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Research paper

Fast therapeutic DNA internalization – A high potential transfection system based on a peptide mimicking cationic lipid

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ABSTRACT

The delivery of nucleic acids into cells is a determining factor for successful gene therapy. In this study we investigate the uptake and time dependent processing of a lipid-based non-viral nucleic acid delivery system composed of a peptide-mimicking cationic lipid (*N*-{6-amino-1-[*N*-(9*Z*)-octadec-9-enylamino]-1-oxohexan-(2*S*)-2-yl}-*N*′-{2-[*N*,*N*-bis(2-aminoethyl)amino]ethyl}-2-hexadecylpropandiamide - OH4) and a phospholipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine - DOPE). Studies by confocal laser scanning microscopy (CLSM) indicate a rapid internalization of fluorescent labelled DNA within 1 h. Furthermore, vesicular structures on the lipid surface were reported, which are associated with the application of the lipid-based non-viral vector. Time dependent investigations of the gene expression of a reporter gene encoding for enhanced green fluorescent protein (eGFP) or luciferase in 4 different cell lines demonstrate an initial gene expression soon after 4 h followed by a boost in gene expression beginning from 12 h to 24 h. Investigations with selective blocking of endocytic pathways using low molecular weight inhibitors suggested clathrin-mediated endocytosis as main internalization route in 3 cell lines. Our research presents a new horizon in rapid gene therapy using non-viral vectors; due to the modifications of the lipid components, fast nucleic acid internalizations could be achieved using our delivery systems.

1. Introduction

In recent decades, gene therapy moved into the focus of scientific interest, occasionally [1]. The CRISPR-Cas9 technology has once again brought gene therapy into light [2,3]. The current research trend shows that gene therapy will be one of the most potent therapies in the near future. With this therapy, diseases like cancer, cardiovascular disorders, or primary immune deficiencies can be possibly cured [4,5]. Besides the development of suitable therapeutic nucleic acids, the development of safe, effective and economical carrier systems (vectors) capable of protecting the nucleic acids against degradation by DNases or RNases and transporting them across the cell membrane to their desired cell-organelle, represents another key parameter. Recently, the gene therapeutic agent "Glybera®" (Alipogen tiparvovec), based on an adeno-associated virus vector and indicated for the treatment of lipoprotein lipase deficiency has been approved by the EMA. Nevertheless, these systems have an immunogenic potential and an oncogenic residual risk and are also extremely expensive due to their complex manufacturing processes [6,7].

Lipid-based vectors offer a safe alternative to viral vectors. These vectors are characterized by a significantly lower immunogenic and oncogenic potential and significantly lower manufacturing costs. However, these systems have a lower efficiency than their viral counterparts despite having been studied for more than 30 years [8]. In order to produce efficient lipid vectors, it is essential to acquire detailed knowledge of all involved mechanisms (cell attachment, cellular uptake, intracellular transport and release of nucleic acids). So far the exact uptake-, release- and transport-mechanisms are not fully understood. The currently accepted scientific theory is that the lipid-DNA complexes (lipoplexes) attach via glycosaminoglycans (GAG) (e.g. heparanes) due to electrostatic interactions at the outer cell membrane followed by an uptake via endocytosis. But the exact endocytic pathway depends on different factors like size, shape, and zeta potential of the vector or the cell line [9]. However, in this context other routes such as membrane fusion should not be disregarded [10]. A clear understanding into

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the exact mechanism could not be achieved due to technical problems. For instance, co-localization of fluorescently labelled lipids, nucleic acids and endosomes is difficult, because the usual sizes are often below the resolution limit of optical microscopes. Also experiments which inhibit various endocytose pathways, via siRNA knockdown or chemical inhibitors are not always selective or can interfere with these sensitive processes [11]. However, all observations should be collected and critically discussed in order to solve the puzzle of these mechanisms in future.

In our research group, we have specialized on the development of liposomal gene delivery systems, and presented some efficient cationic lipid mixtures in previous studies [12–16]. In the present work, we get insights into the time dependent uptake of a recently described cationic lipid formulation (OH4/DOPE, 1/1, n/n; where n/n means the molar ratio, Fig. 1) by using confocal laser scanning microscopy (CLSM). Furthermore we investigate the time dependent reporter gene expression by enhanced green fluorescent protein (eGFP)-based and luciferase-based cellular assays. In particular, a rapid uptake of these lipoplexes was observed, while a rapid low gene expression occurred after 4 h, there was a substantial boost in gene expression after a long lag time. The specific inhibition of different endocytic pathways indicated clathrin-mediated endocytosis as main internalization route for the delivery systems used. Furthermore, vesicular structures where observed on the cell surface, whose function is yet to be investigated.

2. Materials and methods

2.1. Materials

If not mentioned otherwise, chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). DOPE and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rhod-DOPE) (λ_{abs}^{max} 560 nm/λ_{em}^{max} 583 nm) were bought from Avanti Polar Lipids (Alabaster, AL, USA). OH4 was synthesized as described previously [12,17]. The plasmids peGFP-C2 and pCMV-luc were acquired from Clontech (Mountain View, CA, USA) and Plasmid Factory (Bielefeld, Germany). Plasmid isolation kit was purchased from QIAGEN (Hilden, Germany). Both plasmid DNAs were transformed in *Escherichia coli* DH5α (Invitrogen GmbH, Darmstadt, Germany) and isolated following the manufacturer's instructions mentioned in the isolation kit. The purity of pDNA was verified by the absorbance-quotient A_{260}/A_{280} and 1% agarose gel electrophoresis. A549 (human lung carcinoma), HeLa (human cervix adenocarcinoma epithelial cells), and LLC-PK1 (pig kidney epithelial cells) cells were acquired from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). SK-OV-3 (human ovarial adenocarcinoma) cells were purchased from ATCC (Manassas, USA). Cell culture media and fetal bovine serum (FBS) were supplied by Biochrom (Berlin, Germany). YoYo®-1 Iodide (λ_{abs}^{max} 491 nm/ λ_{em}^{max} 509 nm) (1 mM in DMSO), wheat germ agglutinin (WGA) – Alexa Fluor® 633 conjugate (λ_{abs}^{max} 632 nm/ λ_{em}^{max} 647 nm) and Lipofectamine™ 2000 were obtained from Thermo Fischer Scientific. ATTO Rho6G-*N*-hydroxysuccinimidester (λ_{abs}^{max} 533 nm/λ_{em}^{max} 577 nm) was purchased from Atto-TEC (Siegen, Germany).

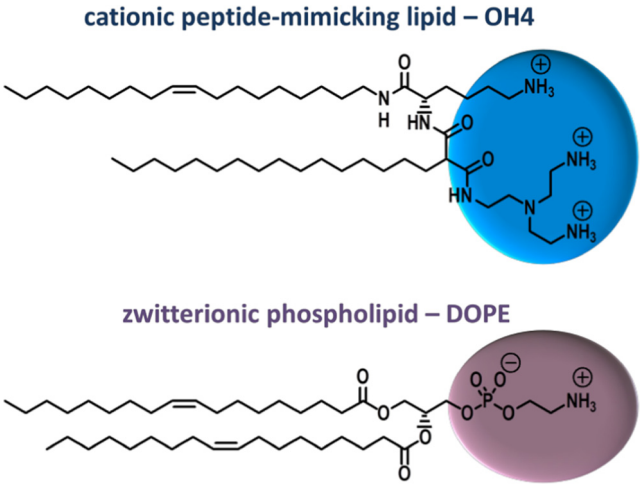

Fig. 1. Chemical structures of the two lipids used in the cationic lipid formulation for nucleic acid transfer. The cationic lipid component is *N*-{6-amino-1-[*N*-(9*Z*)-octadec-9-enylamino]-1-oxohexan-(2*S*)-2-yl}-*N*′-{2-[*N*,*N*-bis(2-aminoethyl)amino] ethyl}-2-hexadecylpropandiamide (OH4) bearing three primary amino groups in the head group of which the vast majority is protonated at physiological pH value. In addition, the phospholipid 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was used as co-lipid.

2.2. Preparation of cationic vesicles

Vesicles were prepared by the film hydration procedure [18]. OH4 and DOPE were separately dissolved in chloroform/methanol (8/2, v/v) and mixed to a molar ratio of 1/1. The solvent was evaporated for 1 h at 200 mbar and a further 3 h at 10 mbar. After formation of dry lipid films, a sterile filtrated 10 mM MES buffer solution (pH 6.5) was added to give a final concentration of 1 mg·mL^{-1}. Afterwards, the lipid dispersions were incubated at 50 °C while shaking (1400 rpm) for 30 min (Eppendorf Thermomixer 5436) followed by sonication at 37 kHz for 3 min at 25 °C.

2.3. Lipoplex formation

The plasmid DNA was complexed at different N/P ratios (ratio of the primary amines in the cationic lipid to the phosphate groups of the DNA) with the cationic OH4/DOPE vesicles by adding plasmid DNA to the lipid dispersion in a one-step mixing procedure. The samples were incubated for 15 min at 25 °C while shaking gently (Eppendorf Thermomixer 5436).

2.4. Transfection experiments

2.4.1. eGFP assay

A549, HeLa, or LLC-PK1 cells were cultured in 75 cm^2 tissue culture flasks in Dulbecco's modified eagle medium (DMEM) adjusted to contain 4.5 mg·mL^{-1} glucose supplemented with 10% FBS at 37 °C and 5% CO_2. The cells were grown ~90% confluent and were passaged three times a week. For experiments, cells within passage numbers 10–30 were used. Cells were seeded onto 96-well plates at a density of 10,000–11,000 cells per well 24 h before transfection. Cells were washed once with PBS buffer (pH 7.4) containing Ca^{2+} and Mg^{2+}. Lipoplexes were added to the cells (0.1 μg DNA per well) and filled up with DMEM containing 10% FBS to the final volume of 100 μL per well. After 4 h, 8 h, 24 h, or 48 h the cells were washed twice with PBS and the wells were filled up with 100 μL PBS. The eGFP fluorescence was measured using a BMG 10 filter (λ_{abs} = 485 nm, λ_{em} = 520 nm) with a fixed gain. The transfection efficiency is auto-fluorescence corrected and represented in relative fluorescence units (RFU) normalised to the cell count.

2.4.2. Luciferase assay

SK-OV-3 cells were cultured in 75 cm^2 tissue culture flasks in Iscove's modified Dulbecco's eagle medium (IMDM) supplemented with 10% FBS at 37 °C and were grown in monolayers in 7% CO_2 under humid conditions and were passaged upon reaching 80% confluence. The cells were seeded onto 96-well plates at a density of 10,000 cells per well and incubated for 24 h before transfection.