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Novel hyperbranched polyamidoamine nanoparticle based gene delivery: Transfection, cytotoxicity and *in vitro* evaluation

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ABSTRACT

In this study, hyperbranched polyamidoamine (hPAMAM) was developed as a novel non-viral gene vector for the first time. The hPAMAM was synthesized using a modified "one-pot" method. DNA was then bound to hPAMAM at different weight ratios (w_{hPAMAM}/w_{DNA}). The higher weight ratio could bring larger particle size and higher zeta potential of hPAMAM–DNA complexes. The encapsulated DNA was protected by hPAMAM from degradation for over 3 h. Under the optimal condition, high gene transfection efficiency could be achieved in COS7 (47.47 ± 1.42%) and HEK293 (40.8 ± 0.98%) cell lines. And hPAMAM showed rather minor cytotoxicity *in vitro* (cell viability = 91.38 ± 0.46% in COS7 and 92.38 ± 0.61% in HEK293). The hPAMAM mediated human vascular endothelial growth factor 165 ($hVEGF_{165}$) gene transfected cells could express $hVEGF_{165}$ stably for 14 days, with the peak expression at day 2. In conclusion, hPAMAM based gene delivery was economical, effective and biocompatible, and may serve as a promising non-viral vehicle for gene therapy.

1. Introduction

The most demanding task in gene delivery systems is optimization for gene vectors. The modified viral vectors can mediate highly efficient gene transfer (Edelstein et al., 2007). Nevertheless, there are significant concerns about the safety of viral vectors, such as the potential for unwanted immune responses and aberrant gene expression (Lehrman, 1999; Bonetta, 2002; Marshall, 2002), which prompted parallel pursuit for non-viral vectors. However, complicated and costly synthesis procedure, limited transfection efficiency and high cytotoxicity also prohibit the widespread application of the current explored non-viral gene vectors. Therefore, development of an economical, effective and biocompatible non-viral vector is necessary for gene therapy. As a type of cationic dendrimer, polyamidoamine (PAMAM) dendrimer is a promising non-viral gene vector at present (Gao et al., 2008). It could form nano-complexes with DNA, whose size is suitable for endocytic uptake by non-phagocytic cells. The intracellular studies demonstrated that PAMAM could induce the so-called "proton sponge effect", which allowed for the complex to escape from endo-/lysosomal trafficking pathway (Kumar et al., 2010). The encouraging gene transfection efficiency and minor cytotoxicity of PAMAM attract more and more attention (Yang et al., 2011). However, the synthesis procedure of PAMAM requires stepwise convergent approaches and repeated purification steps, which are extremely time-consuming and costly (Tomalia and Fréchet, 2002). As an analog to PAMAM, hyperbranched polyamidoamine (hPAMAM) has similar chemical and physical properties to PAMAM but can be synthesized using a simple "one-pot" method (Gao and Yan, 2004). And we modified the "one-pot" synthesis procedure to improve the reliability at an earlier study (Cao et al., 2007). Therefore, hPAMAM may serve as a novel gene vector and a suitable substitute for PAMAM.

In the present study, we synthesized hPAMAM using our modified "one-pot" method, optimized the transfection condition for hPAMAM nanoparticles based gene delivery into COS7 and HEK293 cell lines, investigated its cytotoxicity *in vitro* and evaluated the gene expression after transfection. We anticipate that hPAMAM mediated gene transfection will enable a novel, economical, effective and biocompatible approach for gene therapy.

2. Materials and methods

2.1. Materials

Monkey African green kidney fibroblast-like cell line (COS7) and human embryonic kidney cells (HEK293) were donated by Chinese Academy of Science. Polyethylenimine (PEI) (Mw = 25 kDa) and Lipofectamine 2000 were purchased from Sigma Aldrich (USA).

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Plasmid of enhanced green fluorescent protein (pEGFP) and human vascular endothelial growth factor 165 ($phVEGF_{165}$) were purchased from Gene Chem (China).

2.2. Synthesis of hPAMAM

Using diethylene triamine (DETA) and methyl acrylate (MA) as raw materials, hPAMAM was synthesized economically by a modified "one-pot" method. In a flask, diethylene triaminse (DETA) (20.6 g) was dissolved in 25 mL methanol. And methyl acrylate (MA) (20.6 g) was dropwise added into the flask. The mixture was stirred for 48 h at room temperature. Then a rotary evaporator was equipped onto the flask. Under the vacuum, the remaining methanol was removed from the mixture at room temperature, followed by the reaction for 1 h at 60 °C, 1 h at 80 °C, 1.5 h at 100 °C, 1.5 h at 120 °C, and 3 h at 140 °C. The product was precipitated in ethyl oxide three times and then kept in a sealed container.

2.3. Binding of hPAMAM nanoparticles and pEGFP

The hPAMAM was diluted in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Invitrogen, USA). DNA (pEGFP or $phVEGF_{165}$) was then added to the hPAMAM solution at different weight ratios (w_{hPAMAM}/w_{DNA} = 2, 4, 6, 8, 10, 12 and 14). The mixture was vortexed gently to form hPAMAM–DNA complexes. Particle size distribution and zeta potential of hPAMAM–DNA complexes were detected with a Zetasizer instrument (Malvern instruments, UK). Shape of the complexes was visualized using a transmission electron microscopy (TEM, JEM-1230, JEOL, Japan).

2.4. Protection and release assay of DNA

For determine the resistance of hPAMAM–DNA complexes to nuclease digestion, 50 μL DNase I buffer was mixed with 350 μL solution containing 8 μg naked DNA or hPAMAM–DNA complexes. Then 2 U DNase I (Thermo Fisher Scientific, USA) was added to the mixture and incubated at 37 °C. Samples (100 μL) were collected at 0 h, 1 h, 2 h and 3 h during incubation. To quench DNase I degradation, all samples were treated with 10 μL ethylene diamine tetraacetic acid (25 mM) for 10 min at 65 °C. Finally, 10 μL sodium doclecyl sulfate (10%, w/v) was added to each sample and incubated for 2 h at 65 °C to facilitate DNA dissociation. All samples were then placed into an ice bath and electrophoresed using 1% agarose gel for analysis on the Gel Document System (Bio-Rad, USA).

2.5. Cell culture

COS7 and HEK293 cells were cultured and expanded in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, USA) at 37 °C under a humidified atmosphere containing 5% CO_2.

2.6. Transfection efficiency in COS7 and HEK293 cell lines

COS7 and HEK293 cells were seeded at a density of 1×10^5 cells/cm^2 in 6-well tissue culture plates with a medium volume of 2 mL per well. After 24 h, the culture medium was replaced by DMEM. And the hPAMAM–pEGFP complexes on different weight ratios were then added into the plates. Four hours later, transfection medium was replaced by fresh DMEM containing FBS. The transfection efficiency was evaluated by a flow cytometer (FACSAriaII, BD, USA) at 24 h after transfection. Non-transfected cells were used for setting the auto-fluorescence baseline. Data were analyzed using Flowjo 7.6 software with gating at 1% for the optimal w_{hPAMAM}/w_{DNA} ratio during transfection. To determine the optimal plasmid dosage for transfection, 1, 3, 4 and 5 μg DNA were transfected into cells using the above process simultaneously.

Lipofectamine 2000 and PEI (25 kDa) were also used as gene vectors for pEGFP delivery into COS7 and HEK293 cell lines according to manufacturer protocols. The transfection efficiency of Lipofectamine 2000 and PEI were compared with that of hPAMAM nanoparticles.

2.7. Cytotoxicity during transfection

After gene transfection at different weight ratios and DNA dosage, the apoptotic and dead cells were stained by Annexin V-PE apoptosis detection kit (Beyotime, China). Cell viability was then evaluated by flow cytometry.

2.8. Gene expression after transfection

The COS7 and HEK293 cell lines were transfected by hPAMAM–$phVEGF_{165}$ complexes on chamber slides. At 24 h after transfection, cells were immunostained by anti-VEGF primary antibody and Cy-3 conjugated second antibody, and 4,6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Non-transfected cells were used as controls. The immunostained cells were then visualized using a confocal microscope (Olympus, Japan).

To detect the intracellular residual hPAMAM–$phVEGF_{165}$ complexes, the transfected cells were cultured and then harvested at 14 days after transfection. TEM was then used to search the intracellular complexes.

The transfected cells on 2, 4, 8 and 14 days after transfection were collected to quantify $hVEGF_{165}$ gene expression using real-time polymerase chain reaction (PCR). The primers for $hVEGF_{165}$ amplification were (forward) 5′-ATGAACTTTCTGCTGTCTTGGGTG-3′; (reverse) 5′-TCACCGCCTCGGCTTGTCACA-3′; and 18s expression was used as an internal control. After isolation of total RNA and cDNA synthesis, the PCR thermal cycling program was: 1 cycle of enzyme activation at 95 °C for 30 s, then annealing at 58 °C for 30 s and extension at 72 °C for 30 s for 40 cycles.

At 2, 4, 8 and 14 days after transfection, the cell supernatant samples of transfected cells were collected for $hVEGF_{165}$ protein quantification using a $hVEGF_{165}$ enzyme-linked immunosorbent assay (ELISA) kit (R&D, USA) according to supplier's instructions. The $hVEGF_{165}$ protein secreted from non-transfected cells was used as a control.

2.9. Statistical analysis

The data were analyzed with SPSS 17.0 software (SPSS, USA). All values were expressed as the mean ± standard error of the mean. The significant difference between two groups was evaluated using the Student *t*-test.

3. Results

3.1. Characterization of hPAMAM–DNA particles

The average particle size and zeta potential of hPAMAM–DNA complexes at different w_{hPAMAM}/w_{DNA} ratios were shown in Fig. 1A and B. Under TEM, the hPAMAM–DNA complexes appeared oval-shaped between 100 and 500 nm, with a w_{hPAMAM}/w_{DNA} ratio of 8 using 2 μg DNA (Fig. 1C). The encapsulated DNA was protected from degradation by hPAMAM for over 3 h, in contrast to the naked DNA, which was fully degraded by DNase I in the first hour (Fig. 1D).

3.2. Optimization of hPAMAM based transfection

Maximum transfection efficiency (47.47 ± 1.42% in COS7, 40.8 ± 0.98% in HEK293), could be achieved when w_{hPAMAM}/w_{DNA}

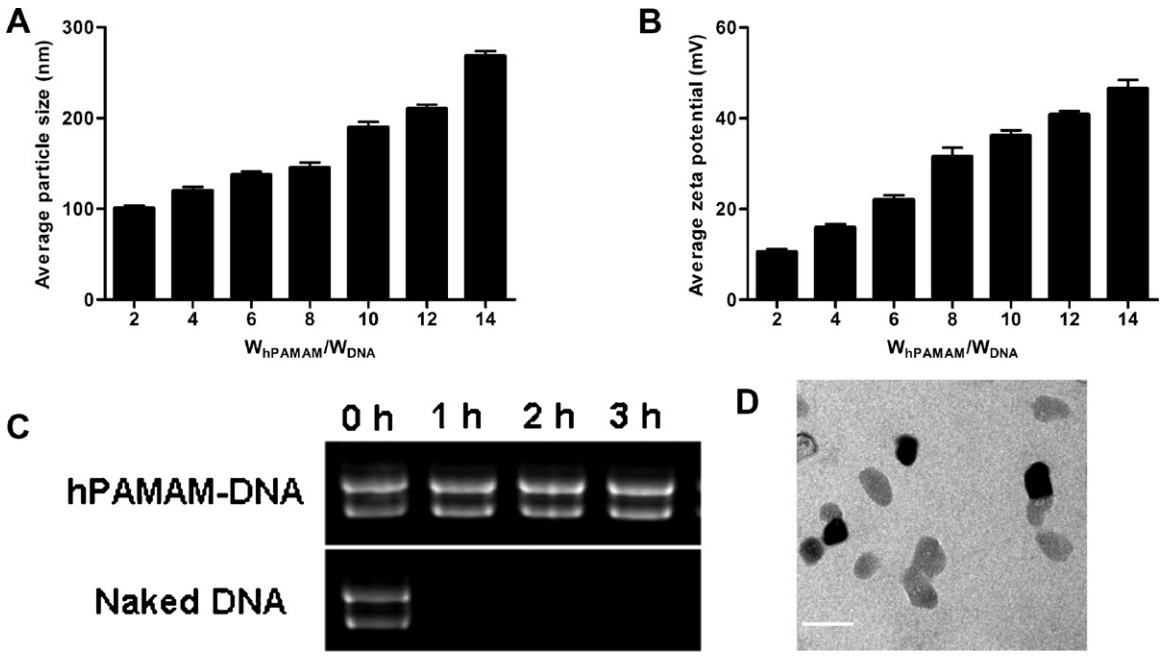

Fig. 1. Characterization of hPAMAM–DNA complexes. (A) Average particle size of hPAMAM–DNA at various w_{hPAMAM}/w_{DNA} ratios using 2 μg DNA. (B) Average zeta potential of hPAMAM–DNA at various w_{hPAMAM}/w_{DNA} ratios using 2 μg DNA. (C) TEM image of hPAMAM–DNA at w_{hPAMAM}/w_{DNA} ratio of 8 using 2 μg DNA. (D) hPAMAM encapsulated DNA showed stability against DNase I for up to 120 min, scale bar = 200 nm.

ratio was 8 using 2 μg DNA per 1×10^5 cells (Fig. 2A). And changing DNA dosage could not enhance transfection efficiency at w_{hPAMAM}/w_{DNA} ratio of 8 (Fig. 2B). Therefore, the optimal transfection condition was w_{hPAMAM}/w_{DNA} of 8 using 2 μg DNA per 1×10^5 cells. Under this condition, hPAMAM could achieve higher transfection efficiency than Lipofectamine 2000 ($37.63 \pm 1.36\%$ in COS7, $31.63 \pm 0.64\%$ in HEK293, $p < 0.01$) and PEI ($17.1 \pm 1.1\%$ in COS7, $15.43 \pm 0.44\%$ in HEK293, $p < 0.01$) (Fig. 2C–E).

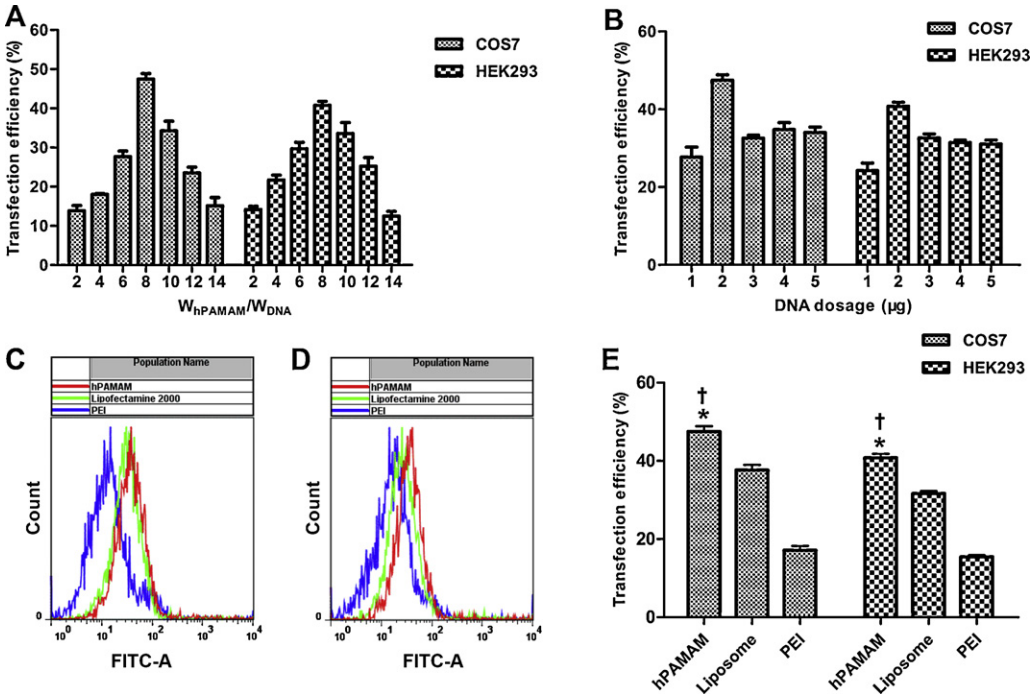

Fig. 2. Optimization of hPAMAM based transfection. (A) Transfection efficiency of hPAMAM in COS7 and HEK293 cell lines at various w_{hPAMAM}/w_{DNA} ratios. (B) Transfection efficiency of hPAMAM in COS7 and HEK293 cell lines at various DNA quantities. (C) Flow cytometry demonstrated the comparison of transfection efficiency of hPAMAM, Lipofectamine 2000 and PEI in COS7 cell lines. (D) Flow cytometry demonstrated the comparison of transfection efficiency of hPAMAM, Lipofectamine 2000 and PEI in HEK293 cell lines (red: hPAMAM; green: Lipofectamine 2000; blue: PEI). (E) Compared with Lipofectamine 2000 and PEI, hPAMAM could achieve higher transfection efficiency under optimal condition. * vs. Lipofectamine 2000, $p < 0.01$; † vs. PEI, $p < 0.01$.

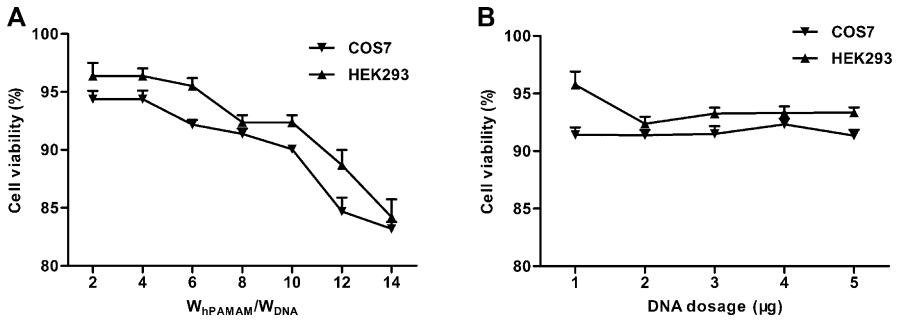

Fig. 3. Cytotoxicity of hPAMAM. (A) Cell viability on 24 h after transfection in COS7 and HEK 293 cell lines at various w_{hPAMAM}/w_{DNA} ratios. (B) Cell viability at 24 h after transfection in COS7 and HEK 293 cell lines at various DNA quantities.

3.3. Cytotoxicity of hPAMAM

With the enhancement of w_{hPAMAM}/w_{DNA} ratio, the cell viability decreased gradually at 24 h after transfection. DNA quantity did not influence the cell viability significantly. Under the optimal transfection condition, the cell viability was 91.38 ± 0.46% in COS7 and 92.38 ± 0.61% in HEK293 (Fig. 3A and B).

3.4. Gene expression under optimal transfection condition

Immunostaining showed that COS7 and HEK293 could express $hVEGF_{165}$ protein under the optimal transfection condition (Fig. 4A–D). Under microscope, the percentage of the $hVEGF_{165}$ expressing cells was 43.74 ± 0.93% (Fig. 4A), 0.56 ± 0.34% (Fig. 4B), 39.13 ± 2.07% (Fig. 4C) and 0.47 ± 0.38% (Fig. 4D). There was no

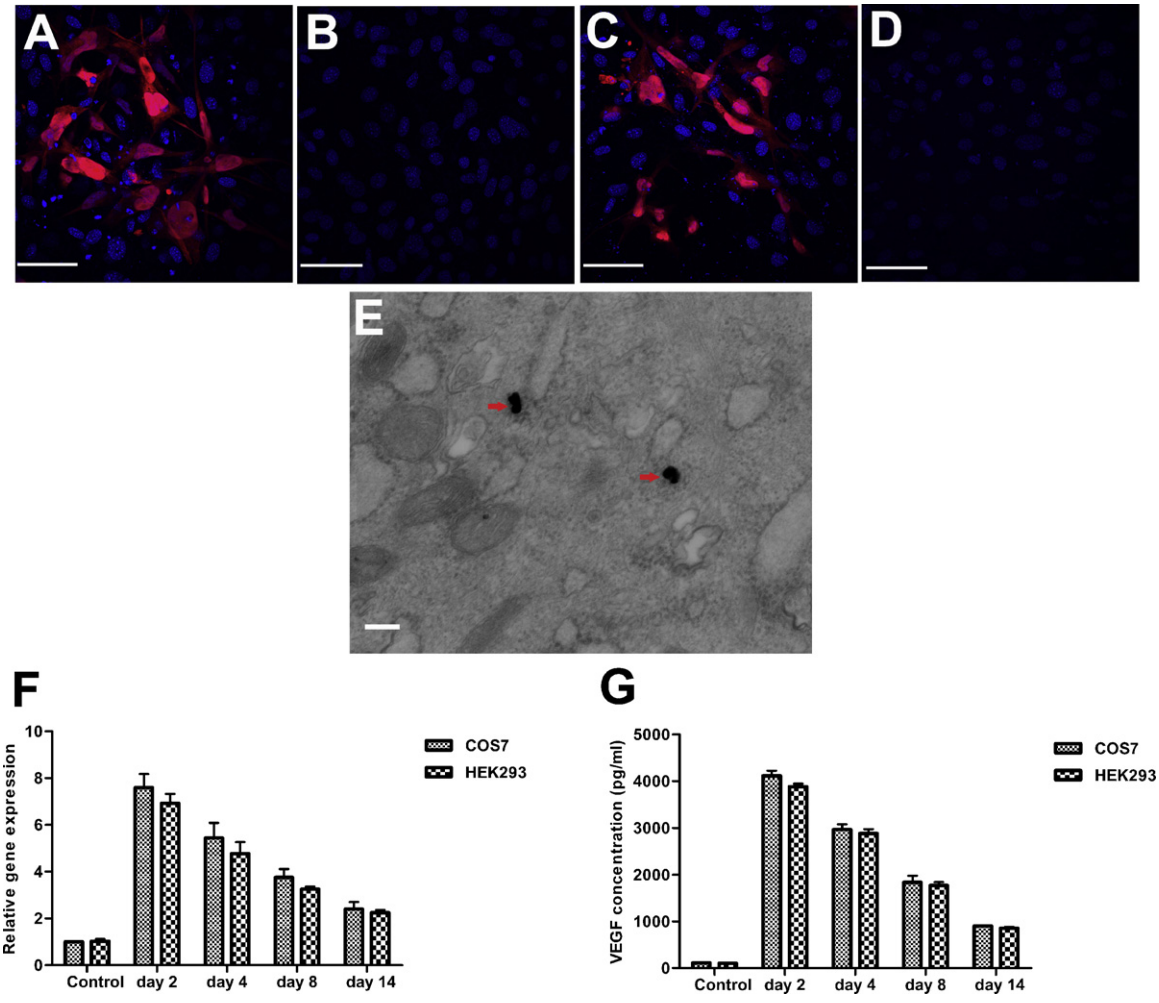

Fig. 4. Evaluation of gene expression under optimal transfection condition. (A) $hVEGF_{165}$ expression (red) from hPAMAM–$phVEGF_{165}$ transfected COS7 cell lines. Nuclei were counterstained with DAPI (blue). (B) The non-transfected COS7 cell lines were used as a control. (C) $hVEGF_{165}$ expression (red) from hPAMAM–$phVEGF_{165}$ transfected HEK293 cell lines. Nuclei were counterstained with DAPI (blue). (D) The non-transfected HEK293 cell lines were used as a control. (E) The residual hPAMAM–$phVEGF_{165}$ complexes (red arrow) on day 14 after transfection. (F) Gene expression of transfected cells during 14 days after transfection. (G) ELISA assay of $hVEGF_{165}$ concentration during 14 days after transfection, scale bar = 50 μm (A–D), 200 nm (E).

significant difference between the pEGFP based transfection efficiency on flow cytometer and percentage of $hVEGF_{165}$ expressing cells under microscope ($p > 0.05$). Under the TEM, the intracellular hPAMAM–$phVEGF_{165}$ complexes were observable at 14 days after transfection (Fig. 4E). Gene expression increased 7.60 ± 0.58 times in COS7 and 6.93 ± 0.4 times in HEK293 at day 2, 5.44 ± 0.65 times in COS7 and 4.77 ± 0.49 times in HEK293 at day 4, 3.75 ± 0.36 times in COS7 and 3.25 ± 0.11 in HEK293 at day 8, and 2.39 ± 0.31 times in COS7 and 2.24 ± 0.12 times at day 14 compared with that of untransfected ones (Fig. 4F). ELISA demonstrated that the transfected cell stably secreted $hVEGF_{165}$ protein for 2 weeks, with the peak expression level (4116.35 ± 108.04 pg/mL in COS7 and 3883.02 ± 61.53 pg/mL in HEK293) at day 2 after transfection (Fig. 4G). And there was no significant difference between COS7 and HEK293 cells on $hVEGF_{165}$ gene and protein expression ($p > 0.05$).

4. Discussion

For a non-viral gene delivery system to be clinically effective, it should be economical in synthesis, highly efficient in gene transfection, and compatible with biological components. With this goal in mind, hPAMAM was developed as a novel non-viral gene delivery vector in the study. We modified the synthesis procedure of hPAMAM nanoparticles, optimized the transfection conditions, and investigated the cytotoxicity and efficiency of this gene transfer system.

The whole synthesis procedure of hPAMAM was simple and economical. Complicated synthesis procedure of the current non-viral gene vectors, PAMAM for example, could lead to expensive cost and hinder the widespread application (Kumar et al., 2010). Using DETA and MA as raw materials, we synthesized hPAMAM successfully by a modified "one-pot" method, which was rather reliable and time-saving. Therefore, hPAMAM may serve as an economical vector for gene delivery.

After combined with hPAMAM, DNA could be protected from degradation of nuclease. As cationic polymer, the presence of primary and tertiary amines of PAMAM could be loaded with DNA and allowed for the microparticles to resist acidification in acid titration experiments. This buffering capacity against pH changes is purported to lead to endophagosomal escape into the cytoplasm through a "proton sponge mechanism" (Santos et al., 2009; Sun and Zhang, 2010). In our study, as an analog with PAMAM, hPAMAM also have a large number of primary and tertiary amines and enable the protection for DNA, which was confirmed by the integrity of DNA after nuclease digestion.

As an effective gene vector, hPAMAM exhibited encouraging gene transfection efficiency. One of the drawbacks of non-viral gene vehicle is the limited transfection efficiency (Al-Dosari and Gao, 2009). In the present study, hPAMAM also showed higher transfection efficiency than Lipofectamine 2000 and PEI (25 kDa), which have been used as commercial non-viral vectors. However, in the present study in COS7 and HEK293 cell lines, which are two model cell lines commonly used to evaluate gene transfection efficiency (Xu et al., 2008; Intra and Salem, 2010; Ping et al., 2011), the transfection efficiency of hPAMAM was $47.47 \pm 1.42\%$ in COS7 and $40.80 \pm 0.98\%$ in HEK293 under optimal condition. The hPAMAM showed higher transfection efficiency than Lipofectamine 2000 ($37.63 \pm 1.36\%$ in COS7 and $31.63 \pm 0.64\%$ in HEK293) and PEI ($17.10 \pm 1.1\%$ in COS7 and $15.43 \pm 0.44\%$ in HEK293).

The high transfection efficiency may be related to the suitable particle size and zeta potential of hPAMAM–DNA complexes at the w_{hPAMAM}/w_{DNA} ratio of 8 using 2 μg DNA. Particle size is a critical determinant of transfection efficiency and is influenced by vector/DNA ratio (Ross and Hui, 1999). Previous studies showed size-dependent internalization of particles through clathrin- and caveolae-mediated endocytosis pathways. And microspheres with a diameter of <200 nm are taken up predominantly via clathrin-mediated endocytosis and are processed along this pathway to the lysosomal compartment (Rejman et al., 2004). In our study, at the w_{hPAMAM}/w_{DNA} ratio of 8 using 2 μg DNA, the particle size achieved 145.43 ± 5.57 nm, which may bring the optimal endocytosis effect through clathrin-mediated pathway as well as maximum transfection efficiency. However, the mechanism of the suitable particle size (145.43 ± 5.57 nm) for maximum endocytosis still needs further investigation in our future study. Additionally, it was found that higher zeta potential (<26 mV) could benefit for binding hPAMAM–DNA complexes to cell membrane and result in higher transfection efficiency. But zeta potential above 26 mV could not give more binding rate and higher transfection efficiency (Ye et al., 2007, 2008). In our study, zeta potential of complexes achieved 31.58 ± 1.93 mV at the w_{hPAMAM}/w_{DNA} ratio of 8, and it continued to rise gradually with the increment of weight ratio. It is speculated that higher zeta potential (>31.58 ± 1.93 mV) as well as higher w_{hPAMAM}/w_{DNA} ratio (>8) would not enhance the transfection efficiency significantly, since as small as 26 mV is sufficient for binding the complexes to the cell membrane for endocytosis.

The hPAMAM showed minor cytotoxicity during gene transfection. Cytotoxicity was one of the major concerns in clinical application of gene vehicles (Xu et al., 2009). In our study, cell viability decreased with the enhancement of hPAMAM dosage during transfection. However, cell viability was still higher than 80% when w_{hPAMAM}/w_{DNA} ratio was 14. And under the optimal transfection condition (w_{hPAMAM}/w_{DNA} ratio = 8), the cell viability could be above 90% (91.38% in COS7 and 92.38% in HEK293), which represented the minor toxicity for cells and possibility of clinical application.

Using hPAMAM as a gene vector to deliver $phVEGF_{165}$ into cells, we observed the stable and long-term hVEGF165 expression *in vitro*. The duration and level of gene expression after transfection is critical for gene therapy and intervene (Ye et al., 2008). In our study, hPAMAM overexpressed for 14 days stably with the peak expression level (4116.35 ± 108.04 pg/mL in COS7 and 3883.02 ± 61.53 pg/mL in HEK293) at day 2 after transfection. Moreover, we observed the intracellular hPAMAM–$phVEGF_{165}$ complexes at 14 days after transfection. We speculate that DNA might be slow-released from hPAMAM nanoparticles after transfection and could overexpressed for a long time stably.

5. Conclusion

In summary, we developed hPAMAM as a novel non-viral gene delivery vector with economical synthesis procedure, high transfection efficiency and low cytotoxicity and stable gene expression. The hPAMAM is therefore likely to have significant translational potential for applications in gene delivery.

Acknowledgments

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