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Factors influencing the transfection efficiency and cellular uptake mechanisms of Pluronic P123-modified polypropyleneimine/pDNA polyplexes in multidrug resistant breast cancer cells

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ABSTRACT

Generally, the major obstacles for efficient gene delivery are cellular internalization and endosomal escape of nucleic acid such as plasmid DNA (pDNA) or small interfering RNA (siRNA). We previously developed Pluronic P123 modified polypropyleneimine (PPI)/pDNA (P123-PPI/pDNA) polyplexes as a gene delivery system. The results showed that P123-PPI/pDNA polyplexes revealed higher transfection efficiency than PPI/pDNA polyplexes in multidrug resistant breast cancer cells. As a continued effort, the present investigation on the factors influencing the transfection efficiency, cellular uptake mechanisms, and intracellular fate of P123-PPI/pDNA polyplexes is reported. The presence of P123 was the main factor influencing the transfection efficiency of P123-PPI/pDNA polyplexes in MCF-7/ADR cells, but other parameters, such as N/P ratio, FBS concentration, incubation time and temperature were important as well. The endocytic inhibitors against clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis were involved in the internalization to investigate their effects on the cellular uptake and transfection efficiency of P123-PPI/pDNA polyplexes *in vitro*. The data showed that the internalization of P123-PPI/pDNA polyplexes was obtained from both CME and CvME. Colocalization experiments with TRITC-transferrin (CME indicator), Alexa Fluor 555-CTB (CvME indicator), monoclonal anti-α-tubulin (microtubule indicator), and LysoTracker Green (Endosome/lysosome indicator) were carried out to confirm the internalization routes. The results showed that both CME and CvME played vital roles in the effective transfection of P123-PPI/pDNA polyplexes. Endosome/lysosome system and skeleton, including actin filament and microtubule, were necessary for the transportation after internalization.

1. Introduction

Pluronic modified cationic polymers have been regarded as potential non-viral vectors for gene delivery *in vitro* and *in vivo* [1–3]. Pluronic triblock copolymers composed of poly (ethylene oxide) – poly (propylene oxide) – poly (ethylene oxide), are particularly attractive pharmaceutical agents for transfection application as they are non-ionic and have been shown to increase the exogenous gene expression *in vivo* when intramuscularly co-injected with plasmid DNA (pDNA) [4–6]. To our knowledge, no reports have so far been published regarding Pluronic-mediated transfection of pDNA *in vitro* or *via* intravenous administration. However, Pluronic can increase pDNA transfection mediated by cationic lipids and cationic polymers *in vitro* and *in vivo* [2,7–9]. Among these studies, cationic polymers including polyethylenimine (PEI), polypropylenimine (PPI), and poly (2-*N*-(dimethylaminoethyl) methacrylate) (pDMAEMA) were modified with Pluronic (P123, P85, F38, F127, or L92) and achieved unexpected high transfection efficiency [3–11]. The extensively studied and most effective Pluronics are P123 and P85. Furthermore, the important biological activities of Pluronics are their ability to incorporate into membranes followed by subsequent translocation into the cells, affecting various cellular functions, such as ATP synthesis, the activity of drug efflux transporters, and apoptotic signal transduction [12]. Specifically, Pluronics can inhibit P-glycoprotein (P-gp), a drug efflux protein

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that hinders distribution of many drugs. As a result, they can enhance drug cellular uptake, nuclear translocation, and transcriptional activation of gene expression, leading to drastic sensitization of multidrug-resistant (MDR) tumors to various anticancer agents [3,12–14].

Understanding how uptake mechanisms, endosomal escaping, and intracellular trafficking affect transfection of non-viral vectors is prerequisite and helpful to design more efficient non-viral gene delivery vectors and improve their efficiency. The evidence from the literatures suggested that it is conceivable that the positively charged polymer/pDNA polyplexes are generally internalized into cells by endocytosis [15–17]. Classically, the uptake pathways are divided into two groups in eukaryotic cells: endocytic pathways and non-endocytic pathways [18]. The mechanisms of endocytosis can be distinguished: phagocytosis, macropinocytosis (MP), clathrin-mediated endocytosis (CME), and caveolae-mediated endocytosis (CvME) [17]. CME and CvME are the most relevant mechanisms in the internalization of non-viral gene delivery systems. Selective inhibition of the different internalization pathways has been shown to be a powerful method to investigate uptake mechanisms of nanoparticles and infer the relevance of the blocked pathway in the internalization mechanism [19–22].

In our previous work, Pluronic P123-modified cationic polypropylenimine dendrimer (P123-PPI) was designed and synthesized as a potential non-viral gene delivery vector. The results showed that P123-PPI/pDNA polyplexes was effective for pDNA delivery and had a broader transfection spectrum of cell lines when compared to current commercially available Lipofactamine™ 2000 [8,9]. Pluronic P123 is a poly (ethylene oxide) (EO)-poly (propylene oxide) (PO)-poly (ethylene oxide) (EO)-based triblock copolymer EO20-PO70-EO20, not only exhibits important biological activities mentioned above but also acts as an inhibitor of P-glycoprotein (P-gp), which has been found to recuperate the sensitivity of MDR tumor cells to doxorubicin, paclitaxel, and other anti-cancer agents [2–24].

Although more efforts were put into the design and synthesis of Pluronic-modified cationic polymers for improving the transfection efficiency, detailed work to establish the cellular uptake mechanisms and influence factors underlying the high transfection efficiency is currently lacking, which distinguishes them from the common cationic polymers without Pluronic modification. Therefore, it is necessary to get insight on the uptake mechanisms for P123-PPI mediated gene transfection and study whether the different internalization pathways might influence the intracellular fate of the polyplexes and their transfection efficiency.

In this study, influence factors on transfection efficiency, the cellular uptake mechanisms, and intracellular fate and distribution of P123-PPI/pDNA polyplexes were investigated in MCF-7/ADR cells. Specific endocytic inhibitors of different endocytic pathways were used in the cellular uptake study of polyplexes to determine which routes were involved in the effective transfection of the polyplexes. In addition, fluorescence-labeled indicators of CME and CvME, endosome-lysosome system, and microtubule skeleton had been used to study intracellular trafficking of the polyplexes by confocal microscopy.

2. Materials and methods

2.1. Materials

Pluronic P123 (MW 5750) was kindly provided by BASF Co., Ltd. (Shanghai, China). Polypropylenimine (G3, MW 1684) was purchased from SyMO-Chem (Eindhoven, The Netherlands). *N*, *N*-Carbonyldiimidazole (CDI) was obtained from Sigma–Aldrich (St. Louis, MO, USA). YOYO-1, Alexa Fluor 555-cholera toxin B subunit (CTB), LysoTracker Green DND26, and Lipofectamine™ 2000 were obtained from Life Technologies (Burlington, ON, USA). Rhodamine nucleic acid labeling kit was purchased from Mirus Bio LLC (Madison, WI, USA) and used according to the manufacturer's instructions. Human transferrin (Tf), Chlorpromazine hydrochloride (CPZ), Filipin III, Genistein, 5-(*N*, *N*-Dimethyl) amiloride hydrochloride (DMA), Cytochalasin D (Cyto D), Nocodazole (NCZ), Paclitaxel (PTX), Monensin and mouse monoclonal anti-α-tubulin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM, high glucose) and Fetal Bovine Serum (FBS) were purchased from Gibco BRL Life Technologies (Gaithersberg, MD, USA).

2.2. Synthesis of P123-PPI

The synthesis of P123-PPI was reported in detail in our previous publications [8,9]. A brief synthesis scheme of P123-PPI is shown in Fig. 1. P123 (0.5 mmol) and CDI (0.5 mmol) were dissolved in 25 mL of anhydrous acetonitrile, respectively. Then CDI solution was added dropwise to P123 solution in the presence of nitrogen gas. The mixture was stirred for 4 h at 40 °C, diluted with 75 mL of water and dialyzed against 20% ethanol. For P123-PPI conjugation, mono-imidazolylcarbonyl-activated P123 was added dropwise with constant stirring to PPI (210 mg) dissolved in 20 mL of water. The reaction mixture was incubated at 25 °C for 48 h. The products were dialyzed against water to remove unbound PPI. The resulting product, denoted P123-PPI, was collected, lyophilized, and stored at 4 °C.

2.3. Preparation of P123-PPI/pDNA polyplexes

P123-PPI/pDNA polyplexes were prepared self-assembly by electrostatic interaction between the positively charged P123-PPI and negatively charged pDNA. To prepare P123-PPI/pDNA polyplexes at N/P ratio of 20, 24 μL of 2.5 mg/mL P123-PPI stock solution was diluted with 26 μL deionized water and then added into 50 μL of 40 μg/mL pDNA solution. The samples were mixed slightly, and incubated at room temperature for 20 min to ensure polyplexes formation. The particle size, polydispersity index and zeta potential of the P123-PPI/pDNA polyplexes were measured by dynamic light scattering (DLS) using a nano-Zetasizer (Malvern Instruments, UK) after the samples were appropriately diluted in distilled water.

2.4. Cell culture and transfection in vitro

Human doxorubicin-resistant breast adenocarcinoma cell line (MCF-7/ADR) was cultured in complete medium [high glucose DMEM supplemented with 10% (v/v) FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL)] at 37 °C in a humidified 5% CO_2 incubator. For transfection, the MCF-7/ADR cells were seeded in 24-well plates at a density of 5×10^4 cells/well and incubated for 24 h. When the cells reached approximately 70–80% confluency, 450 μL of fresh DMEM and 50 μL of P123-PPI/pDNA polyplexes (1 μg pGL3-promoter) or Lipofectamine™ 2000/pDNA complexes were added at equivalent pDNA to each well. The plate was immediately rocked back and forth to uniformly distribute the polyplexes in the medium and incubated for 4 h. After 4 h, the transfection medium containing polyplexes was replaced with 500 μL of fresh complete medium. Then the cells were incubated for total 48 h at 37 °C before assessing the level of luciferase expression. Luciferase assay was performed according to the manufacturer's instructions (Promega, Madison, WI, USA). The cell protein was determined by BCA protein assay kit (Beyotime Institute of Biotechnology, China).