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Self-assembled human serum albumin-coated complexes for gene delivery with improved transfection

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The efficiency and safety of gene delivery vectors were important factors for gene therapy. To enhance gene transfection efficiency and to incorporate biocompatible components to the polyamidoamine (PAMAM) dendrimer mediated gene delivery systems, human serum albumin (HSA) was introduced to dendriplexes of PAMAM dendrimer and DNA via electrostatic interactions to form self-assembled PAMAM/DNA/HSA complexes (HSA-dendriplexes). The self-assembled complexes were characterized by gel retardation assay and particle size and zeta potential analysis. Meanwhile, the toxicity of HSA-dendriplexes was evaluated by the MTT assay and haemolysis test, which indicated that the complexes exhibited decreased cytotoxicity with the incorporation of HSA. As compared to dendriplexes, the ternary HSA-dendriplexes increased the enhanced green fluorescent protein gene (EGFP) expression *in vitro* by 1.7-fold. In addition, HSA-dendriplexes showed a significantly higher luciferase gene expression than dendriplexes or naked DNA in the liver, kidney, lungs and spleen of mice. Our results demonstrated that HSA-dendriplexes increases PAMAM mediated gene transfection efficiency and decreases the cytotoxicity and haemolysis, which made the ternary complexes a promising targeting gene delivery system.

1. Introduction

Gene therapy is a promising approach in disease therapy. The safety and the efficiency of the gene delivery vector were important factors for gene therapy in a wide variety of illnesses (Scollay 2001; Soo et al. 2011). Thus, different gene delivery vector systems such as recombinant virus vectors (Chtarto et al. 2003), polycationic polymers (Merdan et al. 2002), cationic liposomes (Wanlop et al. 2009) and carbon nanotubes (Cyrille et al. 2009; Rui et al. 2010) have been developed in recent years to achieve gene transfection of high-efficiency and low-toxicity. Compared to viral vectors, nonviral vectors are safer, more versatile and easy of manufacture (Jung-hua et al. 2010). Among these nonviral vectors, cationic nonviral vectors such as cationic polyamidoamine (PAMAM) dendrimers have unique nanocharacteristics, promising a potential gene delivery vector for gene therapy in the future (Kukowska-Latallo et al. 1996).

PAMAM dendrimers were macromolecules with a regular and highly branched spherical structure which have a unique surface of primary amino groups (Kukowska-Latallo et al. 1996). Therefore, PAMAM dendrimers showed high charge densities due to the surface of the molecule (Frechet 1994). Thus, PAMAM dendrimers have received most attention as potential transfection agents for gene delivery because those macromolecules endow unique characteristics of functional groups and form a self-assembled structure with nucleic acids (Froehlich et al. 2009).

However, the transfection efficiency and cytotoxicity is a dilemma for PAMAM mediated gene transfection (Jevprasesphant et al. 2003). Many efforts have been made to increase the transfection efficiency by modification of the amino groups of PAMAM with a poly-amino group such as arginine (Dipak et al. 2008; Choi et al. 2004) or ornithine (Ajay et al. 2010) to increase the cationic surface charge. Nevertheless, with the increase in transfection efficiency, surface modified PAMAM dendrimers showed a higher toxicity than native PAMAM. Conjugating with the poly(ethylene glycol) (PEG) chains has been considered as a method to reduce the toxicity and to increase the biocompatibility of PAMAM dendrimers (Jevprasesphant et al. 2003). That was because PEG is nontoxic, nonimmunogenic, water-soluble, and has favorable pharmokinetics with tissue distribution. However, PEG modification can alter the binding affinity of PAMAM to DNAs, drug components, and proteins in general (Froehlich et al. 2009). In addition, all the modification strategies developed above were through chemical reaction, which were complex, inflexible, and made if difficult to maintain the bioactivity of the ligands. Thus, a better approach is needed to reduce the toxicity and increase the biocompatibility and transfection efficiency for the PAMAM mediated gene transfection at the same time.

Molecular self-assembly is a convenient, rapid and powerful strategy for fabricating nano-sized complexes with various supramolecular architectures. Compared to chemical reactions, the self-assembly strategy is more convenient, flexible, and favorable for keeping the bioactivity of the biomacromolecule (Zhang et al. 2010). Recent studies have demonstrated that polyamidoamine (PAMAM) dendrimers can bind human serum albumin (HSA) due to the electrostatic interaction of polymers and protein (Froehlich et al. 2009; Shcharbin et al. 2005, 2007). HSA has always been the center of attention of pharmaceutical industry due to its excellent biocompatibility (Kratz 2008). Moreover, HSA is an essential nutrient for the highly proliferative tumor cells. Reports have demonstrated the cancer cells' overexpression of specific human serum albumin (HSA)

Table 1: Size and zeta potential of dendriplexes with PAMAM G1-G5 dendrimers at N/P ratio 10*

Dendrimer generation	Surface amino groups	Molecular weight (Da)	Particle size (nm)	Zeta potential (mV)
G1	8	1430	735.2 ± 75.7	−11.8 ± 0.5
G2	16	3256	542.4 ± 52.7	4.5 ± 0.2
G3	32	6909	482.7 ± 30.7	9.1 ± 0.6
G4	64	14215	422.6 ± 58.0	12.8 ± 0.8
G5	128	28826	322.5 ± 5.11	18.8 ± 0.9

* DNA concentration within the complexes was 5 μg/mL. Data was expressed as the mean ± standard deviation (n = 3).

receptors and their ability to internalize large amounts of albumin through the mechanism of caveolae-mediated endocytosis (Hawkins et al. 2008; Bunuales et al. 2011; Di et al. 2005; Kratz 2010).

Above-mentioned studies have propelled us to hypothesize that the addition of HSA to PAMAM mediated gene delivery system may increase the cellular uptake due to specific interactions between HSA and HSA receptors on tumor cells resulting in a high transfection efficiency. In addition, the net positive charge of the dendriplexes may decrease after the addition of negatively charged HSA so that the HSA-dendriplexes have a lower toxicity. To test this hypothesis, we prepared self-assembled HSA-dendriplexes via electrostatic adsorption of PAMAM-DNA complexes to negatively charged HSA. HSA-dendriplexes were characterized by transmission electron microscope (TEM) dynamic light scattering (DLS) and zeta-potential measurement. The toxicity of HSA-dendriplexes was evaluated by the MTT assay and haemolysis test. The ability of HSA-dendriplexes to enhance transfection efficiency was evaluated *in vitro* and *in vivo*.

2. Investigations, results and discussion

2.1. Optimization of N/P ratio and PAMAM generation

The complexes formed by different N/P ratios and different generations were used to transfect HEK 293T cells with a luciferase gene to determine the optimal N/P ratio and PAMAM generation. As shown in Fig. 1, the luciferase activity increased significantly with increasing the N/P ratio of dendriplexes in all PAMAM generations. Additionally, compared with dendriplexes formed by different generations of PAMAM at the same N/P ratio, a generation–dependent increase of the luciferase activity has been observed. Among all studied groups, dendriplexes formed by PAMAM G5 at the N/P ratio 10 and 15 showed a high efficiency of luciferase expression. However, significant cytotoxicity was observed at the N/P ratio 15.

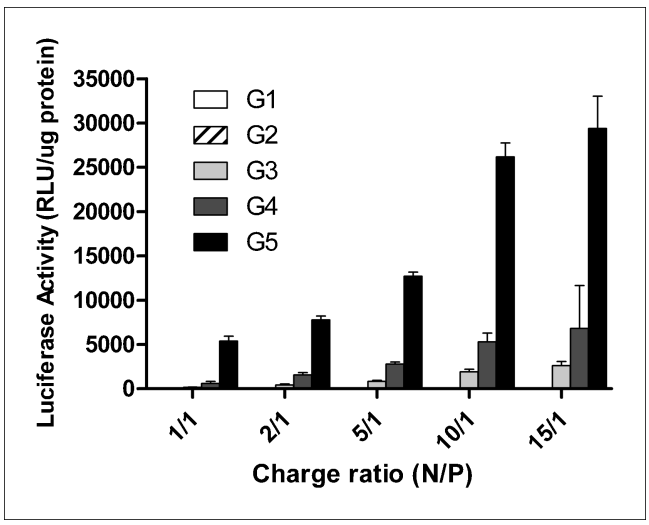


Fig. 1: Gene transfection effeciency of different PAMAM generations at various charge ratios (N/P). The results were expressed as mean ± standard deviation (n = 3)

As shown in Table 1, particle size of the dendriplexes decreased from 735.2 ± 75.7 to 322.5 ± 5.11 nm with an increase in the generation of PAMAM dendrimer. DNA was well condensed by PAMAM G4 and G5, while only partially condensed by lower generations (G1 to G4). A gradually increase of zeta potential was observed from lower generation to higher generation. It is known that the transfection efficiency of dendriplexes is related to their surface charge and size. Thus higher surface charge has been considered stable and also uneasy to form the aggregation of dendriplexes which leads to a low transfection effects (Ahmed et al. 2006). Thus, the N/P ratio 10 and generation 5 PAMAM were used for the preparation of the dendriplexes in the subsequence experiments.

2.2. Characterization of HSA-dendriplex

To study the influence of the HSA participation on particle size and surface charge, HSA-dendriplexes were characterized by dynamic light scattering, zeta-potential measurements and TEM.

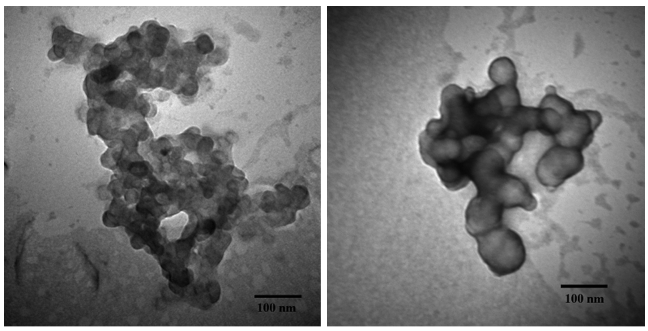
As shown in Table 2, the particle size of the HSA-dendriplex increased with adding of HSA at a HSA/DNA weight ratio from 0.002 to 2. The particle sizes of the dendriplexes were in the range of 318.3 ± 9.1 to 334.8 ± 31.9 nm. At the HSA/DNA weight ratio of 20, the largest HSA-dendriplexes (630.7 ± 20.8 nm) can be reached due to the binding of negative charged HSA to positive charged dendriplex and the lowest zeta potential. Regarding of the zeta-potential of HSA-dendriplex, all the simples were net positive charge. Increasing the HSA/DNA weight ratio from 0 to 20 resulted in significant decrease of zeta potential from 18.8 ± 0.9 to 2.5 ± 0.2 mV, suggesting that HSA can bind the dendriplex and partly neutralize of over positive charge which may cause the cytotoxicity and haemolysis *in vitro* and *in vivo*.

TEM image of the morphology of HSA-dendriplexes prepared at charge ratio (N/P) 10:1 and HSA/DNA weight ratio 2 were

Table 2: Size and zeta potential of HSA-dendriplex*

HSA/DNA weight ratio	Particle size (nm)	Zeta potential (mV)
0	322.5 ± 5.11	18.8 ± 0.9
0.002	318.3 ± 9.1	17.1 ± 0.8
0.02	319.2 ± 6.2	16.0 ± 1.1
0.2	327.9 ± 9.7	10.7 ± 2.3
2	334.8 ± 31.9	7.1 ± 1.7
20	630.7 ± 20.8	2.5 ± 0.2

* DNA concentration within the complexes was 5 μg/mL. Data was expressed as the mean ± standard deviation (n = 3).



(a) (b)

Fig. 2: TEM image of different complexes: (a) dendriplexes at PAMAM/DNA charge ratio (N/P) of 10, (b) dendriplexes at PAMAM/DNA charge ratio (N/P) of 10 and HSA/DNA weight ratio of 2. (200 000 X)

shown in Fig. 2. The size observed was similar to the measurements obtained by dynamic light scattering. Compared to dendriplex (Fig. 2a), the surface topography of HSA-dendriplex (Fig. 2b) was smoothes, suggesting that HSA was coated on the surface of dendriplex.

2.3. Formation of HSA-dendriplexes

When ethidium bromide (EB) intercalates DNA (2–4 base pairs for EB), a significant increase in fluorescence intensity has been observed. Dendrimers such as PAMAM with higher affinity for DNA can displace EB and decrease the fluorescence. This made EB a useful probe to measure PAMAM-DNA interactions. As shown in Fig. 3, the relative fluorescence decreased dramatically at the N/P ratio 10 as compared with free DNA. No significant difference in the relative fluorescence uints has been observed between dendriplex and HSA-dendriplex at different weight ratios of HSA/DNA at the N/P ratio till 10, indicating that adding of HSA has no effect on the binding capability of dendrimers to DNA.

As shown in Fig. 4a, an electrophoretic mobilization of DNA was retarded completely in lane 9 to lane 12, suggesting that HSA-dendriplex of the N/P ratio from 5 to 20 could completely bind plasmid DNA and complexes were positively charged and physically stable at neutral pH. Similarly, for HSA-dendriplexes with a PAMAM/DNA charge ratio (N/P) of 10 and different HSA/DNA weight ratio (Fig. 4b), the ternary complexes were able to efficiently bind DNA at the HSA/DNA weight ratio from

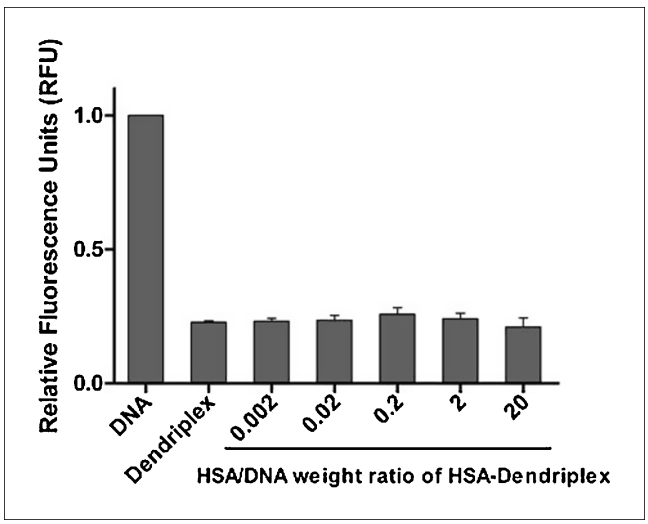


Fig. 3: DNA condensation assay of HSA-dendriplex with a range of HSA/DNA weight ratio (expressed on the x-axis). Data was presented as mean ± standard deviation (n = 3)

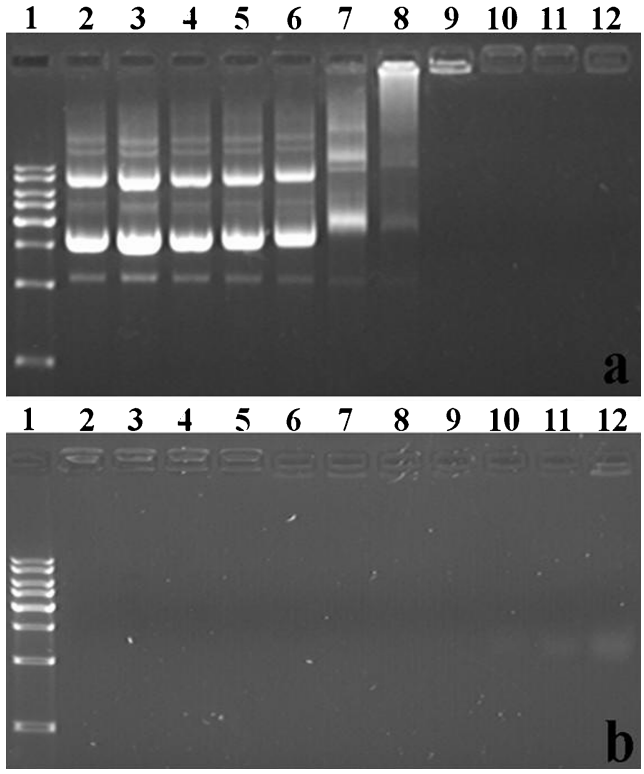


Fig. 4: Agarose gel retardation assay of different complexes: (a) HSA-dendriplexes with a HSA/DNA weight ratio of 2 and different PAMAM/DNA charge ratio (N/P). Lane 1: DNA ladder; Lane 2: plasmid DNA; Lane 3–12: HSA-dendriplex at PAMAM/DNA charge ratio of 0.05, 0.1, 0. 2, 0.5, 1, 2, 5, 10, 15, 20. (b) HSA-dendriplexes with a PAMAM/DNA charge ratio (N/P) of 10 and different HSA/DNA weight ratio. Lane 1: DNA ladder; Lane 2–12: HSA-dendriplex at HSA/DNA weight ratio of 0.002, 0.02. 0.2, 1, 2, 5, 10, 20, 40, 60, 80

0.002 to 20. However, for the HSA-dendriplex at the HSA/DNA weight ratio above 40, the mobilization of DNA could not be retarded completely, indicating that, the HSA/DNA weight ratio should be less than 40 in order to load plasmid DNA completely.

2.4. Stability of the HSA-coated dendriplex

To investigate whether HSA-dendriplex can protect plasmid DNA against the degradation from DNase I, naked DNA and HSA-dendriplex at HSA/DNA weight ratio of 20 were incubated with DNase I and their stability was analyzed by 0.8% agarose gel electrophoresis. As shown in Fig. 5, naked plasmid DNA was completely digested by DNase I, whereas DNA in HSA-dendriplex remained intact. In general, DNase I mediated DNA digestion requires the interaction of enzyme and DNA. However, in our study, DNA was condensed in the HSA-dendriplexes with the binding of PAMAM and DNA. HSA on the surface of HSA-dendriplex the binding of PAMAM and DNA forms steric hindrance to can resist the interactionbetween DNase I and DNA.

2.5. Transfection in vitro

2.5.1. Human serum albumin increases PAMAM-mediated gene transfer into 293T cells

We examined the effect of HSA on transfection mediated by PAMAM/DNA complexes at different HSA/DNA weight ratios. An enhancement of transfection has been observed by an increase of the HSA/DNA weight ratio (Fig. 6). When HSA was added to preformed PAMAM/DNA complexes, the number of GFP-expressing cells, as evaluated by fluorescence microscope

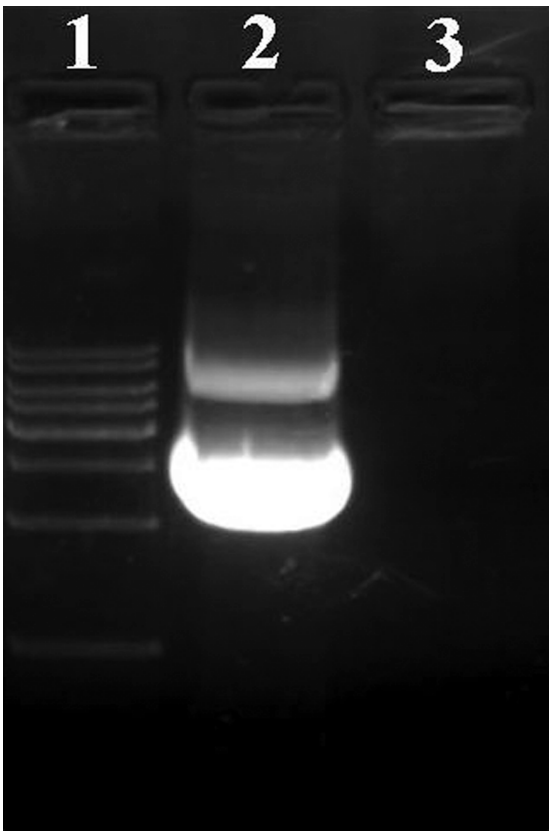


Fig. 5: The stability of HSA-dendriplex against DNase I digestion. Lane 1: DNA ladder; lane 2: HSA-dendriplex at N/P 10 and HSA/DNA weight ratio 2; lane 3: plasmid DNA

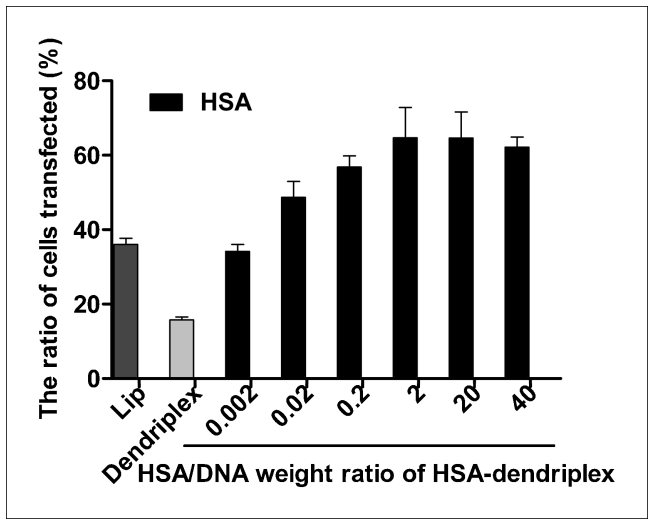


Fig. 7: Comparation of enhanced transfection efficiency of HSA-dendriplexes. Each data point in line represented the mean ± standard deviation (n = 3)

and flow cytometry (Fig. 7), has been highly increased from 34.1 ± 1.9% to 64.9 ± 8.1% whereas the transfection efficiency of lipofectamine 2000 was 36.1 ± 1.6%.

The enhancement of PAMAM-mediated gene transfer by albumin was found in transfected cells with an increase of the HSA/DNA weight ratio. These data suggested that the more albumin is available on the surface of the complexes, the higher gene expression will be. However, a plateau and slightly decrease in transfection occurred with higher albumin amounts. For nonviral gene vectors, intracellular uptake is a key influence in transfection efficiency. Pathways feasible for the cellular uptake of nonviral gene delivery vectors include: clathrin-mediated pathways, caveolae-mediated pathways, and clathrin-, caveolae-independent pathways (Peng et al. 2010; Khalil et al. 2006). Dendriplexes bind to the phospholipid membranes at the cell surface by electrostatic interaction and are uptaken by clathrin-mediated endocytosis (Mislick et al. 1996; Mounkes et al. 1998). In this route, gene vectors are finally transported to lysosomes. Even though a dendrimer can act as a 'proton sponge' to protect plasmids (Tang et al. 1996), major plasmid breakdown occurs, contributing to low transfection efficiencies (Sabah et al. 2004). Therefore, methods of escaping the clathrin-mediated pathway will be advantageous for nonviral gene delivery vectors. Human serum albumin coated nanoparticles are able to enter cells via the caveolae mediated pathway, and escape endosome/lysosome entrapment (Mo et al. 2007). In this route, no extremely low pH environment organelle can be reached (Ma and Diamond 2001). Thus, with HSA coating on the surface of dendriplex, HSA-dendriplex may change the intracellular trafficking route to escape endosome/lysosome entrapment, which resulted, therefore, more preferable for gene delivery.

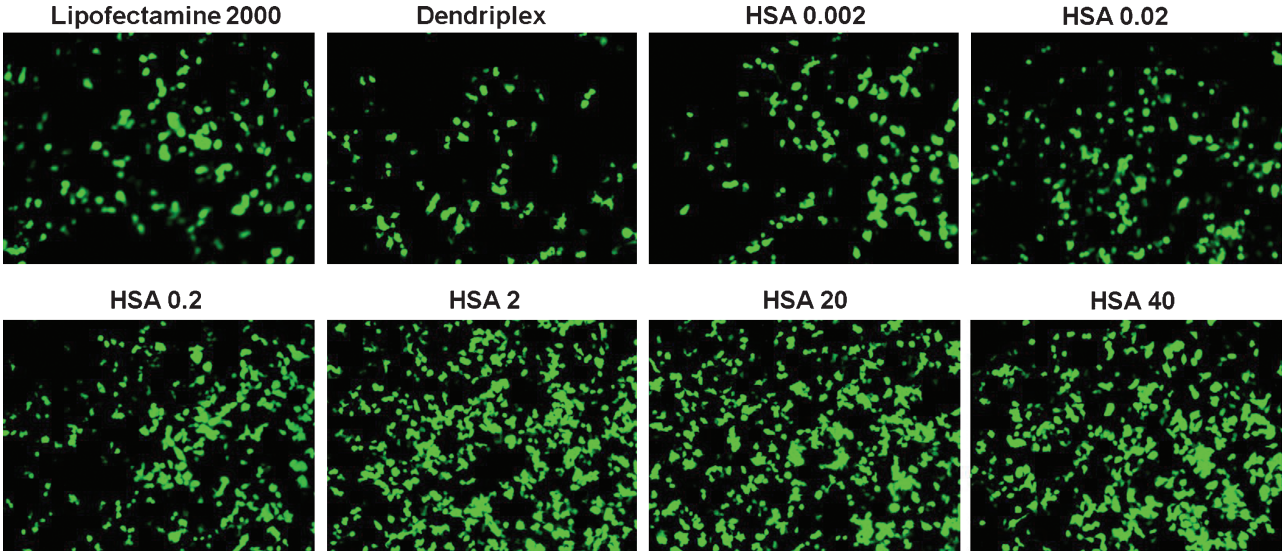


Fig. 6: Expression of enhanced green fluorescent protein (EGFP) in 293T cells transfected with EGFP-N3 seen through a fluorescence microscope

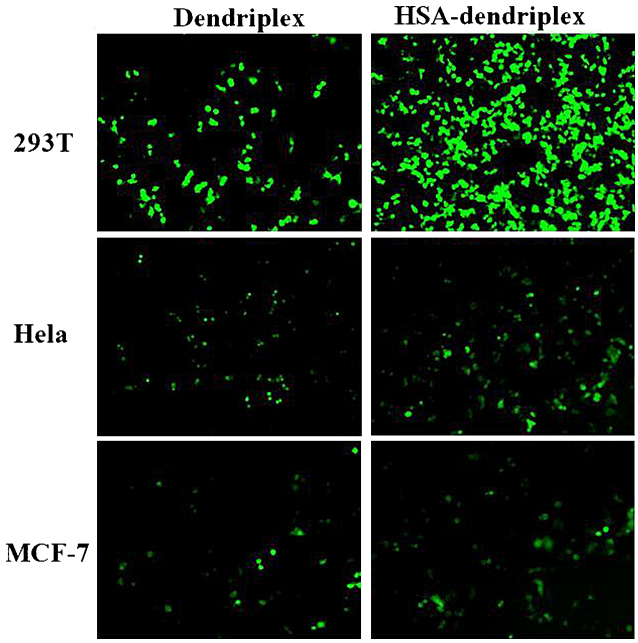


Fig. 8: Transfection efficiency of dendriplex at N/P 10 and HSA-dendriplex at N/P 10 of HSA/DNA weight ratio of 2 in 293T, Hela, MCF-7 cell lines

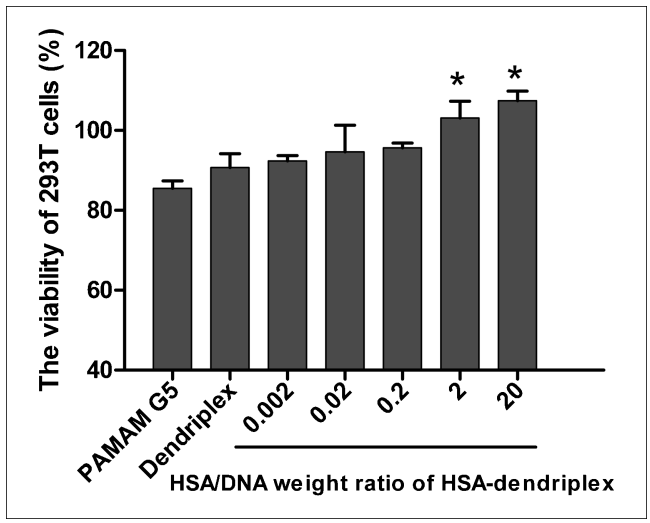


Fig. 9: Cytotoxicity of HSA-dendriplexes at N/P 10 and various HSA/DNA weight ratios. PAMAM concentration within the complexes was 40 μg/mL. Results were expressed as mean ± standard deviation (n = 3). *$p < 0.05$ versus the dendriplex group

2.5.2. Enhanced gene transfection efficiency in various cell lines

It is well known that transfection efficiency of the synthetic vectors depends on the nature of the cell line. This may be because of the difference in cellular binding, internalization mechanism and the properties of the cell line such as doubling time, rate of uptake of nutrients and related biochemical processes (Douglas et al. 2008; Mo and Lim 2004; Panyam et al. 2002; Qaddoumi et al. 2003). In our study, the percentage of transfection efficiency and GFP expression levels with PAMAM/DNA/HSA complex were significantly higher than the simple PAMAM/DNA complexes in all tested cell lines (Fig. 8). GFP expression was higher in HEK 293T cells than Hela cells and MCF-7 cells. Our results clearly indicated that HSA-dendriplexes were better transfecting agents as dendriplexes in all of the tested cell lines.

2.6. Cytotoxicity of PAMAM dendrimers–DNA complexes

The cytotoxicity of dendriplexes and HSA-dendriplexes were evaluated by the MTT assay and the results are illustrated in Fig. 9. As compared to PAMAM G5 dendrimer (85.5 ± 2.5%) and dendriplex (90.4 ± 3.5%), the viability of HEK 293T cell was increased relatively, from 91.7 ± 2.4% to 107.7 ± 1.9%. The cytotoxicity of PAMAM dendrimers was known to be generation dependent; low-generation dendrimers showed significantly less cytotoxicity than high-generation dendrimers (Jevprasesphant et al. 2003). Additionally, the nature and density of charged groups are other factors that determine the toxicity of dendrimers (Dufes et al. 2005). With the coating of HSA, the over positively charged surface of the dendriplex was coated by negatively charged HSA, which made them be more biocompatible. Meanwhile, the decrease of zeta potential, see Table 2, can be attributed to the decrease in cytotoxicity of dendriplex.

2.7. Haemolysis study

One of the most convenient ways to deliver a gene based on dendrimers *in vivo* is intravenous injection. However, if that could be done, blood constituents would be likely to be the first and unwanted targets of their action; in particular, haemolysis can occur. It is well known that haemolysis can be caused by various factors, e.g. hypotonic solutions and toxic compounds (Klajnert et al. 2010).

As illustrated on Fig. 10b, aggregations of red blood cells were observed and very few cells maintained normal physiological shape after 3 h incubation at 37 °C with dendriplex prepaired at N/P 10. When erythrocytes were incubated with HSA-dendriplex, most of erythrocytes maintained their normal shape and no aggregation of red blood cells was observed (Fig. 10c).

As shown in Fig. 11, time-dependent haemolysis was observed in all samples. The longer the erythrocytes were incubated with complexes, the more haemolysis was observed. The decrease of haemolysis was observed with increased HSA added. Adding HSA to the system significantly reduced the amount of haemolysis caused by PAMAM G5 dendrimers. HSA-coated dendriplex leads to the least haemolytic and G5 PAMAM dendrimer leads the most haemolytic in our study.

Haemolysis test has been widely used in evaluating red blood cell lysis and the amount of haemoglobin release. The data obtained in such assays also gave a qualitative indication of the interaction between biomaterials and red blood cells. High haemolytic data suggest that certain biomaterials may potentially cause damage when administered intravenously. In general, the membrane of red blood cells is negatively charged. High-positive charged PAMAM dendriplexes can interact with both the lecithoid bilayer and certain membrane proteins of red blood cells, which causes aggregations of erythrocytes. The cationic surface group numbers of dendriplexes determines the ability of those ineractions which may lead to the haemolysis activity. Our study clearly showed that the coating of HSA party neutralized of positive charge of dendriplex and diminished the membrane damage caused by dendrimers.

2.8. Transfection in vivo

Finally, to examine the potential application of HSA-dendriplexes for *in vivo* gene delivery, we determined the expression levels of Renilla luciferase gene in the heart, liver, spleen, lungs and kidney of mice via intravenous administration. Plain dendriplexes and naked DNA were used as controls. HSA-dendriplexes mediated significantly higher levels of luciferase gene expression in the liver, kidney, lungs and spleen, compared

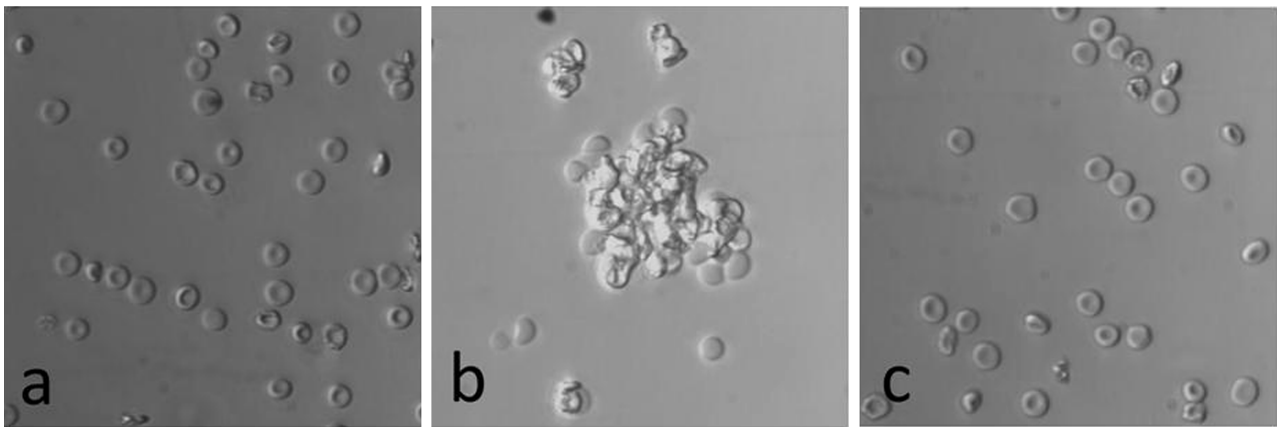


Fig. 10: Optical micrographs of control erythrocytes (haematocrit 0.2%) (a); erythrocytes (haematocrit 0.2%) after 3 h incubation at 37 °C with dendriplex prepaired at N/P 10 (b); erythrocytes (haematocrit 0.2%) after 3 h incubation at 37 °C with HSA-dendriplex prepaired at N/P 10 at PAMAM G5 and at HSA/DNA weight ratio 20 (c). Magnification 400 ×

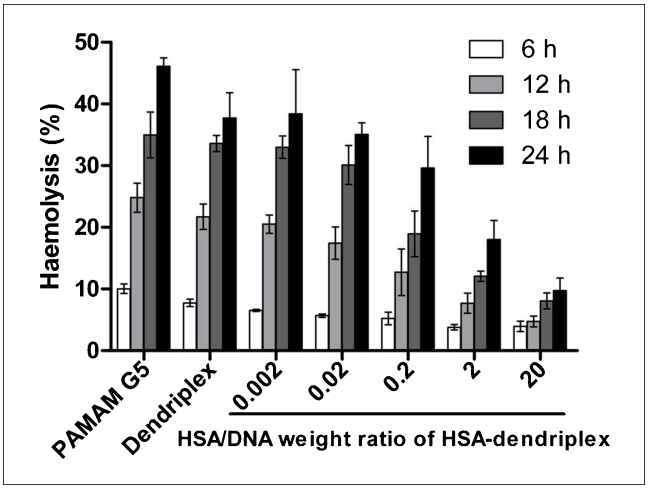


Fig. 11: Haemolysis induced by HSA-dendriplex, the concentration of PAMAM dendrimer is 3 mg/mL. Results were expressed as mean ± standard deviation (n = 3)

to plain dendriplexes or naked DNA. Luciferase gene expression in the heart did not show a significant difference.

As what has been observed *in vitro*, HSA-dendriplexes showed higher levels of gene expression than dendriplexes. It is likely that the presence of a major component of serum, HSA, on the dendriplexes minimizes their interaction with other serum components, including oleic acid and heparin which were shown to promote the dissociation of genetic material from the complexes (Zelphati et al. 1998).

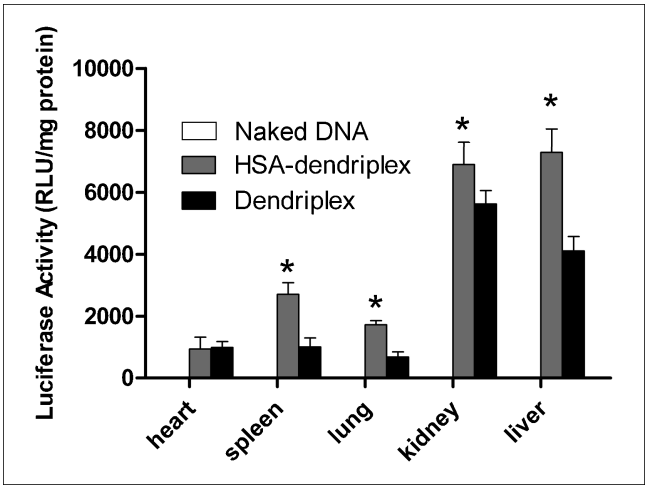


Fig. 12: *In vivo* gene expression after intravenous injection of naked DNA, dendriplexes at N/P 10, HSA-dendriplex at N/P 10 and HSA/DNA weight ratio of 20 and dendriplexes at N/P 10. The bars represented the mean ± standard deviation (n = 6). *$p < 0.05$ versus the dendriplex group

2.9. Conclusions

In conclusion, the HSA-dendriplexes in this study were shown to generate nanosized particles with plasmid DNA. The carrier had a remarkably low toxicity and good transfection performance *in vitro* and *in vivo*. Because of the abundance and the easy preparation and purification, low immunogenicity, low toxicity and low cost, we believe that HSA-dendriplex is a novel nano gene delivery system. Further studies to understand the mechanism of HSA-dendriplexes uptake and distribution of HSA-dendriplexes *in vivo* are in progress.

3. Experimental

3.1. Materials

PAMAM dendrimers of different generations were purchased from Sigma-Aldrich (Shanghai, China). Lipofectamine^{TM} 2000 Transfection Reagent was purchased from Invitrogen. The plasmids pLC-3.0, (Promega, Beijing, China) encoding luciferase, pRL-CMV encoding renilla luciferase (Promega, Beijing, China) and pEGFP-N3 encoding EGFP (Clontech, US) were used in the transfections studies. All other reagents were obtained from Biodee Reagent Company, Beijing, China.

3.2. Cell culture

HEK 293T (human embryonic kidney cells), Hela (human epithelioid cervical carcinoma cells) and MCF-7 (human breast cancer cells) cells were obtained from China Center for Type Culture Collection (Wuhan, China). The cells were maintained at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle's Medium (Dulbecco's modified Eagle's medium (DMEM), Gibco, Invitrogen), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL) (Gibco, Beijing, China).

3.3. Plasmid preparation

Plasmid was amplified in *Escherichia coli* (strain DH5α), isolated and purified using an endotoxin-free Plasmid Giga Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. Concentration and purity of plasmid were assessed using UV-6300 Spectrophotometer (Mapada, Shanghai, China) at 260 nm and 280 nm. Plasmid integrity was confirmed by 0.8% agarose gel electrophoresis and stored at −20 °C until further use.

3.4. Preparation of dendriplexes and HSA-dendriplex

Dendriplexes of different charge ratios (N/P) were formed by incubating the two components together in PBS (150 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM NaH_2PO_4, pH 7.4) for 15 min at 37 °C. Charge ratios (N/P) were

calculated based on the number of terminal amine groups on a PAMAM dendrimer and the number of phosphate groups in the plasmid DNA. HSA-dendriplexes were prepared by adding HSA to the preformed dendriplexes at N/P ratio 10. The required amount of HSA was added to the preformed dendriplexes and vortexed in PBS. Then, HSA-dendriplexes were formed after incubated for another 15 min at 37 °C. The weight ratio of HSA and plasmid DNA was from 0.002 to 20.

3.5. DNA condensation

DNA condensation was monitored by ethidium bromide (EB) interaction assay (Shcharbin et al. 2009). Briefly, 1 μL EB solution (0.5 mg/mL) was added to 100 μL blank solution (PBS, 50 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM NaH_2PO_4, pH 7.4), 100 μL dendriplexes and HSA-dendriplexes solutions. After incubation for 2 min at room temperature, the fluorescence was measured and analyzed by using *TECAN* Safire2 Multimode Reader (*TECAN*, Shanghai, China) with excitation and emission at 260 and 600 nm, respectively. Results were expressed as relative fluorescence (%) to DNA control, and were corrected for background fluorescence of free EB in solution.

3.6. Gel retardation assay

Different HSA-dendriplexes were prepared by incubating in PBS at room temperature for 30 min. Each sample was analyzed by electrophoresis on a 0.8% agarose containing EB (0.5 μg/mL) at 80 V for 1 h. The location of the DNA was identified under UV irradiation.

3.7. Characterization

Zeta potential and size of the dendriplexes prepared at different N/P ratios and HSA-dendriplexes prepared at different HSA/DNA weight ratios were measured by using Nano-ZS90 Zetasizer (Malvern, UK). All measurements were carried out on the dendriplexes with 5 μg/mL plasmid DNA in PBS at pH 7.4. The morphology of the samples was examined by transmission electron microscopy (TEM) using a H-7650B microscope (HITACHI, Japan).

3.8. HSA-dendriplex stability

HSA-dendriplexes were prepared at different HSA/DNA weight ratios (0.002–20), and the final DNA concentration was 50 μg/mL in 100 μL of total volume. Each HSA-dendriplex solution was incubated with DNase I (5 U/μg of plasmid DNA) at 37 °C for 30 min. 3 μL of EDTA (0.5 M) solution was added to stop the DNA degradation, and SDS was added to 1% final concentration in order to disassemble the complexes. Then, all the samples were incubated for 1 hour and analyzed by 1% agarose gel electrophoresis to evaluate the integrity of DNA in the dendriplexes.

3.9. Determination of optimal N/P ratio and generation

For transfection, cells were seeded at a density of 10^5 cells/well in a 24-well culture plate, grown for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity using DMEM (pH 7.4) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin solution. After this, the medium was removed and washed with PBS twice. Subsequently, 50 μL of the DNA complexes (containing 1 μg of pCL-3.0 plasmid or pEGFP-N3 plasmid) and 0.2 mL DMEM were added to each well, and incubated for 5 h at 37 °C with growth medium for another 48 h. Levels of luciferase activity in the transfected cells were assayed by luciferase assay kit (promega), according to the manufacturer's introduction.

3.10. Determination of percentage of cells transfected

HSA-dendriplexes were prepared by complexing 1 μg of pEGFP-N3 at N/P of 10 at different HSA/DNA weight ratios (0.002–40). Cells were transfected as described above. After transfection, cells were detached by tripsin (Gibco). Cell suspensions were then transferred to microtubes (BD), fixed by 0.2 mM EDTA. The percentage of cells transfected was quantitatively assessed at 48 h after transfection by flow cytometry by FITC (emission 530/30 bandpass) filter using fluorescence-activated cell sorting (FACS) machine (BD FACSAria system, argon ion laser 488 nm). For each sample, 10,000 events were collected and fluorescence was detected. Transfection efficiency was calculated based on the percentage of the cells that expressed pEGFP (positive cells) in the total number of cells.

3.11. Transfection efficiency in various cell lines

HEK 293T, Hela and MCF-7 cell lines Cells were transfected as described above. Qualitative assessment of pEGFP expressed in transfected cells was done by observing the cells under a fluorescent microscope (LEICA DMIL).

3.12. In vitro cytotoxicity

HEK 293T cells were seeded at a density of 1×10^4 cells per well in 200 μL medium in a 96 well plate. After an overnight culture at 37 °C in a 5% CO_2 humidified atmosphere, cells were treated with PAMAM dendrimer, dendriplexes and HSA-dendriplexes (containing 1 μg of plasmid) prepared at N/P ratio 10. After 5 h incubation, 20 μL of MTT (5 mg/mL) was added and the cells were incubated for approximately 4 h. The growth medium was removed and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the MTT crystals and the optical density was read using *TECAN* Safire2 Multimode microplate reader (*TECAN*, Shanghai, China) with 570 nm as excitation wavelength and 630 nm as the background. Viability of cells exposed to dendriplexes was expressed as a percentage of the viability of cells grown in the absence of dendriplexes.

3.13. Haemolysis test

In vivo toxicity of the dendriplexes and HSA-dendriplexes was evaluated by haemolysis test (Klajnert et al. 2010). Blood from rats from the experimental animal center of Tsinghua University was anticoagulated with 3 per cent sodium citrate. Erythrocytes were separated from the plasma and leucocytes by centrifugation (1000 *g*, 5 min) at 4 °C and washed three times with PBS. Erythrocytes at a haematocrit of 2% (the proportion of the blood volume occupied by red blood cells) were suspended in different HSA-dendriplex solutions at concentration of 4.5 mg/mL and incubated at 37 °C for 6, 12, 18 and 24 h. HSA-dendriplex solutions were dissolved in PBS.

For microscopy, 10-fold diluted samples were then viewed after incubated at 37 °C for 3 h under a fluorescent microscope (LEICA DMIL) at a magnification of 400 ×.

To measure the haemolysis caused by the dendrimers, the erythrocyte suspensions were centrifuged (1000 *g*, 5 min) and the percentage haemolysis was determined on the basis of haemoglobin released into the supernatants, measured spectrophotometrically at 540 nm. For reference (100% haemolysis), red blood cells were treated with distilled water.

3.14. In vivo studies

Female BALB-c mice (8 weeks of age), were purchased from the center of experimental animal (Tsinghua University, Beijing). All animals were approval by the Committee on Animal Research at the Tsinghua University, Beijing. Gene expression *in vivo* was assessed by injecting HSA-dendriplexes at a HSA/DNA weight ratio of 20, dendriplexes (both at a N/P ratio of 10), naked DNA or saline into 8-week-old individual mice in groups of six via the tail vein in a total volume of 200 μL. Twenty-four hours following intravenous injection, the mice were killed by breaking the neck. The heart, liver, spleen, lungs and kidneys were collected and washed twice with cold saline. The organs were homogenized with lysis buffer (Promega) using the homogenizer (DY89-II, Huadr, Beijing) and centrifuged at 12,000 g for 5 min at 4 °C. Levels of renilla luciferase activity in the transfected cells were assayed by renilla luciferase assay kit (promega), according to the manufacturer's introduction.

3.15. Statistical analysis

Statistical analysis was performed using two-way ANOVA (Origin software, 7.5 version, Origin Lab, USA). $p < 0.05$ was considered statistically significant.

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