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# Retinal gene delivery enhancement by lycopene incorporation into cationic niosomes based on DOTMA and polysorbate 60

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ABSTRACT

The present study aimed to evaluate the incorporation of the natural lipid lycopene into niosome formulations based on cationic lipid DOTMA and polysorbate 60 non-ionic surfactant to analyze the potential application of this novel formulation to deliver genetic material into the rat retina. Both niosomes with and without lycopene were prepared by the reverse phase evaporation method and physicochemically characterized in terms of size, zeta potential, polydispersity index and capacity to condense, release and protect the DNA against enzymatic digestion. *In vitro* experiments were performed in ARPE-19 cells after complexion of niosomes with pCMS-EGFP plasmid at appropriate cationic lipid/DNA ratios. At 18/1 mass ratio, nioplexes containing lycopene had nanometric size, positive zeta potential, low polydispersity and were able to condense, release and protect DNA. Percentage of transfected cell was around 35% without compromising cell viability. The internalization pathways studies revealed a preference to caveolae mediated endocytosis and macropinocytosis, which could circumvent lysosomal degradation. Both subretinal and intravitreal administrations to the rat retina showed that nioplexes were able to transfect efficiently the outer segments of the retina, which offer reasonable hope for the treatment of many inherited retinal diseases by a safe non-viral vector formulation after the less invasive intravitreal administration.

## 1. Introduction

The abnormal expression or activity of numerous retinal proteins has been linked to the pathogenesis of several blinding retinal disorders with a genetic background, such as Leber congenital amaurosis [1], age-related macular degeneration [2] or retinitis pigmentosa [3]. Unfortunately, most of these devastating conditions do not have effective treatment at the moment. Although novel approaches, such as enzyme/protein replacement and stem cell-based therapies have shown recently promising results, gene therapy is by far the most well-developed field of research for the treatment of both inherited and acquired retinal disorders [4,5]. The unique anatomical and histological features of the eye provide both benefits and challenges for the progress in gene-based ocular therapeutics [6].

In the last decade, many viral and non-viral gene delivery approaches have been developed for the treatment of many retinal pathologies [7,8]. Compared with their counterparts, non-viral vectors have attracted great attention as safer alternative to deliver genetic material, since they can circumvent many safety issues that are still associated with viral gene delivery systems, such as immunogenicity, mutagenicity and oncogenic effects [9]. Consequently, the use of non-viral vectors in clinical trials has increased since 2004, while that of viral vector has decreased significantly [10]. Actually, cationic lipids and cationic polymers are the most commonly used non-viral vectors [10,11]. However, to date, one of the main problems that non-viral formulations have to face, in order to reach the clinical practice, is their limited transfection efficiency. Therefore, research activity on this area merits special attention for the scientific community [12].

As drug delivery system, niosomes have received growing attention by time for being osmotically active and chemically stable formulations [13]. Besides, when it comes to easy handling and low toxicity, they are considered quite advantageous over the well-known liposomes [14].

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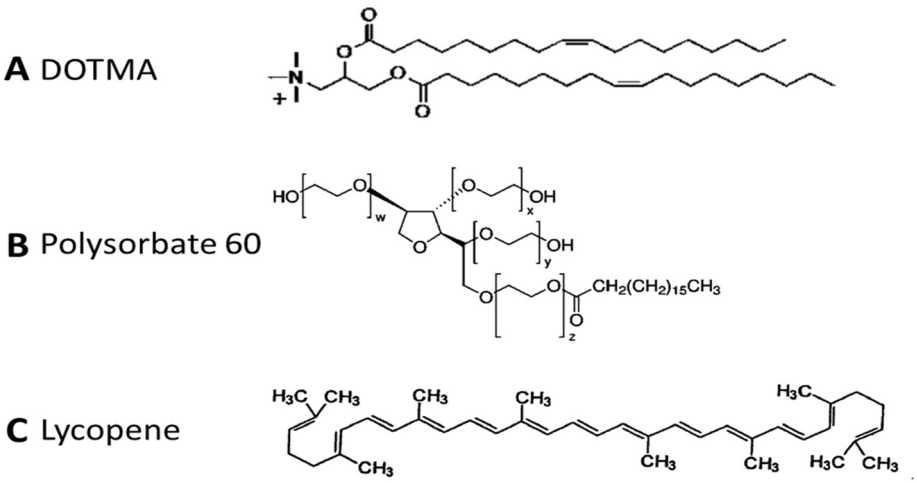


**Fig. 1.** Chemical structures of the cationic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) (A), Polysorbate 60 (B), and Lycopene (C).

However, their use as gene delivery systems has been poorly studied, although some recent results have revealed their appealing properties to transfect efficiently brain and retinal cells in rats [15–17].

Niosomes, for gene delivery purposes, are self-assembled vesicular nano carrier systems composed typically by non-ionic surfactant, "helper" and cationic lipids [18]. The non-ionic "electrically neutral" surfactants enhance the stability of niosome formulations [19]. Additionally, cationic lipids form complexes by electrostatic interactions upon the addition of negatively charged genetic material [15,16], and "helper" lipids have a marked influence on both the physico-chemical and biological properties of niosome gene carriers [15,17,20].

Recently, it has been reported on the literature the flattering properties of the "helper" lipid squalene (a natural lipid that belongs to the terpenoid family) in cationic niosome gene delivery formulations. Therefore, we decided to investigate the effect that lycopene, another natural lipid, could have on a niosome formulation based on cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and non-ionic surfactant polysorbate 60. Lycopene is a carotenoid that contains 40 carbon and 56 hydrogen atoms (Fig. 1-C). Classically, it is known to be one of the most potent natural antioxidants that mediate cytoprotective, immunomodulatory and anticancer activities [21]. Additionally, lycopene can be found at high concentration levels in the eye, where it has shown both anti-inflammatory and anti-angiogenic effects [22].

We designed two novel niosome vector formulations for retinal gene delivery purposes based on the same cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and the same non-ionic surfactant polysorbate 60, in the absence/presence of lycopene (DP60 and DP60L, respectively). Both niosomes were elaborated by the solvent emulsification-evaporation technique and compared in terms of particle size, polydispersity index (PDI) and zeta potential. Upon the addition of the reported pCMS-EGFP plasmid at different cationic lipid/DNA ratios (w/w), nioplexes were obtained and characterized by size, PDI, morphology, and the ability to condense, release and protect the DNA from enzymatic digestion. *In vitro* experiments were performed to compare the behavior of both vectors in ARPE-19 cells regarding their cellular uptake, transfection efficiency, viability, and internalization mechanism. Following the *in vitro* characterization, the most promising formulation was administered to rat eyes *via* intravitreal and subretinal injection in order to evaluate transfection efficiency by confocal microscopy in both whole-mount and sagittal cross sections of the retina.

## 2. Materials and methods

### 2.1. Production of cationic niosomes

Niosomes were elaborated with slight modifications of the previously described reverse phase evaporation method [23]. Briefly, 5 mg of cationic lipid DOTMA D (Avanti Polar Lipids Inc., Alabama, USA) and 26 mg of polysorbate 60 P60 (0.5%, w/v, Sigma-Aldrich, Madrid, Spain) with/without 1 mg lycopene L (Sigma-Aldrich, Madrid, Spain) were dissolved in 1 ml of organic solvent, dichloromethane (Panreac, Barcelona, Spain). The emulsions were obtained by sonication of such organic phase with 5 ml milliQ water for 30 s at 45 W (Branson Sonifier 250®, Branson Ultrasonics Corporation, Danbury, USA). Dichloromethane was removed from emulsions by evaporation under magnetic agitation for 2 h leaving the cationic nanoparticles in the aqueous medium. The corresponding molar ratios of both DP60 and DP60L formulations were 1:4 and 1:4:0.4, respectively.

### 2.2. Plasmid propagation and elaboration of nioplexes

pCMS-EGFP plasmid (5541 bp, Plasmid Factory, Bielefeld, Germany), was propagated in *Escherichia coli* DH5-α and purified using the Qiagen endotoxin-free plasmid purification Maxi-prep kit (Qiagen, California, USA) according to the manufacturer's instructions. The purified plasmid DNA was quantified by measuring absorbance at 260 nm in a NanoDrop® Spectrophotometer (Thermo Fisher Scientific Inc. Denver, USA). The purity of the plasmid was verified by agarose gel electrophoresis (Bio-Rad, Madrid, Spain) in Tris Borate-EDTA buffer, pH 8.0 (TBE buffer). DNA bands were detected using GelRed™ (Bio-Rad, Madrid, Spain) to stain DNA, and images were observed with a ChemiDoc™ MP Imaging System (Bio-Rad, Madrid, Spain). The stock solution of pCMS-EGFP plasmid (0.5 mg/ml) was estimated to be around 0.14 μM.

Both DP60 and DP60L nioplexes (niosome/DNA complexes) were elaborated by mixing an appropriate volume of a stock solution of pCMS-EGFP plasmid (0.5 mg/ml) with different volumes of the niosome suspensions (1 mg cationic lipid/ml) to obtain different cationic lipid/DNA mass ratios (w/w). The mixture was left for 30 min at room temperature to enhance electrostatic interaction between the cationic niosomes and the negatively charged plasmid.

### 2.3. Characterization of niosomes/nioplexes

Particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) with Zetasizer Nano ZS (Malvern Instruments, UK). Determination of zeta potential by Laser Doppler