Biomaterials 77 (2016) 267–279

Contents lists available at ScienceDirect

Biomaterials

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

The influence of the polar head-group of synthetic cationic lipids on the transfection efficiency mediated by niosomes in rat retina and brain

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ARTICLE INFO

Article history:
Received 30 July 2015
Received in revised form
2 November 2015
Accepted 6 November 2015
Available online 14 November 2015

Keywords:
Niosomes
Gene delivery
Non-viral vector
Cationic lipid
Retina
Brain

ABSTRACT

The development of novel non-viral delivery vehicles is essential in the search of more efficient strategies for retina and brain diseases. Herein, optimized niosome formulations prepared by oil-in water (o/w) and film-hydration techniques were characterized in terms of size, PDI, zeta potential, morphology and stability. Three ionizable glycerol-based cationic lipids containing a primary amine group (lipid **1**), a triglycine group (lipid **2**) and a dimethylamino ethyl pendent group (lipid **3**) as polar head-groups were part of such niosomes. Upon the addition of pCMS-EGFP plasmid, nioplexes were obtained at different cationic lipid/DNA ratios (w/w). The resultant nioplexes were further physicochemically characterized and evaluated to condense, release and protect the DNA against enzymatic digestion. *In vitro* experiments were performed to evaluate transfection efficiency and cell viability in HEK-293, ARPE-19 and PECC cells. Interestingly, niosome formulations based on lipid **3** showed better transfection efficiencies in ARPE-19 and PECC cells than the rest of cationic lipids showed in this study. *In vivo* experiments in rat retina after intravitreal and subretinal injections together with in rat brain after cerebral cortex administration showed promising transfection efficiencies when niosome formulations based on lipid **3** were used. These results provide new insights for the development of non-viral vectors based on cationic lipids and their applications for efficient delivery of genetic material to the retina and brain.

1. Introduction

Gene therapy is a challenging field that is emerging as a promising strategy for the treatment of several diseases [1]. Concretely, non-viral vectors have attracted the interest of the scientific community because, compared to viral vectors, they offer a safer way to deliver genetic material, as they do not exhibit antigen-specific immune and inflammatory response, are cheaper, easy to elaborate and the size of DNA inserted is theoretically unlimited [2]. Nevertheless, their low transfection efficiencies and the transient gene expression are the main concerns that these carriers have to overcome to reach clinical practice. There is a wide range of non-viral vectors described in the literature, such as those composed of polymers, lipids or peptides [3–5]. Among lipidic systems, liposomes are the most common vectors. However, our research group has previously described that niosomes are a promising alternative to liposomes for gene delivery purposes. Niosomes are carrier systems that form vesicles with a bilayer structure and compared to liposomes they are recognized for their low cost and superior chemical and storage stabilities. Nevertheless, few reports have been focused on their application for gene delivery purposes [6,7].

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http://dx.doi.org/10.1016/j.biomaterials.2015.11.017
0142-9612/

Unlike liposomes, which are elaborated with phospholipids, niosomes for gene delivery purposes are typically based on non-ionic surfactants to form more stable emulsions. In addition, helper lipids are also added to the formulations to enhance the physicochemical properties of the emulsion and finally, the cationic lipids, whose structural and physical properties clearly influence the transfection efficiency and toxicity [8–10].

Cationic lipids for gene delivery purposes usually contain four functional domains (hydrophobic group, linker group, hydrophilic head-group and backbone), which affect some important physicochemical parameters, such as the flexibility, stability, biodegradability, the level of hydration, interaction with DNA and its condensation [11–14]. In our previous work [15] the influence of the polar head-group on transfection efficiency together with cell viability was studied in HEK-293, ARPE-19 and MSC-D1 cells. Although promising results were obtained, such studies were performed with lipids containing a serinol backbone, which due to its low biodegradability reduced significantly cell viability. Therefore in the present manuscript, we have modified the serinol backbone by a glycerol one in order to improve the design of the niosomes formulation for *in vivo* retinal and brain delivery purposes.

Regarding the applications of gene therapy, the eye has favorable characteristics for this type of therapy, such as small size, immune-privileged position and well-defined compartmentalized anatomy, which minimize potential adverse reactions [16]. Additionally, most of the devastating inherent diseases in the eye are well described and their genetic background is also well known. However, at present few effective treatments are available for inherent retinal diseases. Therefore, research on the design of novel formulations for gene delivery to the retina represents a promising approach in order to translate animal research into clinical trials [17]. On the other hand, neurological disorders are the most difficult diseases to treat with clinical pharmacological approaches, mainly due to the complexity of the nervous system and the different brain physical barriers that drugs need to overcome after systemic administration [18]. Gene therapy represents a promising alternative to the traditional pharmacological approaches to face many devastating genetic diseases of the brain, such as Batten disease [19], Canavan disease [20] or Parkinson's disease [21]. In the past few years many gene transfer methods have been developed to treat retinal and brain diseases. However, all gene clinical trials are based on viral vectors that generate moderate optimism to drive the field forward. Therefore, we present an alternative and a safer approach to confront inherent retinal and brain disorders by the use of niosomes as non-viral carriers for gene delivery purposes.

Consequently, in the present study, we designed niosomes vectors for retinal and brain delivery purposes based on three synthetic ionizable cationic lipids containing polysorbate 80, as a non-ionic surfactant and squalene, as a helper lipid. These cationic lipids had three different functional domains: 1) an hydrophobic tail formed by two saturated hydrocarbonated alkyl chains of fourteen atoms of carbons in length; 2) a polar head formed by an amino group (lipid **1**), a glycine triglycine (lipid **2**) and a dimethylaminoethyl group (lipid **3**) and 3) a glycerol-based building block. Niosomes prepared by the oil-in-water emulsion (o/w) and film-hydration techniques were characterized in terms of size, PDI, zeta potential, morphology and physical stability. Upon the addition of the pCMS-EGFP reporter plasmid, we obtained nioplexes at different cationic lipid/DNA ratios (w/w). The influence of cationic lipid/DNA ratios on particle size, zeta potential and the ability to condense, release and protect DNA from enzymatic digestion was analyzed. *In vitro* experiments were performed and analyzed by flow cytometry to evaluate the most promising formulations in terms of transfection efficiency, viability and mean fluorescence intensity (MFI) in human embryonic kidney 293 cells (HEK-293), retinal pigment epithelia 19 cells (ARPE-19) and rat primary embryonic cerebral cortex cells (PECC). In order to move forward and according to the previous characterization results of the niosome formulations and nioplexes, we carried out some preliminary *in vivo* studies by confocal microscopy to evaluate transfection efficiency of the most promising formulation in the rat retina after intravitreal and subretinal injection and in the rat brain after injection in the cerebral cortex.

2. Material and methods

2.1. Material

All reactions were carried out under an inert atmosphere of argon. Flash column chromatography was carried out on silica gel SDS 0.063–0.2 mm/70–230 mesh. ^{1}H and ^{13C} NMR spectra were recorded at 25 °C on a Varian Mercury 400 MHz spectrometer using deuterated solvents. Tetramethylsilane (TMS) was used as an internal reference (0 ppm) for ^{1}H spectra recorded in $CDCl_3$ and the residual signal of the solvent (77.1 ppm) for ^{13}C spectra. For CD_3OD and d_6-DMSO the residual signal of the solvent was used as a reference. Chemical shifts are reported in parts per million (ppm), coupling constants (J) in Hz and multiplicity as follows: s (singlet), d (doublet), t (triplet), q (quadruplet), quint (quintuplet), m (multiplet) and br (broad signal). Electrospray ionization mass spectra (ESI-MS) were recorded on a Micromass ZQ instrument with a single quadrupole detector coupled to an HPLC, and high resolution (HR) ESI-MS on an Agilent 1100 LC/MS-TOF instrument (Servei d'Espectrometría de Masses, Universitat de Barcelona). HEK-293 cells, ARPE-19 cells, Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM) were obtained from the American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's medium Han's Nutrient Mixture F-12 (1:1), Trypsine, Hank's Balanced Salt Solution (HBSS), Neurobasal medium (NB), Fetal bovine Serum (FBS), B27® and Glutamax™ supplements and Penicillin-Streptomycin (Pen/Strep) antibiotics were purchased from Gibco® (San Diego, California, US). The plasmid pCMS-EGFP was purchased from PlasmidFactory (Bielefeld, Germany). The gel electrophoresis materials and gel red solution were acquired from Bio-Rad (Madrid, Spain). DNase I, sodium dodecyl sulfate (SDS), squalene, polysorbate 80, PBS and paraformaldehyde were purchased from Sigma–Aldrich (Madrid, Spain), and dichloromethane (DCM) was purchased from Panreac (Barcelona, Spain). Opti-MEM® reduced medium, antibiotic/antimycotic solution and Lipofectmanine™ 2000 transfection reagent were acquired from Invitrogen (San Diego, California, US). The BD Viaprobe kit was obtained from BD Biosciences (Belgium).

2.2. Synthesis of ionizable cationic amino lipids

The synthesis of the ionizable cationic amino lipids is summarized in Supplementary Data Scheme S1. Synthesis of *tert*-butyl-*N*-[2-[[2-[[2-[2,3-di(tetradecoxy)propylamino]-2-oxo-ethyl]amino]-2-oxo-ethyl]-amino]-2-oxo-ethyl]carbamate (**3**). Firstly, the carboxylic acid activation was carried out as follows: glycine tripeptide (2.0 eq) and *N*-hydroxysuccinimide (2.1 eq) were dissolved in DCM (3 mL) and the solution was stirred for a couple of minutes. Then, EDC (2.2 eq) was added. The reaction was stirred overnight at room temperature. The organic layer was washed with water (3 x 5 mL) and dried over anhydrous $MgSO_4$. The combined organic layers were reduced *in vacuo* and the anticipated crude was used in the next step without further purification. Cationic lipid **1** (compound **2**) (100 mg; 0.206 mmol) was added over the activated tripeptide. Reaction was heated at 60 °C and stirred overnight. The solvent was evaporated and the resultant crude was purified by flash