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Insights on the intracellular trafficking of PDMAEMA gene therapy vectors

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ABSTRACT

It is known that an efficient gene therapy vector must overcome several steps to be able to express the gene of interest: (I) enter the cell by crossing the cell membrane; (II) escape the endo-lysosomal degradation pathway; (III) release the genetic material; (IV) traffic through the cytoplasm and enter the nucleus; and last (V), enable gene expression to synthetize the protein of interest. In recent years, we and others have demonstrated the potential of poly(2-(*N*,*N*′-dimethylamino)ethylmethacrylate) (PDMAEMA) as a gene therapy vehicle. Further optimization of gene transfer efficiency requires the understanding of the intracellular pathway of PDMAEMA. Therefore the goal of this study was to determine the cellular entry and intracellular trafficking mechanisms of our PDMAEMA vectors and determine the gene transfer bottleneck. For this, we have produced rhodamine-labeled PDMAEMA polyplexes that were used to transfect retinal cells and the cellular localization determined by co-localization with cellular markers. Our vectors quickly and efficiently cross the cell membrane, and escape the endo-lysosomal system by 24 h. We have observed the PDMAEMA vectors to concentrate around the nucleus, and the DNA load to be released in the first 24 h after transfection. These results allow us to conclude that although the endo-lysosomal system is an important obstacle, PDMAEMA gene vectors can overcome it. The nuclear membrane, however, constitutes the bottleneck to PDMAEMA gene transfer ability.

1. Introduction

Gene therapy has long been heralded as the hope to evolve from symptomatic care to a cure for genetic diseases. Recent successes in using gene therapy for treating enzyme-deficiency, ocular, and hematopoietic diseases have shown great potential [1–6]. While gene therapy relies mostly on viral vectors, these have limitations. Therefore, there is considerable effort to develop non-viral vectors whose efficiency resembles the one of viral vectors [7,8].

Poly(2-(*N*,*N*-dimethylamino)ethyl methacrylate) (PDMAEMA) is a synthetic polymer that has been shown to transfect different cell lines such as OVCAR-3 and COS-7 [9,10]. The physicochemical properties of PDMAEMA have been extensively studied regarding its interaction with DNA [11] and serum proteins [12], its pH-dependent release of nucleic acid load [13], and its long-term stability [14]. Our previous work has shown for the first time the ability of PDMAEMA to act as a gene carrier targeting retinal epithelium cells [15]. We have shown PDMAEMA to complex with genetic material at the nano-scale with a positive surface charge and to protect its load from nuclease degradation. This vector was able to transfect retinal pigment epithelium cells in a polymer chain, polyplex size, and concentration dependent manner, but still at values lower than those observed for viral vectors.

It is known that a gene delivery system must overcome several barriers to express the gene of interest: (I) enter the cell by crossing the cell membrane; (II) escape the endo-lysosomal degradation pathway; (III) traffic through the cytoplasm and enter the nucleus; (IV) release the genetic material; and lastly (V), express the gene for further translation into the protein of interest [7]. For polymers like polyethylenimine (PEI) there are several studies describing that the prevailing uptake mechanisms are clathrin- and caveolin-dependent endocytosis [16–18]. For PDMAEMA, studies of the intracellular pathway are scarce and incomplete [19,20], and to further optimize the performance of PDMAEMA, we set out to study the cellular internalization and trafficking of our polyplexes to determine the limiting step in the gene transfer. For this, we have chemically modified the polymer with the fluorophore rhodamine-B for its tracking while transfecting retinal pigment epithelium cells and evaluated their intracellular route. Using this strategy, we have determined the main bottleneck and thus

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established the ground for further improvement to the transfection efficiency.

2. Materials and methods

2.1. Cells and plasmid DNA

A retinal pigment epithelium cell line (D407) was used for the in vitro experiments. Cells were maintained in Dulbecco's modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich), and 5% fetal bovine serum (FBS; Sigma-Aldrich). Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

A plasmid encoding the green fluorescent protein (GFP) under the CMV promoter was used in the experiments (pEPiTO-hCMVeGFP, 5245 bp) [21]. The plasmid was grown in *Escherichia coli* GT115 competent cells (Invitrogen), extracted and purified using Qiagen's Maxi-Prep kit according to the manufacturer's instructions. Plasmid concentration and integrity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and by agarose gel electrophoresis, respectively.

2.2. Labeling with rhodamine B isothiocyanate

Firstly, PDMAEMA was synthesized and characterized as described in our previous work [16], but replacing the chain transfer agent (CTA) by 4-cyanopentanoic acid dithiobenzoate (1.3 mg, 4.65×10^{-6} mol, Sigma-Aldrich) [22]. The obtained polymer was found to have M_n = 98 kDa, M_w = 103 kDa and PDI = 1.05. Labeling with rhodamine was performed by an adapted procedure [23], as depicted in Fig. 1. One gram of PDMAEMA was dissolved in 10 mL of dimethylformamide (DMF, Sigma-Aldrich) containing 2.8 μL of triethylamine (Sigma-Aldrich). Rhodamine B isothiocyanate (0.0107 g, RITC, Sigma-Aldrich) was dissolved in 15 mL of DMF and added to the polymer solution. The reaction was allowed to proceed in the dark, at 0 °C, for 20 h. The reaction mixture was evaporated (Buchi® Rotavapor®, Switzerland) and the residue dissolved in a pH 4 HCl solution. To remove unbound rhodamine B, a dialysis was performed in benzoylated cellulose dialysis tubing with MWCO of 2000 (Sigma-Aldrich) against HCl solution (pH 4) until release of free dye was no longer observed (approximately 15 days, with change of the dialysis medium every 12 h). The labeled PDMAEMA (PDMAEMA-RITC) was freeze-dried in a FreeZone 6 Liter Benchtop Freeze Dry System (Labconco) for 48 h and stored for further use.

2.3. Preparation of PDMAEMA/pDNA polyplexes

Both PDMAEMA and PDMAEMA-RITC stock solutions were prepared at a concentration of 1 mg/mL in water and stored at 4 °C until further use.

The polyplexes, composed of polymer and plasmid DNA, were prepared in water at N/P (nitrogen/phosphorus) ratios of 10:1 and 16:1, based on previous studies [15]. For both PDMAEMA and PDMAEMA-RITC these N/P ratios were calculated according to Eq. (1), where m_p is the mass of polymer, m_D is the mass of DNA, $M_{o,D}$ is the average repeat unit molecular weight of DNA, and $M_{o,p}$ is the repeat unit molecular weight of the polymer.

$$\frac{N}{P} = \frac{m_p M_{o,D}}{2 m_D M_{o,P}} \quad (1)$$

2.4. Size and surface charge

The diameter of polyplexes was determined by dynamic light scattering (DLS) and non-invasive back-scatter (NIBS), and the surface charge of the polyplexes was estimated from zeta potential measurements using laser Doppler velocimetry and phase analysis light scattering (M3-PALS). All measurements were done in MilliQ water at 25 °C and using a detection angle of 173° in a Zetasizer Nano ZS (Malvern Instruments).

2.5. Complexation and DNAse I protection assay

DNA complexation with both PDMAEMA and PDMAEMA-RITC was confirmed by gel electrophoresis. Polyplexes were incubated with DNAse I to assess their ability to protect DNA from enzymatic degradation. The polyplexes were prepared as described above and to digest the DNA, 10× reaction buffer (200 mM Tris-HCl, pH 8.3, 20 mM $MgCl_2$; Sigma-Aldrich) was added, followed by DNAse I (1 unit/μL in 50% glycerol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$; Sigma-Aldrich) at a proportion of 1 U DNAse I per 1 μg of plasmid. This mixture was incubated for 15 min at 37 °C and the reaction was stopped

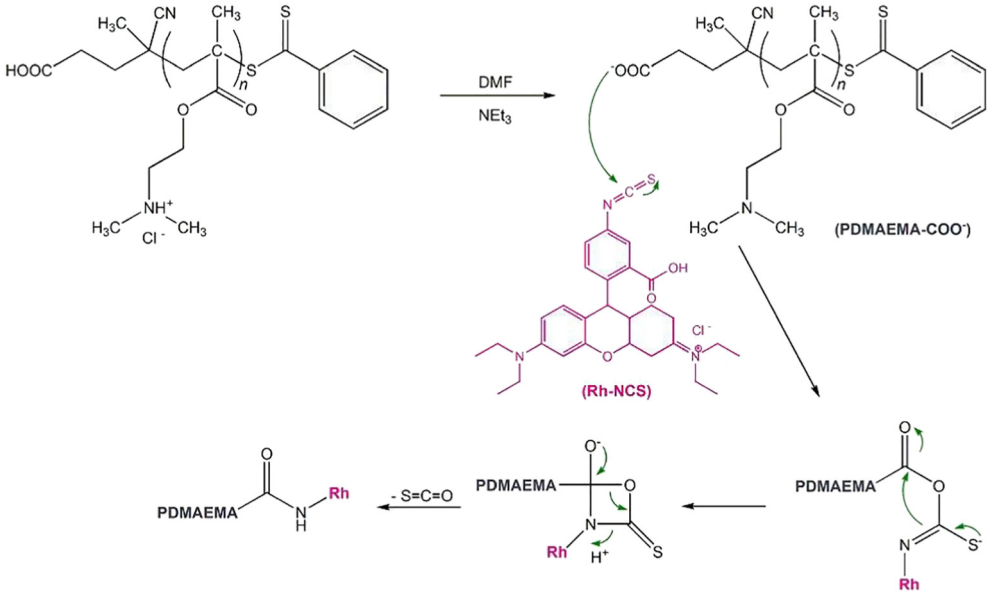

Fig. 1. Schematic representation and proposed reaction mechanism [23,31] of PDMAEMA labeling with rhodamine B.