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Multilayered polyplexes with the endosomal buffering polycation in the core and the cell uptake-favorable polycation in the outer layer for enhanced gene delivery

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ABSTRACT

In the present study, quaternary polyplexes were prepared by sequential addition of polycations (polyethylenimine (PEI) or poly (*N*-(8-aminooctyl)-acrylamide) (P8Am)) for loading pDNA into the core polyplexes and poly (acrylic acid) (PAA) for reversing charges to deposit additional polycation (PEI or P8Am) layer. It was found the cytotoxicity and cellular uptake expression of PEI core polyplexes could be improved by coating a cell uptake-favorable P8Am layer. Conversely, P8Am could not facilitate endosomal release through the proposed proton sponge effect so the PEI core was required for the P8Am-coated quaternary polyplexes to ensure efficient transfection. Consequently, an efficient and safe non-viral gene vehicle was constructed by layer-by-layer deposition, using alternate polyanion and polycation with required functionalities to overcome the obstacles met in the process of transfection. Maximum transfection activity with minimal toxicity was observed when the quaternary polyplex of pDNA/PEI/PAA/P8Am was prepared at a weight ratio of 1/1.5/3/5. Conversely, the same composition in different position such as the cell-favorable P8Am core was externally deposited with the endosome lytic moiety, PEI showed high toxicity and low efficiency. This indicates the pDNA/PEI/PAA/P8Am sequence for a quaternary polyplex is as important as the functional polymer selection for designing safe and reliable gene delivery vehicles. We demonstrate here that gene delivery efficiency may be improved by increasing the uptake level and the endosomal buffering release through an additional layer of cell uptake-favorable polycations associated with the core polycations possessing endosomal release ability.

1. Introduction

Gene therapy offers a potential method to treat disease ranging from inherited disorders to acquired conditions and cancer by transferring exogenous nucleic acids into cells to alter protein expression profiles [1,2]. Generally, cationic nanoparticles would be appropriately applied to cellular uptake because of the electrostatic interaction between nanoparticles and cellular membrane, but it is limited by its low transfection efficiency and high cytotoxicity [3,4]. Except decreasing positive charge by chemical modification, "recharging" of polyplexes using polyanions to reverse the surface charge of polyplexes has been demonstrated to decrease the cytotoxicity of polycation/DNA complexes [5]. We have previously described the formation of ternary polyplexes containing pDNA, synthetic polycations and polyanions [6]. In this study, we extended these studies and found that the further addition of a polycationic layer on the ternary polyplexes could increase the levels of gene expression with reduced toxicity. Two cationic polymers, polyethylenimine (PEI) and poly (*N*-(8-aminooctyl)-acrylamide) (P8Am), and one anionic polymer poly (acrylic acid) (PAA) were used in the polyelectrolyte multilayer (PEM) process, based on layer-by-layer deposition [7]. PEI, one of the most potent polycationic gene delivery vectors, can efficiently complex with pDNA and facilitate endosomal release through the proton sponge effect [8]. However, PEI may cause cytotoxicity and exhibit low uptake level during its practical applications [9–11]. Conversely, P8Am, developed in our laboratory recently, could exhibit high cellular uptake efficiency with minimal toxicity [12], but could not show high transfection ability unless chloroquine [13], a well-known transfection-boosting reagent to promote endosomal escape, was incorporated in the polyplexes. Improvements in gene delivery of multilayered polyplexes may come from their mechanism of action by selecting appropriate cationic polymers and establishing the reasonable arrangement. Therefore, the purpose of this study was to develop efficient non-viral gene delivery vehicles to overcome the obstacles met in the process of transfection by sequential

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deposition of different polycations with high cellular uptake ability and endosome lytic function at different layers simultaneously.

2. Materials and methods

2.1. Preparation of binary, ternary and quaternary pDNA polyplexes

The pDNA used in this study was pCMV-Luc (Promega Co., Cergy Pontoise, France) which was amplified in *Escherichia coli* and purified according to the supplier's protocol (Qiagen GmbH, Hilden, Germany). PEI (branched, Mw = 25,000) and PAA (Mw = 15,000) are purchased from Aldrich and used without further purification. P8Am (Mw = 66,700) was synthesized as described previously [12].

The pDNA vehicle constructed by PEM technique was fabricated sequentially by depositing layers of alternate polyions as schematically illustrated in Fig. 1. The binary core polyplexes of pDNA/PEI (BPI) and pDNA/P8Am (BPII) were prepared in sterile water at a weight ratio of 1/1.5 and 1/3, respectively, by adding equal volume (10 μL) of polymer solution to pDNA solution with calculated amount. Subsequently, PAA was deposited onto the positively charged core polyplexes to form ternary polyplexes at the weight ratios of 1/1.5/3 and 1/3/3 for pDNA/PEI/PAA (TPI) and pDNA/P8Am/PAA (TPII), respectively. Finally, a layer of polycation, PEI or P8Am, was deposited on the ternary polyplexes to form quaternary polyplexes TPI/P8Am, TPII/P8Am, TPI/PEI, and TPII/PEI at outer-layer polycation/pDNA weight ratio of 1–7.

2.2. Particle sizes and surface charges

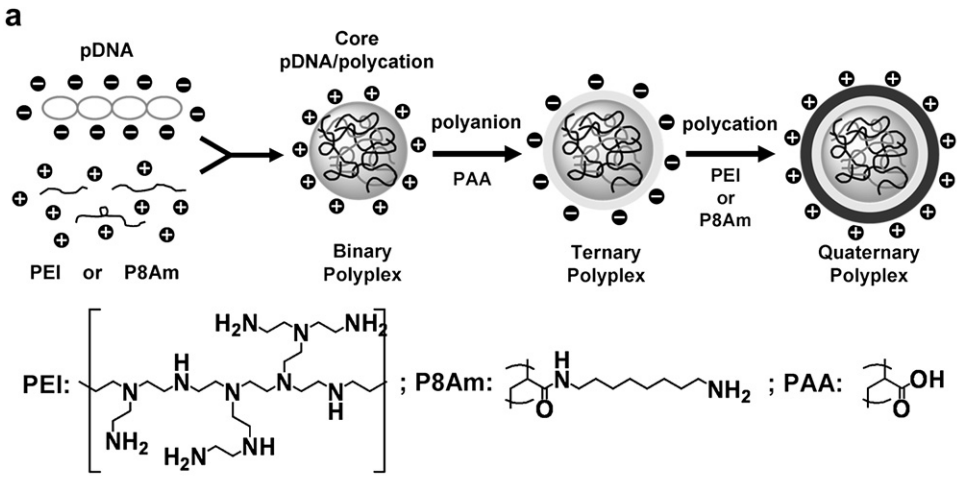
Particle sizes and surface charges of the PEM polyplexes (each containing 1 μg pDNA) were determined by a 90Plus/BI-MAS analyzer instrument (Brookhaven Instruments Corporation, Germany). The data presented reveal the average of two analyses which was performed in automatic mode using the same sample.

2.3. Agarose gel electrophoresis assay

Agarose gel (1.0%, w/v) containing ethidium bromide (0.06 μg/mL, Sigma) was prepared in TAE buffer (40 mmol/L Trisacetate, 1 mmol/L EDTA). The PEM polyplexes (each containing 0.4 μg pDNA) were prepared as described above. After the polyplexes were loaded to gel electrophoresis at 100 V for 30 min and pDNA bands were visualized by using a UV transilluminator.

2.4. Cellular toxicity

The cytotoxicity of the PEM polyplexes was evaluated using the MTT assay in human cervix carcinoma cell line (HeLa cell, BCRC number: 60005) and human hepatoma cell line (HepG2 cell, ATCC). In brief, cells were seeded in 96-well tissue culture plates (Costar, Cambridge, UK) at a density of 10^4 cells/well. Before transfection, the used medium was Dulbecco's Modified Eagle Medium (DMEM, Gibco)



b

Binary polyplex	Ternary polyplex	Quaternary polyplex
BP I (pDNA/PEI)	TP I (BP I/PAA)	TP I/P8Am
		TP I/PEI
BP II (pDNA/P8Am)	TP I (BP II/PAA)	TP II/P8Am
		TP II/PEI

Fig. 1. (a) Schematic representation of the formation of PEM polyplexes (binary, ternary, and quaternary polyplexes). (b) Summary of symbols and sequences of PEM polyplexes.