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Electrostatically assembled dendrimer complex with a high-affinity protein binder for targeted gene delivery

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ABSTRACT

Although a variety of non-viral gene delivery systems have been developed, they still suffer from low efficiency and specificity. Herein, we present the assembly of a dendrimer complex comprising a DNA cargo and a targeting moiety as a new format for targeted gene delivery. A PAMAM dendrimer modified with histidine and arginine (HR-dendrimer) was used to enhance the endosomal escape and transfection efficiency. An EGFR-specific repebody, composed of leucine-rich repeat (LRR) modules, was employed as a targeting moiety. A polyanionic peptide was genetically fused to the repebody, followed by incubation with an HR-dendrimer and a DNA cargo to assemble the dendrimer complex through an electrostatic interaction. The resulting dendrimer complex was shown to deliver a DNA cargo with high efficiency in a receptor-specific manner. An analysis using a confocal microscope confirmed the internalization of the dendrimer complex and subsequent dissociation of a DNA cargo from the complex. The present approach can be broadly used in a targeted gene delivery in many areas.

1. Introduction

Gene delivery systems have found wide applications in many areas such as gene therapy, genome editing, and cell transformation. Viral and non-viral vectors have been developed as gene delivery vehicles (Nayerossadat et al., 2012). Although viral vectors are widely used for gene delivery owing to their relatively high delivery efficiency compared to non-viral systems, they have certain drawbacks, including insertional mutations, immunogenicity, and a limitation with regard to the gene size. Recently, a variety of non-viral systems, including liposomes, and polymeric and inorganic nanoparticles, have been developed. Gene delivery systems based on an electrostatic interaction between anionic nucleic acids and cationic polymers or lipids have been of significant interest owing to their easy and simple processing (Wolfert et al., 1999). Diverse cationic polymers have been employed for gene delivery, including polyethylenimine (PEI), poly (amidoamine) (PAMAM) dendrimers, and poly L-lysine (Choi et al., 1998; Dufes et al., 2005; Liang et al., 2016). In particular, PAMAM dendrimers have attracted significant attention owing to their low cytotoxicity and high delivery efficiency (Cloninger, 2002; Dufes et al., 2005; Liu and Frechet, 1999). PAMAM dendrimers are known to facilitate an endosomal escape through their buffering capacity, namely, a proton sponge effect, of the inner tertiary amine groups to rupture the endosomal membrane, leading to an efficient DNA transfer into the nucleus (Cho et al., 2003). However, certain cationic polymers have been revealed to cause cytotoxicity owing to a disruption of the cell membrane (Lv et al., 2006; Pack et al., 2005). In addition, polymer-based delivery systems give rise to low delivery efficiency. To improve the delivery efficiency, a conjugation of a targeting moiety to nucleic acids and polymers has been attempted. Cationic polymers displaying amine groups can usually be modified through direct chemical conjugation with small molecules, peptides, or proteins (Blessing et al., 2001; Chan et al., 2007; Chiu et al., 2004; Wood et al., 2005). Nonetheless, such modifications have been known to cause a disruption of the original biophysical properties of the polymers and their complex with DNA.

Herein, we present an electrostatic assembly of a dendrimer complex with a DNA cargo and a targeting moiety for targeted gene delivery. A previously developed PAMAM dendrimer modified with histidine and arginine (HR-dendrimer) was used to enhance the proton sponge effect for endosomal escape and transfection efficiency (Bae et al., 2016; Yu et al., 2011). An epidermal growth factor receptor (EGFR)-specific repebody was employed as the targeting moiety (Lee et al., 2015). A repebody has much smaller molecular size (30 kDa) than an antibody, and it was shown to be more suitable as a targeting

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moiety in terms of tissue penetration compared to an antibody (Lee et al., 2017). A polyanionic peptide (PAP) was genetically fused to the repebody, and the resulting repebody (PAP-repebody) was reacted with HR-dendrimers and a DNA cargo to assemble the dendrimer complex through an electrostatic interaction. We demonstrated the utility and potential of the present approach by showing a high delivery efficiency of a target gene with negligible cytotoxicity in a receptor-specific manner. Details are reported herein.

2. Materials & methods

2.1. Gene expression and protein purification

Anti-EGFR repebody (rEgH9) was cloned into a pET21a vector (Novagen, USA) with polyhistidine and polyanionic tags at the N-terminal and C-terminal ends, respectively. The constructed vector was transformed into an *E. coli* host strain (Origami B (DE3), Novagen, USA)). A single colony was picked into an LB medium with ampicillin (100 μg/mL), kanamycin (50 μg/mL), and tetracycline (15 μg/mL), followed by incubation at 37 °C overnight. Cultured cells were diluted 1000-fold into fresh LB, and induced through the addition of IPTG for the protein expression, as described elsewhere (Lee et al., 2014). After the cells were harvested by centrifuging at 6080*g*, the cell pellet was resuspended in a lysis buffer (20 mM Tris, 150 mM NaCl, 10 mM Imidazole, and pH 8.0), and the resuspended cells were subjected to ultrasonication for disruption. Cell lysate was centrifuged at 20,800*g* and 4 °C for 1 h, and the supernatant was collected and filtered through a 0.2 μm syringe filter (Millipore, USA). A polyanionic peptide-fused repebody (PAP-repebody) was purified using a Ni-NTA agarose resin (Qiagen, USA). Briefly, the filtered cell supernatant was incubated with the Ni-NTA column, followed by treatment with a washing buffer (20 mM Tris, 150 mM NaCl, 20 mM Imidazole, and pH 8.0). The PAP-repebody was eluted using an elution buffer (20 mM Tris, 150 mM NaCl, 250 mM Imidazole, and pH 8.0). The eluted proteins were further purified through size-exclusion chromatography using an S200 Superdex column (GE healthcare, USA) with a PBS buffer. The purified fractions were stored at 4 °C for further study.

2.2. Assembly of dendrimer complex

The assembly of the dendrimer complex was obtained by incubating an HR-dendrimer, a plasmid DNA (pDNA), and the PAP-repebody. Briefly, HR-dendrimers were dissolved in distilled water, and pDNA as a DNA cargo was gently mixed at various pDNA/dendrimer weight ratios, followed by dilution in a 1 × PBS buffer (pH 7.4) at room temperature for 30 min. The PAP-repebody dissolved in a 1 × PBS buffer (pH 7.4) was added to the mixture of pDNA and HR-dendrimer at a predetermined ratio at room temperature for 30 min. For the agarose gel retardation assay, the dendrimer complex was loaded in a 1% agarose gel and subjected to electrophoresis for 30 min at 100 V. After the agarose gel running, the gel was stained with a RedSafe™ nucleic acid solution (Intron, Korea) and analyzed using a Gel Doc™ EZ imager (Bio-rad).

2.3. Measurement of hydrodynamic size and zeta potential

The size distribution and zeta potential of the dendrimer complex were measured at 25 °C using a Zetasizer nano zs (Malvern).

2.4. Transmission electron microscopy (TEM)

The dendrimer complex with an optimal weight ratio was stained with phosphotungstic acid for TEM imaging. Briefly, the sample was prepared by dropping a dendrimer complex solution on a copper grid, and the solution was evaporated under ambient conditions. The sample was stained for 1 min with phosphotungstic acid (2%, pH 7.4), and the solution was completely removed using a filter paper. Images were obtained using a 200 kV field-emission transmission electron microscope (JEM-2100F, JEOL Ltd., Japan) following further drying of the grid under ambient conditions.

2.5. Cell culture and luciferase transfection assay

For transfection, the luciferase expression plasmid (pCN-Luci) and HR-dendrimer were prepared as previously reported (Lee et al., 2002; Yu et al., 2011). MDA-MB-231 (human breast cancer) and HeLa (human cervical cancer) cells were maintained in a DMEM medium, and HepG2 (human liver cancer) cells were grown in an RPMI 1640 medium with 10% fetal bovine serum (FBS) (GE Healthcare Hyclone, UK) in a 5% CO_2 incubator (MCO-5AC, Sanyo, Japan) at 37 °C. For transfection of the dendrimer complex, MDA-MB-231 and HepG2 cells were seeded at 2×10^4 cells/well, and the HeLa cells were seeded at 1×10^4 cells in a 96-well plate. After incubation for 24 h to reach adequate confluency, the dendrimer complex was prepared with 0.5 μg of pDNA at various weight ratios of the pDNA, HR-dendrimer, and PAP-repebody, and added to each well and incubated for 4 h at 37 °C in a complete medium containing 10% FBS. Polyethyleneimine (branched, 25 kDa; PEI) (Sigma, USA) was used as a control with an optimized weight ratio of pDNA/PEI of 1:3. The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), and incubated with a fresh medium containing 10% FBS for 24 h at 37 °C. The cells were washed twice with DPBS and lysed for 30 min using a lysis buffer (Promega) to evaluate the luciferase activity. The cell lysates were spun down by centrifugation at 15,900*g* for 10 min, and the supernatant was collected. The protein concentration in the supernatant was measured using a BCA Protein Assay Kit (Thermo scientific), and the luciferase activity was determined to evaluate the transfection efficiency using a Luciferase Assay System (Promega) according to the manufacturer's protocol. The luminescence was recorded using a TriStar2 LB 942 multidetection microplate reader (Berthold). To assess the target specificity, cells were incubated using a medium containing an EGFR-specific repebody at various concentrations or 100 μg/mL Cetuximab (EGFR-specific antibody) for 1 h. The cells were transfected with the dendrimer complex at a predetermined weight ratio of pDNA/HR-dendrimer/PAP-repebody of 1:8:16, and analyzed according to the procedure described above.

2.6. Cell imaging using a confocal microscope

Fluorescent labeling of pDNA and the PAP-repebody was conducted to obtain cellular confocal images. The PAP-repebody was labeled with fluorescein (FITC) by incubating with a 20-fold molar excess of fluorescein-NHS (Thermo) in 1 × PBS (pH 7.4) for 2 h at room temperature, and the FITC-labeled PAP-repebody was purified using a desalting column (PD-10, GE Healthcare). In addition, pDNA was conjugated using Alexa Fluor 647 with a Ulysis Nucleic Acid Labeling Kit (Life Technology) according to manufacturer's protocol. HeLa cells were seeded at 1×10^4 cells per chamber of the culture slide (SPL) and grown for 24 h. The dendrimer complex was assembled by mixing 0.5 μg of pDNA at a weight ratio of pDNA/HR-dendrimer/PAP-repebody of 1:8:16, and added to the cells. After incubation at 37 °C for 4 h, the remaining complexes were removed by washing twice with DPBS, and a fresh medium containing 10% FBS was used for further incubation. The cells were washed with DPBS at different points in time during the incubation, namely, at 4 and 24 h, and fixed with 4% paraformaldehyde for 10 min, followed by washing with DPBS and DAPI staining. The images were acquired using a confocal microscope (LSM 710, Carl Zeiss).

2.7. Evaluation of cell cytotoxicity

MDA-MB-231 and HepG2 cells were seeded at 2×10^4 cells/well, and HeLa cells were seeded at 1×10^4 cells in a 96-well plate and