African Journal of Pharmacy and Pharmacology Vol. 5(17), pp. 2007-2012, 8 November, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.331
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

Cationic liposomes as gene delivery system

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Accepted 27 July, 2011

Delivery of oligonucleotides (ONs) and genes to their intracellular targets is a prerequisite for their successful use in medical therapy. Cationic liposomes are among the most commonly used and promising delivery system for ONs and genes. Cationic-liposomes and their complexes with ON were characterized according to complex size, zeta potential measurements, transmission electron microscope (TEM) and confocal laser scanning microscope (CLSM). It is successfully demonstrated that cationic liposomes, dispersed in 10% serum-containing growth medium, efficiently delivered ON to HeLa cells. Indeed, intact ON was found in the cytoplasm and nucleus only when delivered by cationic liposomes. The results suggest that cationic lipid-based delivery systems can be efficient for gene delivery if their biophysical properties can be properly controlled.

Key words: Cationic liposomes, HeLa cells, gene delivery, characterization.

INTRODUCTION

Gene therapy is based on the introduction of specific exogenous sequences of deoxyribonucleic acid (DNA) into the target cells for production of the therapeutic gene product (Crystal, 1995; Lasic and Templeton, 1996). The prerequisite for successful gene therapy is efficient and safe delivery of DNA into the cells. Because of the fast progress of nucleic acid-based technologies in the treatment of diseases, the call for appropriate delivery vehicles becomes increasingly important. The ideal vehicle should avoid immediate uptake by the mononuclear phagocyte system and have prolonged circulation in blood, thus increasing the probability of reaching the desired targets. In addition, the vehicle should be able to deliver its contents efficiently into the cell cytoplasm, avoiding lysosomal degradation (Shepushkin et al., 1997). The most widely used types of vehicles for gene delivery are: viral (e.g., adenovirus, retrovirus and adeno-associated virus) and non-viral (for example, liposomes, polymer and peptides) (Lasic, 1999). Viral vectors are often highly efficient, but safety and immunogenicity are issues of potential concern, and the limited transgene size often possesses a serious obstacle (Mady et al., 2004). Nonviral vectors, on the other hand, frequently face the problem of low transduction efficiency.

Among the non-viral vectors, cationic liposomes are the most widely used vectors. Although less efficient in delivering the genes than the virus, they have many important qualities such as being much less or non-immunogenic, have no known limitation in the size of the DNA, can be custom-synthesized for targeting and easily scalable for large-scale production. Moreover, the liposome can deliver different kinds of DNA (super coiled or linear) or ribonucleic acid (RNA) with or without proteins, even to non-dividing cells and are usually composed of biodegradable lipids. Also, covalent attachment of target specific ligands on the liposome can facilitate targeted delivery of genes (Mady et al., 2004). These advantages have prompted researchers to explore the applications of cationic liposomes in gene therapy clinical trials (Li and Huang, 1997; Lasic and Papahadjopoulos, 1998; Mady, 2007).

Cationic lipids were used for the first time, for gene delivery, by Felgner et al (1987), and for ON delivery a few years later by Bennett (1993). When cationic liposomes are used, no encapsulation is needed because the lipids bind electrostatically to negatively charged

Abbreviations: ONs, Oligonucleotides; **TEM,** transmission electron microscope; **CLSM,** confocal laser scanning microscope; **DNA,** deoxyribonucleic acid; **DOTAP** 1, 2 - dioleoyl-3 trimethylammonium-propane; **FCS,** fetal calf serum; **PI,** propidium iodide; **DOPE,** dioleoylphosphatidylethanolamine.

nucleotides. Despite their widespread use as transfection reagents, information about the interactions of cationic lipids and polynucleotide is sparse (Mönkkönen and Urtti, 1998). Very little is understood about the events which take place when cationic liposomes interact with mammalian cells or the processes which result in the delivery of nucleic acids. Three model of the interaction of cationic lipid / polynucleotide complex with cells have been proposed: (i) direct fusion with the plasma membrane (Felgner et al., 1987; Lewis et al., 1996), (ii) endocytosis and subsequent fusion or destabilization of endosome membrane (Legendre and Szoka, 1992; Felgner et al., 1994; Zhou and Huang, 1994; Zabner et al., 1995), and (iii) translocation through pores across the plasma membrane (Engberts and Hoekstra, 1995).

The physicochemical properties, such as particle sizes and surface charges of the liposome-DNA and/or oligonucleotides (ON) complexes may be important factors to obtain a higher transfection efficiency of the liposomal vectors. Although gene transfection of plasmid and/or ON complexed with cationic liposomes is investigated, little attention seems to be paid to understanding their physico- chemical characteristics and cellular uptake mechanisms. The intent in this study was to characterize ON /liposome complexes in terms of ζ potential and particle size and to see whether these physicochemical properties have any influence on their disposition characteristics and cellular uptake process. In the presence of serum, we investigated that cationic liposomes efficiently delivered ON to HeLa cells.

MATERIALS AND METHODS

1, 2 - dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Fetal calf serum (FCS), L-Glutamin (200 nM solution), Penicillin 5000 units/streptomycin 5000 mg, and DMEM (Dulbecco's modified Eagle's medium) was purchased from Bio Whittaker Europe, Verviers, Belgium. HeLa cells were obtained from Children Hospital. DNA- analogues of chimeric ON as the one used here will be referred to as FIXDNA-DNA. A 5´FAM (Eurogentec EGT Group, 4102 Seraing- Belgium) labeled 68-mer of sequence (5´-TGT-CAA-GCA-GAT-CGT-GGG-GGA-CCC-CTT-TTG-GGG-TCC-CCC-ACG-ATC-TCC-TTG-ACA-GCG-CGT-TTT-CGC-GC-3´). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, Oreg. USA). All other reagents were of analytical reagent grade.

Preparation of liposomes

Liposomes were formulated according to well-established methods of extrusion (Olson et al., 1979). In short: the appropriate phospholipid composition was mixed in organic solvent in a 50 ml round flask. The organic solvent was evaporated to dryness by a rotary evaporator. The resulting lipid suspension was extruded through 100 nm polycarbonate membranes using mini- extruder (Liposofast, Avestin Inc., Canada). Size measurement was done by dynamic laser light scattering and the size was in the range of 100 nm.

Condensation of ON

DOTAP liposomes were added separately to 2.5 µg ON to achieve the desired charge ratio (+/-). The fluorescence intensity of ON (excitation at 492 nm and emission at 515 nm) was measured by using Perkin Elmer Spectrofluorometer LS 50B (UK). The hydrodynamic diameters of complex were measured by using Zetasizer 3000 HS, Malvern Instruments, Germany.

Transmission electron microscopy

DOTAP/ON (8:1 +/-) complex was also characterized by using negative stain electron microscope (EM 109, Zeiss, West Germany). On a copper grid, the appropriate concentration from each sample was added. Then add one drop of 20% uranyl acetate; wait for 2 min at room temperature; remove the excess solution with a filter paper; then the sample was examined under the transmission electron microscope.

Zeta potential

In deionized water, we dispersed the pure ONs and their complexes with DOTAP (1:8 -/+) and then measured the corresponding zeta-potential ζ (n=5) by using Zetasizer 3000 HS, Malvern Instruments, Germany.

ON transfection experiment

To investigate the gene expression or transfer efficiency, HeLa cells were grown on glass cover slips in six-well plate (10^5-10^6 cells per well) in DMEM medium supplemented with 10% FCS, 1% glutamine and 1% penicillin-streptomycin solutions. The transfection system (ON/DOTAP 1:8 -/+) complexes and cells were incubated for 6 hours at 37°C in 5% CO_2. The cells were washed away by rinsing three times with cold PBS. Cells nuclei were stained with PI stain. Cells were fixed with formaldehyde. Turn the cover slip containing the cells on a Moviol drop on a glass slide and examine with confocal laser scanning microscope. We used True Confocal Scanner, Leica DM R with A4, L5, N3 and Y5 filters, Leica, Wetzlar, Germany and Leitz DM RXE upright microscope with a Krypton/Argon laser (emission wavelengths of 488, 578, and 647 nm) was used. Images were converted to TIF-format with Scanware 5.1 Scion Corporation, Frederick, MD, USA. Cellular distribution of the FAM-ON, complexed with DOTAP liposomes, was investigated in HeLa cells following the kinetics of this process using confocal laser scanning microscope (CLSM). In general, liposomes were in the range of 100 nm in diameter. ON and liposomes were appropriately diluted in 10 mM tris buffered saline (pH 7.8). The final complex had a size of approximately 150 nm, which was diluted with the appropriate cell cultured medium containing 10% FCS and then added to HeLa cells.

RESULTS AND DISCUSSION

ON condensation

The condensing agents must not only efficiently condense ON but also require the ability to be effectively displaced from ON, allowing for subsequent transcriptional and translational events to occur (Sorgi,

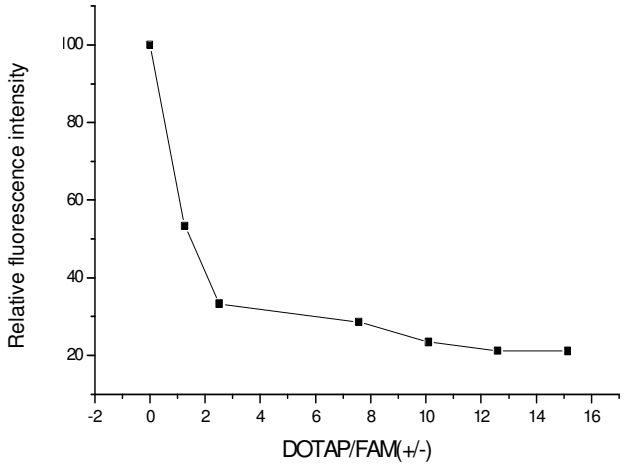

Figure 1. Condensation of FAM by DOTAP liposomes (Excitation wavelength at 492 nm and emission wavelength at 515 nm).

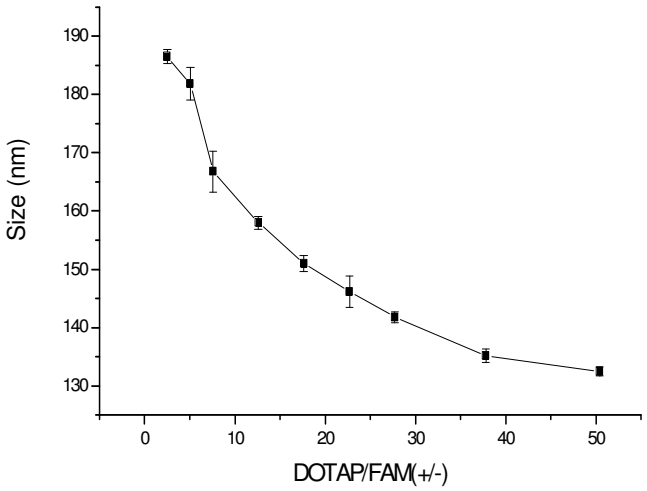

Figure 2. Hydrodynamic diameters of DOTAP/FAM complexes versus different complexes charge ratio (+/-); n = 3.

1998). In order to condense FAM-ON, increasing amounts of DOTAP-liposomes were added to the FAM-ON solution. As seen in Figure 1 the addition of DOTAP to FAM solution resulted in a rapid decrease in fluorescence intensity of FAM-ON. This loss in fluorescence intensity can be attributed to the condensation of ON. This is thought to occur due to electrostatic interactions between cationic lipid and ON, resulting in a charge neutralization of the complex and the formation of a condensed structure. This condensed structure, due to its diminished size, may be more readily endocytosed by the cell, resulting in the increased levels of transgene expression.

The complex is examined by several physical methods to gain insight into the nature of the interaction among the components and the mechanism of the enhanced transfection activity. We first looked at the effect of cationic lipid on the complex size. Figure 2 shows that the size of the complex was dependent on the charge ratio of DOTAP/ON. As the DOTAP content increases, vesicle size is reduced. Results indicate that, at a low charge ratio (2.5:1 +/-), the size of the complex was 186.5 nm. However, as the DOTAP content increases; complex size is reduced to approx. 132 nm at a charge ratio (50:1 +/-). The data indicates the condensation ability of ON by DOTAP liposomes (Sternberg et al., 1994; Li et al., 1998; Perrie and Gregoriadis, 2000).

There are several possibilities that may explain the potentiation effect of the condensing agent on the transfection activity of liposomes. First, on the basis of the current endocytosis model, there is a size limitation for particles to be taken up efficiently by cells (Machy and Leserman, 1983). Direct size measurement of the complexes showed that the condensing agent significantly reduced the size of the complex formed over a wide range of DOTAP/ON ratios.

Transmission electron microscopy

Negative stain electron microscopy is a useful method for addressing questions concerning size distribution of liposome, and although obtaining quantitative data is laborious, negative staining is a reliable technique, which is simple to perform and requires only limited specialized equipment, which should be available in any electron microscopy laboratory (Haschmeyer and Myers, 1972).

Morphological studies demonstrated that spherical DOTAP/ON complexes are formed (Mönkkönen and Urtti 1998). Electron micrograph of DOTAP/ON (8:1 +/-) complex was shown in Figure 3. The majority of the particles in the pictures appeared spherical, small (≤ 100 nm in diameter), electron dense and some of them were associated with low-density lipid membranes. These complexes of small size should be more favorable to enter the cells via an endocytosis pathway than the larger ones.

Zeta potential measurements

Transfection complex formation is based on the interaction of the positively charged lipid with the negatively charged phosphate groups of the nucleic acid. The information of the overall charge of transfection complexes by zeta-potential measurements can be speed up the development of better non-viral DNA delivery vectors for gene therapy (Son et al., 2000; Mady et al., 2004). The following physical chemical parameters were used in the determination of zeta-potential: medium

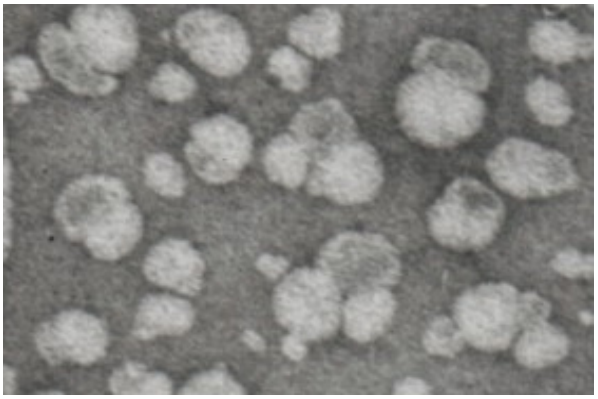

Figure 3. Negative stain electron micrograph of DOTAP/ON (8:1 +/-).

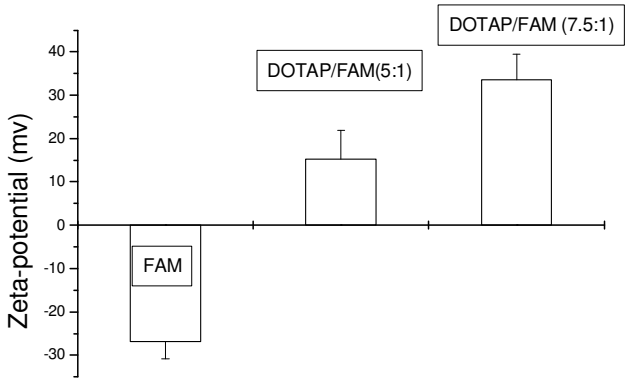

Figure 4. Zeta potential measurements of different formulations (n=5).

viscosity 0.89 cPoise, medium refractive index 1.333, temperature 25°C and dielectric constant 79.

Results in Figure 4 show trends of increasing zeta-potential values with increasing DOTAP content. Values of the zeta-potential of liposomes indirectly reflect vesicle surface net charge and can therefore be used to evaluate the extent of interaction of the liposomal surface cationic charges with ON. On this basis, the zeta-potential of DOTAP liposomes was investigated before and after complexing with FAM-ON. Results show the negatively zeta-potential of the naked FAM (-26.8 ± 4). After addition of DOTAP to FAM in charge ratio of (5:1 +/-), the zeta-potential value becomes positive (15.2 ± 6.6 mV). Also, it was more positive (33.5 ± 6**)** for DOTAP/FAM (7.5:1 +/-).

ON transfection

ONs are promising therapeutic agents against viral infections and cancer. However, problems with their inefficient delivery and inadequate stability have to be solved before they can be used in therapy. Cellular uptake of ON is highly inefficient, as their net negative charge and high molecular weight prohibit efficient transfer across cell membrane without the help of carrier system (Bennett 1993; Ledley, 1995; Welz et al., 2000).

Confocal microscopy is a well-established technique for the investigation of 3D structures in biological and industrial materials (Mady et al., 2009). The basis of this success is the optical sectioning capability of this type of microscopy, which enables one to study 3D-structure of intact specimens in their natural environment. CLSM has the major advantages that it yields a very short depth of focus, its transverse definition and the contrast of the image are better than a standard microscope, the device is very well-suited for optical cross-sectioning and with the use of a laser beam, the intensity of illumination can be very high. With CLSM one can slice incredibly clean, thin optical sections out of thick fluorescent specimens; view specimens in planes running parallel to the line of sight; penetrate deep into light-scattering tissues; gain impressive three-dimensional views at very high resolution; and improve the precision of microphotometry. Figure 5 shows that DOTAP-liposomes effectively transport ON into the nucleus of HeLa cells, in the presence of serum. DOTAP-liposomes improved the cellular delivery of ON by protecting it in FCS, by increasing the total uptake of ON in cells, and by increasing the nuclear localization of ON in cells. Complexation with cationic lipids facilitates the entry of ON into cells. ON-cationic lipid complexes enter the cells via endocytosis and they promote the nuclear entry of ON (Bennet et al., 1992; Zelphati and Szoka, 1996a). After their cellular delivery with cationic lipids, the ONs are mostly seen in the nucleus, whereas the lipids are entirely localized in the cytoplasm and, particulay, in some perinuclear regions (Zelphati and Szoka, 1996a). Zelphati and Szoka (1996b) showed that ONs were released from the complexes as a result of lipid intermixing in the endosomal wall.

The cationic lipid gene delivery system invariably comprises three components: a cationic lipid, a neutral co-lipid, which is most often dioleoylphosphatidylethanolamine (DOPE) and the plasmid DNA that encodes the transgene of interest. Although the liposomal gene delivery systems require a neutral co-lipid, usually DOPE for optimal activity, the aggregation of phosphatidylethanolamine containing liposomes by serum proteins has been observed (Forbes et al., 1984). In the case of cationic lipid based delivery systems, the charge density of the lipid bilayer can significantly affect the colloidal stability of the particles, with mole fractions of helper lipids [for example, (DOPE)] of > 0.4 producing considerable instability at elevated ionic strength. So, in the present work, DOTAP liposomes are only used to investigate how physicochemical methods can be used to

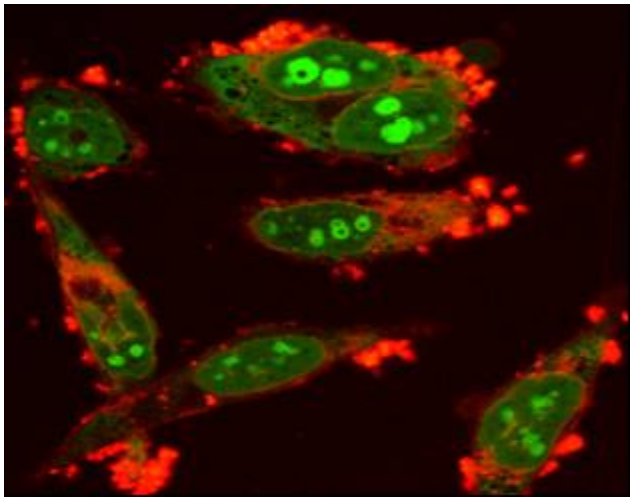

Figure 5. Confocal laser scanning microscopy of HeLa cells incubated with DOTAP/FAM (8:1 +/-) complexes in DMEM supplemented with 10% FCS, at 37°C and 5% CO_2 after 6 h.

assess the functionality of the ON-lipid complexes *in vivo*.

Most mammalian cells require the addition of serum to the culture medium for optimal growth and maintenance of the cell lines *in vitro*. The presence of serum often reduces the transfection efficiency of liposomal vectors (Felgner et al., 1987; Felgner and Ringold, 1989; Gao and Huang, 1995; Lee and Huang, 1997; Mady et al., 2009). This may be due to the premature release of DNA from the complexes or its degradation by the nucleases. So, in the presence of serum, we investigated that cationic liposomes efficiently delivered ON to HeLa cells.

Analytical methods are described that provide a platform for systematically evaluating the effect of formulation variables in the development of ON-lipid non-viral gene therapy complexes. The methods encompass those used to characterize the components, that is, ON and liposomes, as well as those applied to the complexes themselves. Several lines of evidence obtained from studies on complex size; zeta-potential; electron microscope and CLSM indicate that ON is entrapped within the aqueous compartments, in between bilayers, presumably bound to the cationic charges. The focus is on physicochemical methods so that their parameters can be assessed in relationship to the functionality of the ON-lipid complexes *in vivo* (Perrie and Gregoriadis, 2000; Hutchins, 2000).

More studies are needed to compare and investigate the role of fusogenic helper lipids such as DOPE and cholesterol or peptides to disrupt the endosome membrane so as to facilitate the escape of DNA from endosome. Also, covalent attachment of target specific ligands on the liposome can facilitate targeted delivery of genes (Mady et al., 2004). These advantages have prompted researchers to explore the applications of cationic liposomes in gene therapy clinical trials.

ACKNOWLEDGEMENT

Author extends his appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No RGP-VPP-121.

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