International Journal of Pharmaceutics 550 (2018) 388–397

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Polysorbate 20 non-ionic surfactant enhances retinal gene delivery efficiency of cationic niosomes after intravitreal and subretinal administration

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ARTICLE INFO

*Keywords:*
Non-viral vector
Gene therapy
Retina
Non-ionic surfactants
Niosomes

ABSTRACT

The success of non-viral vectors based on cationic niosomes for retinal gene delivery applications depends on the ability to achieve persistent and high levels of transgene expression, ideally from a single administration. In this work, we studied the effect of the non-ionic surfactant component of niosomes in their transfection efficiency in rat retina. For that purpose, three niosome formulations that only differed in the non-ionic tensioactives were elaborated. Niosomes contained: cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), helper lipid squalene and polysorbate 20, polysorbate 80 or polysorbate 85. Niosomes and corresponding nioplexes were fully characterized in terms of size, polydispersity index, zeta potential, morphology and ability to protect and release DNA. *In vitro* experiments were carried out to evaluate transfection efficiency, cell viability and intracellular trafficking pathways of the formulations. Nioplexes based on polysorbate 20 niosomes were the most efficient transfecting retinal cells *in vitro*. Moreover, subretinal and intravitreal administration of those nioplexes *in vivo* showed also high levels of transgene expression in rat retinas. Our results demonstrate that the incorporation of polysorbate 20 in cationic niosomes enhances retinal gene delivery. Thus, this formulation emerges as a potential non-viral candidate to efficiently transfer specific therapeutic genes into the eye for biomedical purposes.

## 1. Introduction

Several monogenic retinal disorders are well characterized, with identified mutations that most often affect to specific genes in retinal pigment epithelium (RPE) cells (Koirala et al., 2013). RPE cells perform pivotal functions required for the maintenance of the neural retina, such as protecting the photoreceptor cells and secreting growth factors (Strauss, 2005). In many retinal diseases, including Leber's congenital amaurosis (LCA), retinitis pigmentosa (RP) and age-related macular degeneration (AMD) blindness occurs due to RPE degeneration, which results in photoreceptor loss or dysfunction (Koirala et al., 2013). Therefore, RPE cells constitute the main target of most ocular gene therapy strategies. Tight junctions between RPE cells form the blood retina barrier, which is responsible of the low ocular bioavailability of systemically administered drugs (Conley and Naash, 2010). However, the same barriers that make ocular administration difficult also help the eye maintain its immune privilege, making intraocularly-delivered molecules less likely to induce severe immune responses than their systemically-delivered counterparts (Andrieu-Soler et al., 2006). The success of retinal gene transfer strategies depends on the ability to achieve persistent and high levels of transgene expression in RPE cells, ideally from a single administration.

Nucleic acids can be delivered into the retina both through viral or non-viral vectors. Some clinical trials using viral vectors have shown

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https://doi.org/10.1016/j.ijpharm.2018.07.035
Received 28 March 2018; Received in revised form 29 June 2018; Accepted 11 July 2018

encouraging results (Bainbridge et al., 2008; Maguire et al., 2008). However, some limitations of viral vectors such as safety concerns, expensive production costs (Pezzoli et al., 2012) and that many disease genes of the RPE and retina are too large to fit into viral vectors (Koirala et al., 2013) have motivated the development of non-viral vectors for ocular gene delivery. Non-viral vectors present low immunogenicity, high nucleic acid packing capacity, ease of fabrication, high reproducibility and acceptable costs compared to their counterparts (Jin et al., 2014). Among the wide plethora of non-viral vectors, recently emerged niosomes are biocompatible, synthetic, non-ionic surfactant vesicles with a closed bilayer structure (Choi et al., 2004; Grimaldi et al., 2016) and they are based on three principal components: cationic lipids, helper lipids and non-ionic tensioactives (Ojeda et al., 2015; Ojeda et al., 2016). The global chemical properties of these components influence on the physicochemical characteristics of niosomes, such as size, surface charge and morphology, which in turn determine their ability to enter the cells, follow a particular endocytic pathway and deliver the DNA cargo into the nucleus (Dabkowska et al., 2012; Liu et al., 1996). Our research group has previously evaluated the effect of cationic lipids (Ojeda et al., 2016) and helper lipids (Ojeda et al., 2016) in retinal transfection, and has reported transgene expression in retinal cells both *in vitro* (Ojeda et al., 2016) and *in vivo* (Ojeda et al., 2016; Puras et al., 2014). In order to optimize the design of niosome formulations for retinal gene delivery applications, in this work we evaluated the effect of the non-ionic surfactant of niosomes on the retinal transfection process and efficiency.

For that purpose, we elaborated three niosome formulations that only differed in the non-ionic surfactant component. Niosomes used in this work contained the cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), combined with the helper lipid squalene and one of the following non-ionic surfactants: polysorbate 20, polysorbate 80 or polysorbate 85, differing in their chemical structure and hydrophile-lipophile balance (HLB) values Fig. 1). The cationic lipid DOTMA has been successfully used in niosome formulations for gene delivery to the retina (Mashal et al., 2017). Squalene is a natural lipid belonging to the terpenoid family (Reddy and Couvreur, 2009) that enhances the stability of niosome formulations (Kim et al., 2003). Polysorbate 80 has been used combined with squalene obtaining effective transgene expression in rat retina *in vivo* (Puras et al., 2014), but the effect of other non-ionic tensioactives such as polysorbate 20 and 85 combined with squalene and DOTMA has not been studied for retinal gene delivery. The three niosome formulations elaborated in this work were named DST20, DST80 and DST85, according to their composition. Niosomes were complexed to the pCMS-EGFP reporter plasmid or to a fluorescein isothiocyanate (FITC) labeled pCMS-EGFP plasmid to form nioplexes. Niosomes and nioplexes were characterized in terms of size, polydispersity index (PDI), zeta potential, morphology and ability to protect and release DNA. *In vitro* experiments were carried out in human embryonic kidney cells (HEK-293) and retinal pigment epithelial cells (ARPE-19) to evaluate cell viability and transfection efficiency. In order to examine the cell entry and intracellular trafficking process of the three formulations in ARPE-19 cells, co-localization analysis were performed between the nioplexes (niosomes/FITC stained DNA) and the stained endocytic pathways. The pathways under study were the clathrin mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and late endosomal compartments (Lys). The best of those formulations was used to transfect primary cultures of rat retinal cells and was administered into rat eyes in order to evaluate the capacity of the formulation to deliver genetic material into the retina after subretinal and intravitreal injections.

## 2. Materials and methods

### 2.1. Preparation of niosomes and nioplexes

Niosomes based on cationic lipid DOTMA (Avanti Polar Lipids, Inc., Alabama, USA), helper lipid squalene (Sigma-Aldrich, Madrid, Spain) and polysorbates 20, 80 and 85 (Sigma-Aldrich, Madrid, Spain) were prepared using the o/w emulsification technique, as previously described (Ojeda et al., 2016). Three different niosome formulations were prepared to a molar ratio of 2 mM cationic lipid – 8 mM helper lipid – 4 mM tensioactive. Briefly, 6.70 mg of the cationic lipid were gently ground with 19 µl of squalene. Then, 1 ml of dichloromethane (DCM) (Panreac, Barcelona, Spain) was added and emulsified with 5 ml of the non-ionic surfactant aqueous solution – at different w/v percentages to obtain the final molar relation- containing either polysorbate 20 (0.49% w/v), 80 (0.52% w/v) or 85 (0.17% w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W. The organic solvent was removed from the emulsion by evaporation under magnetic agitation for 3 h at room temperature, obtaining niosome solutions DST20, DST80 and DST85.

The nioplexes were elaborated by mixing an appropriate volume of a stock solution of either a pCMS-EGFP plasmid (0.5 mg/ml) (Clontech Laboratories, Inc., USA) or a fluorescein isothiocyanate (FITC) labeled pCMS-EGFP plasmid (0.5 mg/ml) (DareBio, Elche, Spain), with different volumes of DST20, DST80 and DST85 niosomes to obtain cationic lipid/DNA (w/w) mass ratios 2/1, 5/1 and 10/1. The mixture was left for 30 min at room temperature to enhance electrostatic interactions between the cationic lipid and the DNA.

### 2.2. Plasmid propagation

The pCMS-EGFP plasmid was propagated in *Escherichia coli* DH5-α and purified using the Qiagen endotoxin-free plasmid purification Maxi-prep kit (Qiagen, Santa Clarita, CA, USA) according to manufacturer's instructions. The concentration of pDNA was quantified by

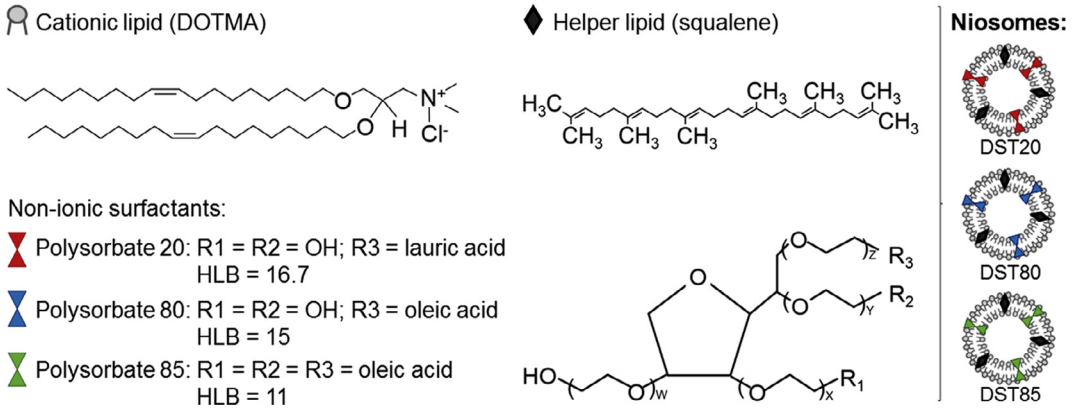


**Fig. 1.** General scheme of niosomes and chemical structures of niosome components.