REVIEW

State Key Laboratory of Bioelectronics[1], Jiangsu Laboratory for Biomaterials and Devices, School of Biological Science and Medical Engineering, Southeast University, Nanjing, P. R. China Pharmaceutical Research Institute[2], China Pharmaceutical University, Nanjing, China

Cationic liposomes as gene delivery system: transfection efficiency and new application

F. Xiong[1], Z. Mi[2], N. Gu[1]

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Ning Gu, State Key Laboratory of Bioelectronics, Jiangsu Laboratory for Biomaterials and Devices, School of Biological Science and Medical Engineering, Southeast University, Nanjing 210009, China
guning@seu.edu.cn

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As it has been generally reported that oppositely charged cationic liposomes (CLs) are superior to either neutral or anionic liposomes as gene delivery carrier, interest in the properties, structures, transfection mechanism of CLs and so forth arises unprecedentedly. However, our understanding about the mechanism of CLs-gene complexes (lipoplex)-cell interaction and factors influencing the transfection efficiency (TE) of CLs remains poor. In this article, we describe some new results aimed at elucidating the relationship between the chemical-physical properties of lipoplex with TE and introducing recent applications of CLs in gene therapy.

1. Introduction

Gene therapy is defined as the introduction of exogenous genetic material, including plasmid DNA (pDNA) (Kang et al. 2008), antisense oligonucleotides (ASODN are short, synthetic, single-stranded DNA, RNA, or their analogs designed to modulate gene expression by selective hybridization (via Watson-Crick base pairing) to their complementary sequences in target or pre-mRNA; Sun et al. 2007), mRNA (Yamamoto et al. 2009), and peptide-nucleic acids (PNAs, synthetic homolog of nucleic acids in which the phosphate-sugar polynucleotide backbone is replaced by a flexible polyamide; Boffa et al. 2007) into cells or tissues in order to cure genetic disorders, AIDS, cancers and other acquired genetic defects. Appropriate gene delivery vectors play a crucial role in gene therapy (Kundu and Sharma 2008). One of the most promising candidate, liposomes (lipid-bilayer membranes composed of natural or synthetic phospholipids that can encapsulate various biologically active compounds such as antibiotics, antigens, proteins and nucleic acids to act as efficient delivery system), have been investigated since the late 1970s (Dimitriadis 1978). However, the poor transfection efficiency (TE) of liposomes limits its long-term therapeutic application as gene delivery vectors. In 1987, Felgner et al. used the synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) for the first time to prepare small unilamellar liposomes, which interact spontaneously with DNA to form lipid-DNA complexes (lipoplex) with 100% entrapment of the DNA and achieve both uptake and expression of the DNA. They later proved the potential of cationic liposomes (CLs) as carriers of RNA in 1989 (Malone et al. 1989). Then these applications of CLs were rapidly followed by numerous pioneering studies, providing a new approach to gene therapy, owing to their potential advantages over viral vectors, such as their safety, versatility and low immunogenicity (Masotti et al. 2009). But the most critical issue about their application is their low TE compared to viral vectors (Ramezani et al. 2009). In this paper, we will introduce CLs briefly and describe recent work that elucidates the relationship between the chemical-physical properties of CL-DNA complexes with TE in mammalian cells.

2. Material, structure and transfection mechanism of cationic liposomes

2.1. Material

Most cationic liposome systems involve the formulation of a cationic lipid and a neutral co-lipid. Cationic lipids are usually composed of cationic head groups such as polyamine structures (Mével et al. 2010), linkers and hydrophobic tails such as a cholesteryl skeleton (Radchatawedchakoon et al. 2010). Commonly used cationic lipids include dioleoyltrimethylammonium propane (DOTAP), *N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium (DMRIE), 2,3-dioleyloxy-*N*-(2-(sperminecarboxamido)ethyl)-*N*,*N*-dimethyl-1-propaniminiumpenta-hydrochloride (DOSPA) and *N*′,*N*′-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide (DODAG). Dioleoyl-L-α-phosphatidylethanolamine (DOPE), cholesterol (Chol) and dioleoyl-phosphatidyl cholin (DOPC) have been widely used in previous study as neutral co-lipid. A selection of these lipids is shown in Fig. 1.

2.2. Structure

Lipoplex particles have been shown to exhibit structural polymorphism; nevertheless, their most efficient form remains unknown (Koumbi et al. 2006). However, X-ray studies have led to two most common types of structures observed in CL-DNA complexes: a multilamellar structure, L^{C}_{a}, with DNA

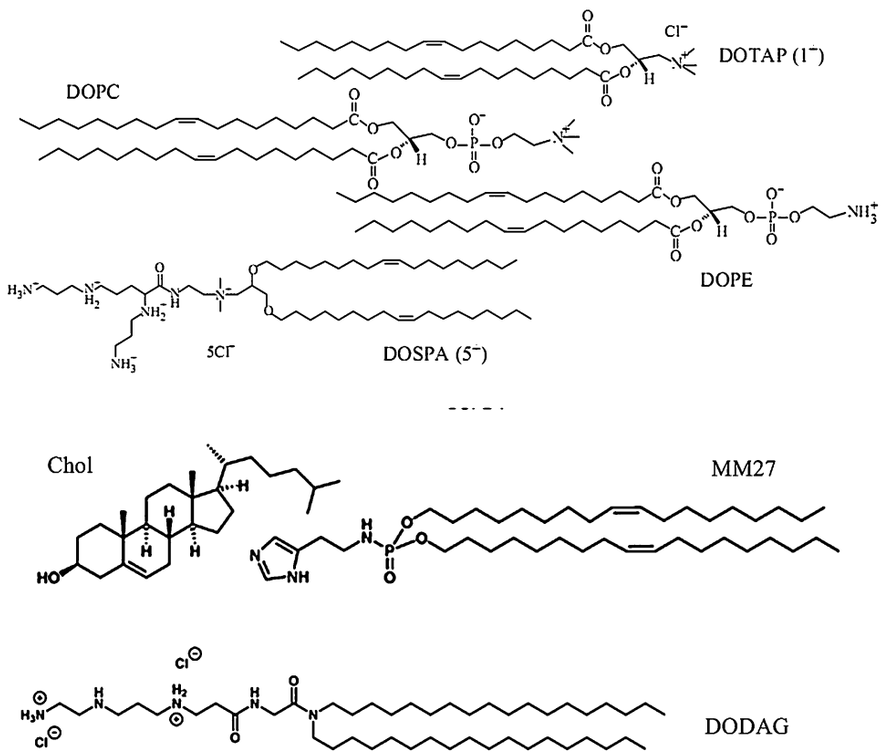


Fig. 1: Some lipids used in the structure and function studies of CL-gene complexes

monolayers sandwiched between cationic membranes as shown in Fig. 2 (Rädler et al. 1997), and an inverted hexagonal structure with DNA encapsulated within the inverse cylindrical micelles, H^C_{II} shown in Fig. 3 (Koltover et al. 1998). Statistical mechanical models have shown that these two phases observed experimentally are indeed equilibrium phases of CL-DNA complexes. DOPE, a natural fusogenic lipid, has a tendency to adopt the inversion hexagonal phase over a wide range of temperatures (Labbé et al. 2009). Such characteristics are typically expected to aid endosomolysis and improve intracellular trafficking of nucleic acids post nanoparticle internalization because the H^C_{II} phase transfect better than the L^C_a phase (Mével et al. 2010; Remaut et al. 2007; Penacho et al. 2010). Conversely, cholesterol apparently did not affect the lipoplex microstructure, but changed the interlamellar spacing (Weisman et al. 2004).

2.3. Transfection mechanism

The mechanism of transfection mediated by CLs has been thought to be related to the electrostatic interaction between the

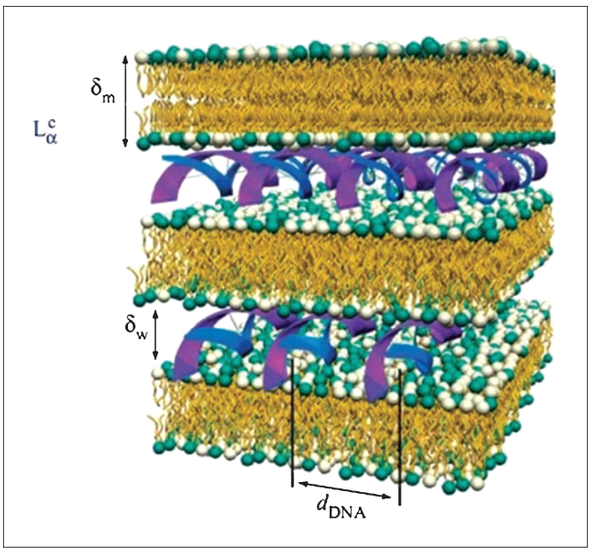


Fig. 2: The lamellar L^c_a phase of CL-DNA complexes with alternating lipid bilayer and DNA monolayer. The L^c_a phase is one of the several possible equilibrium phases, which is formed when CLs are mixed with DNA (Rädler et al. 1997)

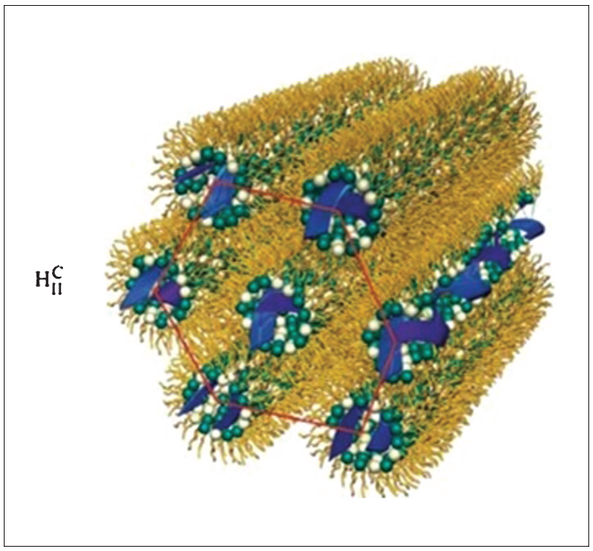


Fig. 3: The inverted hexagonal H^C_{II} liquid crystalline phase of CL-DNA complexes with DNA chains coated with inverse micelles arranged on a hexagonal lattice. The H^C_{II} is one of the several possible equilibrium phases of CL-DNA complexes (Koltover et al. 1998)

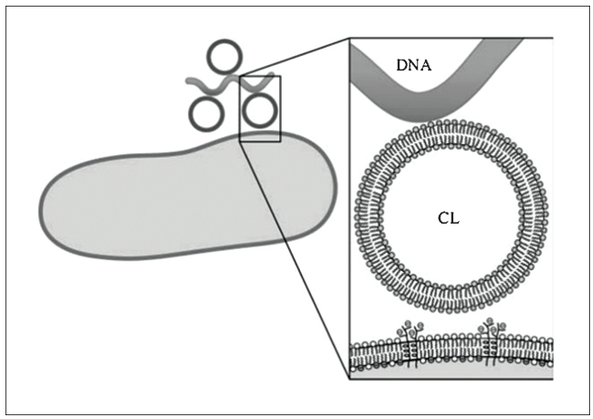


Fig. 4: Cartoon of a "beads-on-a-string" (i.e. CLs attached to a string of DNA) complex electrostatically bound to the surface of an animal cell. The blow-up shows a CL electrostatically binding a section of negatively charged DNA on one side and the plasma membrane containing cell surface proteoglycans with negatively charged sulphated groups on the other side (Safinya et al. 2006)

cationic liposome carriers and the negatively charged phosphate backbone of gene (Oliveira et al. 2009) and cell membranes (Flasterstein et al. 2010). CLs attach to genes to form a liposome-gene complex (lipoplex) with additional cationic charge which is electrostatically bound to mammalian cells surface as depicted in Fig. 4. Then the lipoplex interact with cellular membranes and deliver genes into cells. However, the lipoplex-cell interaction, though well documented, has not been understood completely. One of the first studies in 1973 on liposome-cell interactions utilized cationic liposomes containing stearylamine and suggested that the liposome membrane fuses with the plasma membrane (Papahadjopoulos et al. 1973). In the landmark work of Felgner et al. (1987) fluorescence microscopy was used to reveal that the lipoplex fuses with the cell membrane and that the fluorescent lipid diffuses through the intracellular membranes. Some other authors believed that cationic liposomes deliver lipoplex to cells most likely via an endocytotic process rather than simple fusion (Remaut et al. 2007; Düzgüne and Nir 1999; Stegmann and Legendre 1997). Recently, Zhang et al. (2007) investigated the cellular uptake mechanisms of the co-modified liver-targeting cationic liposomes through antigens inhibition effect assay and confocal laser scanning microscopy (CLSM) analysis. According to their results, the cellular uptake seems to involve both endocytosis and membrane fusion.

3. Factors influencing the transfection efficiency of cationic liposomes

It is well established that lipofection efficiency is multifactorial. In fact, any steps involving gene delivery influence efficiency of lipofection. The factors involved can be subdivided into four categories: 1. cell type (Weisman et al. 2004) and the physiological state of the cell; 2. medium conditions such as medium composition and the presence of serum, etc; 3. lipid composition and type of liposomes; 4. physicochemical and biological effects of the plasmid used for the transfection (Kerner et al. 2001). This article talks about some of them as follows.

3.1. Lipid structure

Research efforts continue to explore the most appropriate lipid structure. Different types of cationic and neutral lipids have been reported to show different degrees of gene transfection ability not only *in vitro* but also *in vivo* in preclinical or clinical studies (Ramezani et al. 2009; Ferrari et al. 1998, Mahato

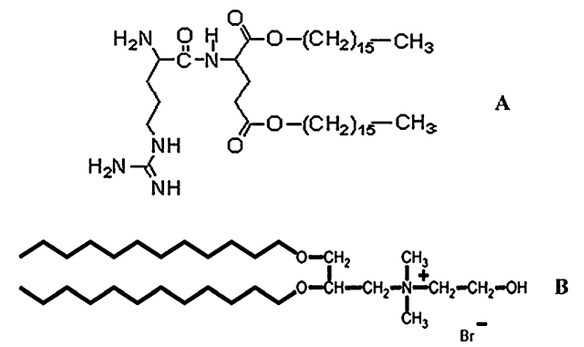


Fig. 5: Chemical structure of Arg-Glu2C16 (A) and DE (B)

2005). For cationic lipids, the linkage between the hydrophobic alkyl side chains, the headgroup of the phospholipids, and the structure of the hydrophobic moieties affects the triumph of conveying the genetic materials into the cells (Simões et al. 2005). Conjugation of a variety of hydrophobic moieties to a variety of polyamine structures, such as lysine or arginine (Obata et al. 2008), is a widely used preparation method. A fourfold higher TE of amino acid-based CLs composed of 1,5-dihexadecyl *N*-arginyl-L-glutamate (Arg-Glu2C16) (shown in Fig. 5) was reported compared to Lipofectamine 2000 when carrying nucleic acids into neuronal cells (Obata et al. 2010). Mével et al. (2010) synthesized a series of novel cationic lipids comprised of cholesteryl-moieties and dialkylglycylamide moiety linked to a polyamine or a guanidinium functional group. Among the CLs formulated by these novel lipids, CLs prepared from DODAG and DOPE were observed to mediate the highest levels of transfection *in vitro* in all three different cell lines studied. In another work, a novel CL formulation based on the recently synthesized cationic lipid (2,3-didodecyloxypropyl) (2-hydroxyethyl) dimethylammonium bromide (DE) (shown in Fig. 5) showed high TE as the delivery system for ASODN (De Rosa et al. 2008). Radchatawedchakoon et al. (2010) designed twenty-four asymmetric divalent head group cholesterol-based cationic lipids (as shown in Fig. 6), and seven of them exhibited higher transfection efficiency than the commercially available transfection agents. 3β-[*N*-(*N*'-Guanidinyl)-2'-aminoethyl)-*N*-(2-aminoethyl)carbamoyl] cholesterol (***5*** in Fig. 6) exhibited highest transfection efficiency. It is reported that the hydroxyethyl group at the cationic headgroup of OH-Chol also improves TE (Ding et al. 2008). As mentioned in 2.3, helper lipids also play important roles in transfection (Ramezani et al. 2008; Mével et al. 2010; Radchatawedchakoon et al. 2010; Zhang et al. 2010; Xu and Anchordoquy 2008).

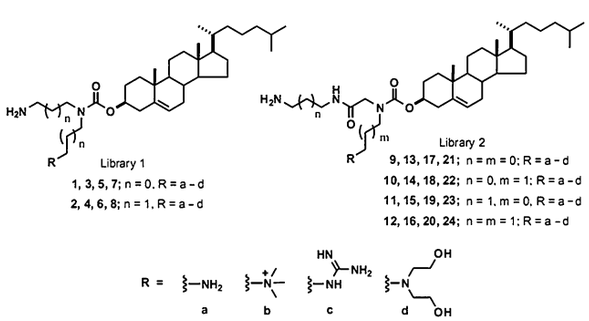
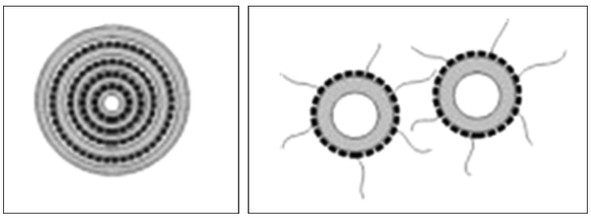


Fig. 6: Structures of new asymmetrical polar head cholesterol-based cationic lipids (Radchatawedchakoon et al. 2010)



(A) (B)

Fig. 7: Structure proposed for the (A) non-pegylated lipoplexes and (B) pegylated lipoplexes. The gray band represents the lipid bilayer from the liposomes, while the black band represent the ONs (Remaut et al. 2007)

3.2. PEGylation

To overcome the rapid clearance of lipoplex, PEGylation of CLs surface has been widely used to provide the lipoplexes with a water shell at the surface (Tagami et al. 2009; Xu et al. 2010; Tatsuhiro and Hiroshi 2008; Koide et al. 2010). While PEGylation has a clear benefit on the systemic level, its benefits on TE at the intracellular level have been doubted (Xu and Anchordoquy 2008). Deshpande et al. (2004) reported that PEGylation lowers the cellular interaction and uptake of the lipoplexes. Zhang et al. (2010) demonstrated that PEGylation severely decreased pDNA or siRNA TE of DC-Chol/DOPE CLs. Remaut et al. (2004) observed that PEGylation lowers the TE of DOTAP/DOPE CLs carrying oligonucleotides (ONs). The failure of PEGylated CLs in establishing an antisense effect was ascribed to different intracellular fate and structural of properties non-PEGylated CLs and PEGylated CLs. Non-PEGylated CLs fuse to form multilamellar lipoplexes (as shown in Fig. 7) that are shielded from the environment and shoùld be protected against enzymatic degradation. Conversely, fusion of PEGylated liposomes does not occur, because the ASODNs are not protected by lipid bilayers. Moreover, PEG-chains prevent contact between neighbouring lipoplexes, resulting in failure of ONs releasing in the surrounding environment (Remaut et al. 2007; Song et al. 2002) (as shown in Fig. 8). To overcome the shortcoming of PEGylation, some modified PEG have been synthesized, such as poly-L-arginine-conjugated polyethylene glycol (PLR–PEG) (Kim et al. 2010) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)2000] (DSPE-PEG) with hexapeptide (antagonist G) at the extremity (Santos et al. 2010). These modifications of PEG contribute to the improvement of TE, providing strategies in the PEGylation of CLs. Also, changing the size of polymer head group and amide chain length of the hydrophobic anchors could improve transfection of PEGylation CLs (Song et al. 2002).

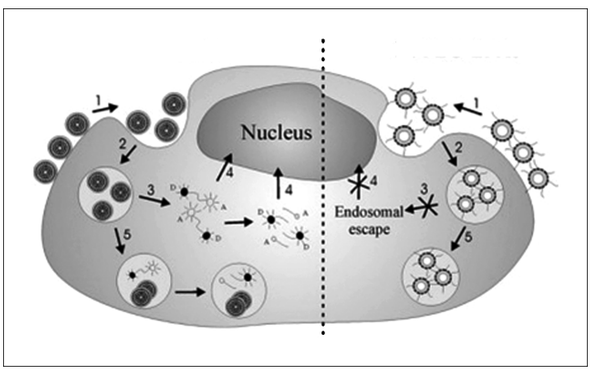


Fig. 8: Proposed intracellular pathway of (A) non-pegylated lipoplexes and (B) pegylated lipoplexes. 1: binding to the cell membrane; 2: internalization by endocytosis; 3: endosomal escape; 4: nuclear accumulation; 5: degradation in the endosomal compartment (Remaut et al. 2007)

3.3. Lipid molar ratio and (+/-) charge ratio

It is worth noting that the ratio of cationic/neutral lipids contributed greatly to TE of lipoplex (Gao and Huang 1995; Plank et al. 1996). Previous studies demonstrated high TE of DC-Chol/DOPE liposomes at 3:2, 1:1 or 1:2 molar ratio of DC-Chol/DOPE (Farhood et al. 1995; Maitani et al. 2007). The most efficient DC-Chol/DOPE liposomes for pDNA or siRNA delivery were at a 1:2 or 1:1 molar ratio of DC-Chol/DOPE, respectively (Zhang et al. 2010).

CLs/gene (+/-) ratio also has an impact on lipofection efficiency (Birchall et al. 1999). However, the degree of impact by this factor differs from lipids and gene type. Masotti et al. (2009) observed that the influence of liposome/DNA molar ratio on TE was very strong for DOTAP/DOPE–DNA, DDAB/DOPE–DNA and DC-Chol/DOPE–DNA lipoplexes, whereas LIPOFECTIN–DNA, DMIRIE–DNA and CELLFECTIN–DNA lipoplexes show a limited dependence of transfection on the molar charge ratio A recent study showed that CLs/gene (+/-) ratio has different influences on DNA and RNA complexed CLs. The siRNA transfection efficiency was positively associated with the weight ratio of DC-Chol/siRNA. In contrast, pDNA transfection efficiency decreased along with the increase of the weight ratio of DC-Chol/pDNA (Zhang et al. 2010). Generally, when the cationic portion provided by CLs exceeded the anionic part provided by the gene phosphates, lipoplex attains maximal transfection (Koumbi et al. 2006). Barron et al. (1999) suggested that the higher final (+/-) lipoplex ratios may correlate in some way with higher gene expression levels. Sometimes decreased TE at high CLs/gene ratio may be ascribed to the lipoplexes toxicity phenomena (Masotti et al. 2009).

3.4. Vesicle size

The influence of liposomal size on gene transfection remains disputed. Some studies indicated that the size of the complexes were not clearly associated with transfection efficiency (Stegmann and Legendre 1997; Xu and Anchordoquy 2008). Koumbi et al. reported that the initial size of the liposome was not always predictive of lipofection potency after comparing TE of CLs composed of $C_{32}H_{69}INO_3P$ (GLB73), $C_{40}H_{81}INO_3P$ (GLB74) and $C_{33}H_{71}INO_3P$ (GLB391) *in vitro* in K562 erythroleukemia cells and 5637 epithelial carcinoma cells by scoring the number of β-galactosidase expressing cells. The highest level of lipofection activity in K562 cells was obtained with the use of unilamellar vesicles GLB.73 (mean diameter of 120 nm), followed by multilamellar vesicles (MLVs) GLB.391 (650 nm) and unilamellar vesicles (100 nm). MLVs GLB.43 of 270 nm average size were significantly more effective than the corresponding unilamellar vesicles of 120 nm mean diameter (Koumbi et al. 2006). There are some other studies showing that the size of lipoplexes did affect the transfection activity of cationic liposomes (Ramezani et al. 2009; Anchordoquy et al. 1997). However, even among authors accepting vesicle size is responsible for the variation of TE, the question whether smaller or larger size correlate with high TE still remains unclear. Some studies reveal that the greater activity of lipoplex is due to the smaller complex size (about 200 nm or less) (Jääskeläinen et al. 2000). For instance, the correlation between TE mediated by lipoplex and a more compact inner DNA structure and smaller particle sizes (around 250 nm) was observed by Penacho et al. (2010). Whereas, additional research shows that large lipoplex particles seem to determine a higher lipofection than small particles do (Masotti et al. 2009). So, MLVs (about 900 nm in diameter) lipoplexes are generally better transfecting agents than small unilamellar vesicles (SUV) (about 25 nm in diameter) due to the larger size of

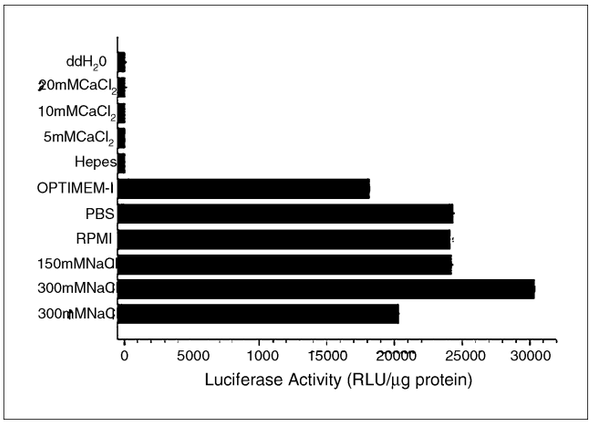


Fig. 9: Impact of lipoplex formation medium upon transgene expression in K562 cells 3 days post-transfection. Bars represent the mean of three independent experiments (±S.E.) (Koumbi et al. 2006)

MLV lipoplexes according to the study of Gonçxalves et al. (2004).

Size of the complexes has been reported to affect the transfection patterns by affecting their biodistribution profile upon administration to the blood circulation, extent of cell association and intracellular trafficking after their internalization (Penacho et al. 2010; Birchall et al. 1999). For example, lipoplexes can gain access into the cells by different sorts of endocytosis; lipoplexes larger than 200 nm may internalize through the caveolin-mediated endocytosis but smaller ones can be internalized via clathrin-mediated endocytosis (Simões et al. 2005). Some studies show that TE is largely dependent upon the lipoplex charge ratio (+/−), and that lipoplex size affect the TE indirectly by affecting final charge ratio of lipoplex (Koumbi et al. 2006).

3.5. Medium conditions

Both complexation ionic strength and solvent medium composition, especially the presence of serum, are reported to exert critical effects on TE. Koumbi et al. (2006) examined the impact of effective unilamellar vesicles GLB.73-gene lipoplex formation medium upon transgene expression in K562 cells by using effective unilamellar vesicles, a pronounced enhancement of transgene expression was detected in solvent systems of 300 mM NaCl (Fig. 9). The CLs-mediated TE was reported to be greatly lowered in the presence of serum in most cases (Radchatawedchakoon et al. 2010; Xu and Anchordoquy 2008), because proteins like serum albumin may change the physicochemical properties of lipoplex, block lipoplex association with cell membranes, reduce their ability to aggregate at the membrane and lead to release and degradation of genetic material (Ross and Hui 1999; Li et al. 1999). However, after intravenous administration, the nanoscopic gene delivery systems need to be stable in serum. Therefore information on the integrity of intravenously injected lipoplex in serum is crucial, and an intense effort to develop CLs that efficiently deliver genes in the presence of serum is in need. It is possible to decrease the lipoplexes susceptibility to serum by varying their size, or composition, i.e. weight ratio, type of co-lipid, or chemical structure of cationic lipid (Esposito et al. 2006). For instance, the presence of serum during lipoplex incubation with the cell culture has a negligible effect on DOTAP/DOPE, DC-Chol/DOPE and DDAB/DOPE lipoplexes at various charge ratio (Masotti et al. 2009, Zhang et al. 2010). Also, CL formulations possessing a higher cholesterol content exhibited a greater resistance to serum-induced aggregation (Xu and Anchordoquy 2008).

4. Recent application of cationic liposomes in gene therapy

CLs are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors, therefore they are potentially attractive for clinical applications (Oliveira et al. 2009; Miller 2004). In this article, we will introduce some most recent application of CLs as gene delivery systems to cure cancer, bone damage, infection and other diseases.

CLs prepared from DOPE and DC-Chol were reported to significantly enhance the growth inhibitory effect of ASODN against the human telomerase transcriptase on human cervical adenocarcinoma cells both *in vitro* and *in vivo* (Han et al. 2008). Surface-complexed DNA-hsp65 in egg phosphatidylcholine /DOTAP/DOPE (50/25/25% molar) liposomes was also found to be effective as a single-dose tuberculosis vaccine (de la Torre et al. 2009). In another work, the potential of CLs-DNA complexes associated with transferrin to mediate gene transfer into osteoblast-like cells was proved (Oliveira et al. 2009). Cardoso et al. (2008) demonstrated that transferrin-lipoplexes can mediate efficient gene silencing in neuronal cells, both *in vitro* and *in vivo*, which may be useful for therapeutic approaches to neuronal protection and repair. Kinet et al. (2009) used a gene transfer method based on cationic liposomes to produce 16K hPRL and demonstrate that 16K hPRL inhibits tumor growth in a subcutaneous B16F10 mouse melanoma model. Transcutaneous immunization by lipoplex-patch based DNA vaccines against Japanese encephalitis virus infection was achieved overcoming the stratum corneum barrier of the skin without carrying out any skin penetration (Cheng et al. 2009).

As it has been generally reported that the CLs are superior to both neutral and anionic liposomes in entrapment efficiency and safety as drug carrier (Flasterstein et al. 2010; Meng et al. 2008; Abu Lila et al. 2009; Henriksen-Lacey et al. 2010; Brgles et al. 2009; Bhowmick et al. 2010), a co-delivery system of gene and drug mediated by CLs has also been developed. Faneca et al. worked on the combined antitumoral effect of vinblastine with *Herpes simplex* virus thymidine kinase gene and ganciclovir (HSV-Tk/GCV) "suicide" gene therapy mediated by human serum albumin (HSA)-associated lipoplexes, in mammary adenocarcinoma cells (TSA cells). The simultaneous addition of vinblastine and HSA-EPOPC:Chol/DNA (+/−) (4/1) lipoplexes to TSA cells improved transgene expression more than 10 times and induced a great enhancement in the antitumoral activity in TSA cells, allowing the use of a much lower dose of the drug to achieve the same therapeutic effect (Faneca et al. 2008). Xiao et al. (2010) simultaneously delivered doxorubicin and the plasmid encoding the phosphorylation-defective mouse survivin threonine 34 → alanine mutant (Msurvivin T34A plasmid) to the same cells through the CLs, which was modified with truncated human basic fibroblast growth factor peptide, and achieved ideal a synergistic/combined anti-tumor effect to same cells *in vitro* and *in vivo.*

5. Conclusion

As a new promising therapeutic method in gene delivery, CLs-gene complexes present unique structures and properties, which always interact with each other and place a significant effect on the transfection behavior of lipoplex. In order to develop more efficient CLs gene carriers, it is necessary to figure out these complex and even contradicting research results, especially intricate influence of particle diameter, serum, lipoplex structure and so on.

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