O.L., et al., *Probing the In Vitro Mechanism of Action of Cationic Lipid-DNA Lipoplexes at a Nanometric Scale*. *Nucleic Acids Research*, 2010. 1: p. 1-15.
- [125] Gregoriadis, G., *Liposome Technology - I - Liposome Preparation and Related Techniques*. 3rd ed, ed. I. Healthcare. 2007, New York (USA). 660.

- [126] Gregoriadis, G., *Liposome Technology - II - Entrapment of Drugs and Other Materials Into Liposomes*. 3rd ed, ed. I. Healthcare. 2007, New York (USA). 424.
- [127] Gregoriadis, G., *Liposome Technology - III - Interactions of Liposomes with the Biological Milieu*. 3rd ed, ed. I. Healthcare. 2007, New York (USA). 464.
- [128] Real-Oliveira, M.E.C.D., et al., *Use of Monoolein as a New Auxiliary Lipid in Lipofection*, in *European Patent n. EP2335687 A2*, E.P. Office, Editor. 2011. p. 1-27.
- [129] Zuhorn, I.S., et al., *Interference of Serum with Lipoplex-cell Interaction*. *Biochimica et Biophysica Acta*, 2002. 1560: p. 25-36.
- [130] Yang, J.P. and L. Huang, *Overcoming the Inhibitory Effect of Serum on Lipofection by Increasing the Charge Ratio of Cationic Liposome to DNA*. *Gene Therapy*, 1997. 4: p. 950-960.
- [131] Zhang, Y. and T.J. Anchordoquy, *The Role of Lipid Charge Density in the Serum Stability of Cationic Lipid-DNA Complexes*. *Biochimica et Biophysica Acta*, 2004. 1663: p. 143-157.
- [132] Conwell, C.C., F. Liu, and L. Huang, *Gene Transfer Activity is Enhanced Significantly by Allowing Cationic Polymer to Interact With Serum Proteins Prior to DNA Addition*. *Molecular Therapy*, 2005. 11: p. pp. S80.
- [133] Arpke, R.W. and P.W. Cheng, *Characterization of Human Serum Albumin-Facilitated Lipofection Gene Delivery Strategy*. *Journal of Cell Science & Therapy*, 2011. 2: p. 108-114.
- [134] Mounkes, L.C., et al., *Proteoglycans Mediate Cationic Liposome-DNA Complex-Based Gene Delivery In Vitro and In Vivo*. *Journal of Biological Chemistry*, 1998. 27: p. 26164-26170.
- [135] Wiethoff, C.M., et al., *The Potential Role of Proteoglycans in Cationic Lipid-mediated Gene Delivery*. *Journal of Biological Chemistry*, 2001. 276: p. 32806-32813.
- [136] Silva, M.E. and C.P. Dietrich, *Structure of Heparin*. *Journal of Biological Chemistry*, 1975. 25: p. 6841-6846.
- [137] Rosenberg, R.D., G. Armand, and L. Lam, *Structure-Function Relationships of Heparin Species*. *Proceedings of the National Academy of Sciences U.S.A.*, 1978. 75: p. 3065-3069.
- [138] Mascotti, D.P. and T.M. Lohman, *Thermodynamics of Charged Oligopeptide-Heparin Interactions*. *Biochemistry*, 1995. 34: p. 2908-2915.
- [139] Zuhorn, I.S., R. Kalicharan, and D. Hoekstra, *Lipoplex-Mediated Transfection of Mammalian Cells Occurs Through the Cholesterol-Dependent Clathrin-Mediated Pathway of Endocytosis*. *Journal of Biological Chemistry*, 2002. 27: p. 18021-18028.
- [140] Habib, N.A., *Cancer Gene Therapy - Past Achievements and Future Challenges*. 1st ed, ed. K.A. Publishers. 2002, New York (U.S.A.). 458.
- [141] Ilarduya, C.T., Y. Sun, and N. Düzgünes, *Gene Delivery by Lipoplexes and Polyplexes*. *European Journal of Pharmaceutical Sciences*, 2010. 40: p. 159-170.
- [142] Resina, S., P. Prevot, and A.R. Thierry, *Physico-Chemical Characteristics of Lipoplexes Influence Cell Uptake Mechanisms and Transfection Efficacy*. *Public Library of Science ONE*, 2009. 4: p. e6058 (1-11).
- [143] Ferrari, M.E., et al., *Trends in Lipoplex Physical Properties Dependent on Cationic Lipid Structure, Vehicle and Complexation Procedure do not Correlate with Biological Activity*. *Nucleic Acids Research*, 2001. 29: p. 1539-1548.
- [144] Hoekstra, D., et al., *Gene Delivery by Cationic Lipids In and Out of an Endosome*. *Biochemical Society Transactions*, 2007. 35: p. 68-71.

- [145] Elouahabi, A. and J. Ruysschaert, *Formation and Intracellular Trafficking of Lipoplexes and Polyplexes*. Molecular Therapy, 2005. 11: p. 336-347.
- [146] Bhushan, B., *Handbook of Nanotechnology*. 2nd ed, ed. S. Verlag. 2010, Berlin (Germany). 1968.
- [147] Caracciolo, G., et al., *Efficient Escape from Endosomes Determines the Superior Efficiency of Multicomponent Lipoplexes*. Journal of Physical Chemistry B, 2009. 113: p. 4995-4997.
- [148] Liu, F., et al., *Effect of Non-Ionic Surfactants on the Formation of DNA-Emulsion Complexes and Emulsion-Mediated Gene Transfer*. Pharmaceutical Research, 1996. 13: p. 1642-1646.
- [149] Tan, Y., et al., *Sequential Injection of Cationic Liposome and Plasmid DNA Effectively Transfects the Lung with Minimal Inflammatory Toxicity*. Molecular Therapy, 2001. 3: p. 673-682.
- [150] Yasuda, S., et al., *Comparison of the Type of Liposome Involving Cytokine Production Induced by Non-CpG Lipoplex in Macrophages*. Molecular Pharmaceutics, 2010. 7: p. 533-542.