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Arginine-chitosan/DNA self-assemble nanoparticles for gene delivery: *In vitro* characteristics and transfection efficiency

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ABSTRACT

Chitosan (Cs) is a natural cationic polysaccharide that has shown potential as non-viral vector for gene delivery because of its biocompatibility and low toxicity. However, chitosan used for gene delivery is limited due to its poor water solubility and low transfection efficiency. The purpose of this work was to prepare Arginine-chitosan (Arg-Cs)/DNA self-assemble nanoparticles (ACSNs), and determine their *in vitro* characteristics and transfection efficiency against HEK 293 and COS-7 cells. Our experimental results showed that the particle size and zeta potential of ACSNs prepared with different N/P ratios were 200–400 nm and 0.23–12.25 mV, respectively. The *in vitro* transfection efficiency of ACSNs showed dependence on pH of transfection medium, and the highest expression efficiency was obtained at pH 7.2. The transfection efficiency increased with the ratio of chitosan-amine/DNA phosphate (N/P ratio) from 1 to 5, and reached the highest level with the N/P ratio 5. Effect of plasmid dosage on the transfection efficiency showed the highest transfection efficiency was obtained at 4 μg/well for HEK 293 cells and 6 μg/well for COS-7 cells. The transfection efficiency of ACSNs was much higher than that of Cs/DNA self-assemble nanoparticles (CSNs). The average cell viability of ACSNs was over 90%. These results suggested that ACSNs could be a safe and effective non-viral vector for gene delivery.

1. Introduction

Gene therapy is considered as a promising approach for the treatment of congenital and acquired diseases by producing bioactive agents or preventing abnormal functions of the cells such as genetic disorder or uncontrollable proliferation of cells (O'Connor and Crystal, 2006; Kim and Rossi, 2007). However, the lack of effective vectors is a major barrier for progress of human gene therapy. The viral vectors are the most common transfection agents in current clinical trails, but safety risk is a major disadvantage (El-Aneed, 2004). Non-viral gene delivery systems have been proposed as safer alternatives to viral vectors because they have the potential to be administered repeatedly with minimal host immune response, are stable in storage, and can be produced easily in large quantities. These advantages would give them impetuses to develop further. Among non-viral systems, cationic polymers have gained increasing attention because they mediate transfection via condensing DNA into particles, which protects DNA from enzymatic degradation and facilitate the cell uptake and endolysosomal escape (Dang and Leong, 2006).

As a non-viral vector for gene delivery, chitosan offers certain advantages such as non-toxic in a range of toxicity tests (Hejazi and Amiji, 2003), soft tissue compatibility (Li and Zhang, 2005), high stability and reasonable cost. However, chitosan shows two major disadvantages: one is poor solubility because the amino groups on chitosan are only partially protonized at physiological pH 7.4. The other is low transfection efficiency. So far, many techniques have been tried to overcome these disadvantages including quaternization of NH_2 groups (Thanou et al., 2002), linking or conjugating cell-specific ligands (Kim et al., 2004), PEG (Park et al., 2001), deoxycholic acid (Kim et al., 2001) or urocanic acid (Kim et al., 2003) to the polysaccharide backbone through NH_2 groups or hydroxyl groups.

Recently, some peptide sequences known as protein transduction domains (PTD) or membrane translocalization signals (MTS) were identified and introduced for the delivery of plasmid DNA (Tung and Weissleder, 2003; Futaki, 2005). Interestingly, it is known that these sequences usually contain positively charged amino acid residues such as arginine and lysine, which have been reported to be able to enhance transportation into cells by several groups (Eguchi et al., 2001; Nakanishi et al., 2003). Oligo-arginine conjugates demonstrated the char-

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acteristics similar to cell penetrating peptides (CPPs) in cell translocation, and the transfection efficiency in Hela cells could be highly improved by conjugating oligo-arginine to PEGlyted lipids (Furuhata et al., 2006). The polyamidoamine dendrimer conjugated with L-arginine also showed to enhance gene delivery potency compared with native dendrimer (Choi et al., 2004).

We are interested in designing a more efficient non-viral gene vector. In the present work, we investigated the characterization of Arginine-chitosan (Arg-Cs)/DNA self-assemble nanoparticles (ACSNs) and transfection efficiency in HEK 293 and COS-7 cells. The results showed that the chitosan linking arginine through NH_2 group could improve its water solubility and enhance its gene transfection efficiency.

2. Materials and methods

2.1. Materials

Chitosan (Cs, MW = 60K) was supplied by Boao Biotechnologies Co., Ltd. (Shanghai, China). The degree of deacetylation (90%) was confirmed by ^{1}H NMR spectroscopy (AM-400 Bruker-Spectrospin, USA). Arginine was purchased from Sinopharm Group Chemical Reagent Co., Ltd. (China). 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from GL Biochem Ltd. (Shanghai, China). Trypsin-EDTA, phosphate buffered saline (PBS), agarose and Lipofectamine^{TM} 2000 were obtained from Gibco-BRL (Burlington, ON, Canada). The Dulbecco's modified Eagle medium (DMEM), antibiotics and fetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Ethidium bromide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Other chemicals if not mentioned were of analytical grade.

Chitosan was modified with arginine according to Liu' method (Liu et al., 2004). The substitution degree of arginine in Cs was 18.3% according to the C/N ratio of the elemental analysis (Elementar Vario EL, Germany). Plasmid EGFP-N1 (4.7 kb) encoding enhanced green fluorescent protein driven by immediate early promoter of CMV was purchased from Clontech (Palo Alto, CA, USA). The plasmid DNA (pDNA) grown in DH5α strain of *E. coli* was isolated with the Qiagen EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). The purity was confirmed by spectrophotometry (A260/A280), and DNA concentration was measured by UV absorption at 260 nm.

The epithelial cell line HEK 293 (Human embryonic kidney) and COS-7 (African green monkey kidney) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.2. Preparation and characteristics of ACSNs

Arg-Cs was dissolved in 20 mM sodium acetate/acetic acid buffer (pH 5.5) and diluted to an appropriate concentration. The concentration of pDNA was 100 μg/ml in 5 mM sodium sulfate. ACSNs were prepared by a complex coacervation technique (Mao et al., 2001), briefly, after heating to 50 °C, equal volume (0.5 ml) of Arg-Cs and pDNA solutions were mixed and immediately vortexed at maximum speed for 30 s. The resulting complexes were allowed to sit at room temperature for 30 min. ACSNs were prepared immediately prior to the experiments. The solution of ACSNs was used for the transfection experiment directly. As control, Cs/DNA nanoparticles (CSNs) were also prepared by the same process as described for ACSNs above.

The DNA binding ability of Arg-Cs was evaluated by agarose gel electrophoresis. The complex containing 0.5 μg of DNA at various N/P ratios were loaded into individual wells with 1.0% agarose gel in Tris-Acetate-EDTA buffer, electrophoresed at 80 V for 45 min, and stained with 0.5 mg/ml ethidium bromide. The resulting DNA migration pattern was revealed under UV irradiation. As control, the DNA binding ability of Cs was also evaluated by the same process as described for Arg-Cs.

The particle size and zeta potential of ACSNs containing 50 μg DNA were determined by the laser light scattering measurement using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA).

2.3. In vitro transfection experiment

Cells (HEK 293 and COS-7) were seeded in 24-well plates at a density of 1×10^5 cells per well in 500 μl of complete medium (DMEM containing 10% FBS, supplemented with 100 U/ml penicillin G and 100 μg/ml streptomysin) and incubated for 24 h prior to transfection. Then, the media was replaced with fresh growth medium with special pH containing ACSNs at various N/P ratios. After 48 h incubation, cells were collected and resuspended in PBS (pH 7.4). The transfection results were measured using a FACSCalibur (Becton Dickinson, USA). An excitation wavelength of 480 nm was used with fluorescence emission measured at 530 nm through fluorescence channel 1 (FL1). As positive control, Lipofectamine^{TM} 2000 was performed according to the manufacture's protocol. CSNs and naked DNA were used on cell cultures and examined as described above. All transfection experiments were performed in triplicate.

2.4. Cytotoxicity assays

HEK 293 and COS-7 cells were seeded at a density of 1×10^4 cells per well in 150 μl growth medium in 96-well plates and incubated for 24 h. Growth media was replaced by fresh serum-free media containing free polymer (Cs or Arg-Cs, 25 μg polymer/well), polyplex (CSNs or ACSNs (N/P ratio 5), 8 μg DNA/well) or Lipofectamine^{TM} 2000. Cells without treatment were used as control. After 12 h, 20 μl MTT solution (5 mg/mL in PBS) was added, and incubated for additional 4 h at 37 °C for MTT formazan formation. Then, the medium was removed and DMSO was added to dissolve the MTT formazan. The plates were mildly shaken for 10 min to ensure the dissolution of formazan. The absorbency values were measured by using microplate reader (Bio-RAD, model 550) at wavelength 490 nm, blanked with DMSO solution. Three replicates were counted for each sample. The mean value of the three times was used as the final result.

2.5. Statistical analysis

Statistical analyses were performed using a Student's *t*-test. The differences were considered significant for p value < 0.05.

3. Results and discussion

3.1. Preparation and physicochemical characteristics of ACSNs

The N/P ratio of ACSN was expressed as the molar ratio of amine group of Arg-Cs to phosphate group of DNA. It should be noted that the amide nitrogens were not counted in calculating the N/P ratio. The mass per charge for Arg-Cs was calculated as an

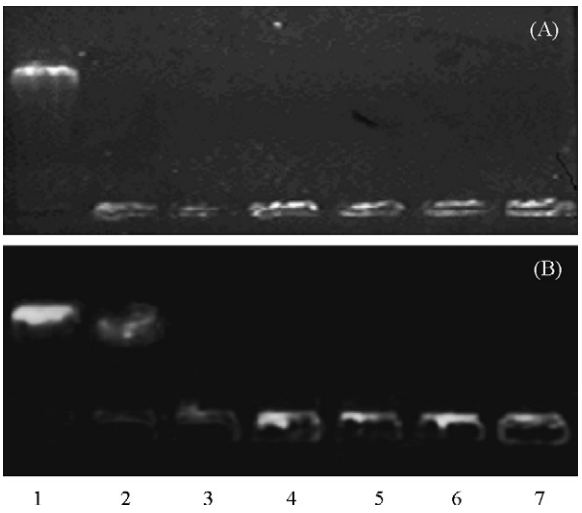

Fig. 1. Gel retarding analysis of ACSNs (A) and CSNs (B). Lane 1, naked DNA control. Lane 2–7, ACSNs or CSNs prepared at N/P ratios of 1, 2, 3, 5, 7 and 10, respectively.

average for whole polymer molecules including arginine. The average mass 330 Da per charge pDNA was used for the calculation of charge ratios (Chan et al., 2007). In order to know the ability of Arg-Cs to condense DNA, the solutions of ACSNs with different N/P ratios were electrophoresed on an agarose gel containing ethidium bromide. At different N/P ratios (N/P ≥ 1), all ACSNs were able to prevent DNA migration (Fig. 1A). As control, the migration of CSNs with different N/P ratios (N/P ≥ 1) was also performed, however, at N/P ratio 1, an dissociative DNA band occurred obviously, which meant some of DNA migrated in the cavity and Cs had not enough ability to condense DNA at this N/P ratio (Fig. 1B, lane 2). These results indicated that Arg-Cs had stronger ability to condense DNA into nanoparticles than Cs (Fig. 1).

Fig. 2A showed that the size of ACSNs was in the range of 200–400 nm with the smallest size at N/P ratio 5. The slight increase of particle size with N/P ratio over 5 could be result from the exclusion of redundant positive charge provided by arginine inside nanoparticles. In this experiment, we also found that ACSNs showed smaller size than that of CSNs when diluted in pure water (data no shown), which suggested that Arg-Cs could induce stronger compaction of DNA due to higher pK_a value (pK_a = 9.04 and 12.48) of arginine compared with that of chitosan (pK_a = 6.5). However, when ACSNs were prepared in acetic acid solution (pH 5.5), the sizes of ACSNs were slightly larger than that of CSNs due to more intra Arg-Cs molecular charge exclusion (data no shown). In addition, we also performed the experiment at N/P ratio < 1, unfortunately, all the complexes flocculated and resulted in that the sizes could not be detected.

The surface charge of gene delivery systems is known to be one of the major factors influencing their transfection efficiency (Romoren et al., 2003). In our experiment, the zeta potential of ACSNs appeared to be a little positive (0.23 mV) at the N/P ratio 1 (Fig. 2A), which was accordant with other reports that the Cs/DNA nanoparticles were electrostatically neutral at the N/P ratio 1 (Mao et al., 2001). It could be because the pH of the measurement solution and the concentration of sodium sulfate produced effects on the zeta potential. Although we performed the complexes in solution containing a little sodium sulfate, the slightly acid pH of measurement solution and the fully protonization of arginine in this pH could make the zeta potential a little positive at the N/P ratio 1. The zeta potential of ACSNs increased with the N/P ratio, and reached the highest value (+12.25 mV) at the N/P ratio 10 (Fig. 2A).

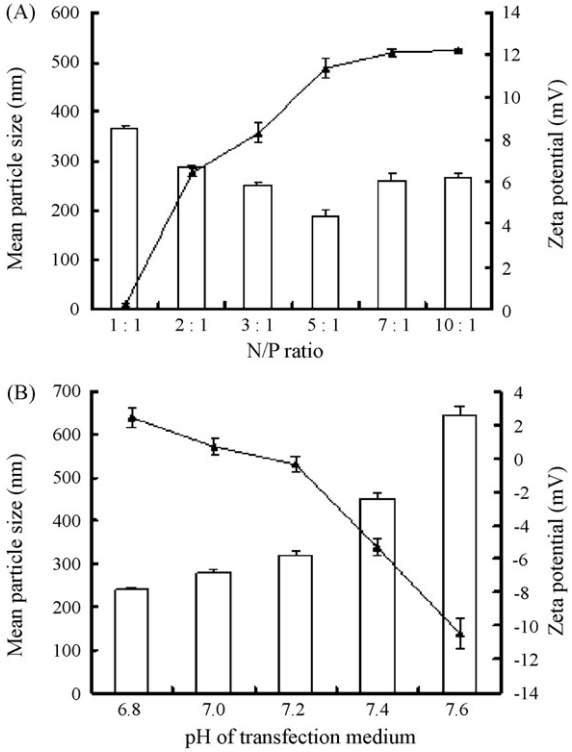

Fig. 2. Effect of N/P ratio (A) and pH of transfection medium (B) on particle size (□) and zeta potential (—■—) of ACSNs.

In order to know the stability of ACSNs in solution, the particle size and the polydispersity indexe of ACSNs (N/P ratio 5) in transfection medium were measured at predetermined time point. The particle size of ACSNs was 188.57 nm with the polydispersity indexe 0.134 at 0.5 h after preparation. At 24 h after preparation, ACSNs showed neglectable change with the particle size 197.46 nm and the polydispersity indexe 0.136, which demonstrated that the ACSNs could be stable in aqueous solution during experiment.

3.2. *In vitro transfection efficiency*

It has been reported that the gene delivery potential of chitosan in human carcinoma cells depends on several factors such as cell type, serum, N/P ratio, pH of transfection medium, and plasmid dosage. In order to know *in vitro* gene transfection efficiency of ACSNs, transfection was carried out against HEK 293 and COS-7 cell lines with different N/P ratio, pH and dosage. It has been known that the physical stability of gene delivery systems in serum is a very important factor for in vivo use (Yi et al., 2000). So far, the effect of serum on transfection efficiency in primary chondrocytes, human-lung carcinoma A549 cells, Hela cells, B16 melanoma cells, and SOJ cells mediated by chitosan has been reported (Zhao et al., 2006; Sato et al., 2001; Ishii et al., 2001). In this work, the effect of serum on the transfection efficiency of ACSNs against HEK 293 and COS-7 cells showed that the presence of 10% FBS resulted in enhancement of the gene transfer efficiency observed through fluorescence microscopy. As a result, our following experiments were carried out in medium with 10% FBS.

It has been reported that transfection efficiency of chitosan/DNA complexes was dependent on pH of transfection medium (Sato et al., 2001; Ishii et al., 2001). This property was considered to be due to the protonation of free amino groups in chitosan because the pK_a of primary amines in chitosan was around 6.5, which sug-

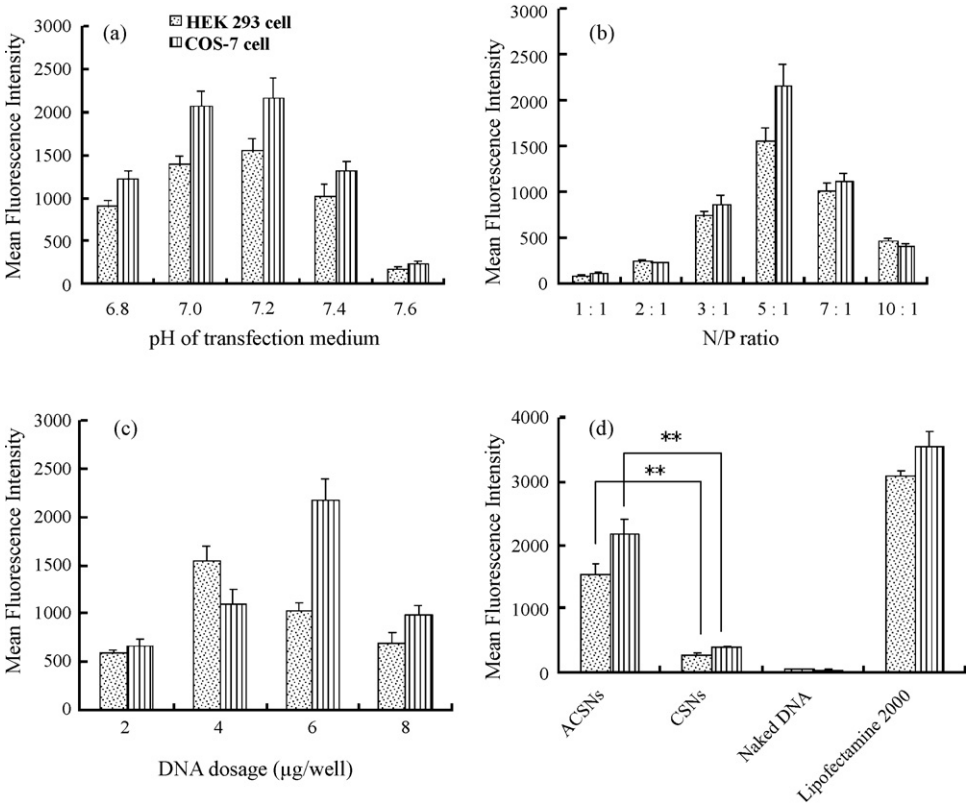

Fig. 3. *In vitro* transfection efficiency of ACSNs against HEK 293 and COS-7 cells in DMEM containing 10% FBS as assessed by mean fluorescence intensity. (a) Effect of pH of transfection medium on the transfection efficiency. ACSNs were prepared at N/P ratio 5, plasmid dosage was 4 μg/well for HEK 293 cells and 6 μg/well for COS-7 cells; (b) effect of N/P ratio on the transfection efficiency of the ACSNs in DMEM (pH 7.2), plasmid dosage was 4 μg/well for HEK 293 cells and 6 μg/well for COS-7 cells; (c) effect of plasmid dose on the transfection efficiency, transfection was performed with ACSNs (N/P ratio 5) in DMEM (pH 7.2); (d) transfection efficiency of ACSNs compared with that of CSNs and naked DNA (pH 7.2). $^{**}p < 0.01$ compared with CSNs. Lipofectamine™ 2000 as positive control.

gested that the free amino groups in chitosan backbone could not be fully protonized and the chitosan/DNA complexes could not be positive enough to bind with the negatively charged cells through electrostatic interaction at pH over 7. To Arg-Cs, the higher pK_a value of arginine could increase the pK_a value of Arg-Cs, and the free amino groups in chitosan backbone still played an important role in condensing DNA into nanoparticles and interacting with cells. As a result, we determined the effect of pH of transfection medium on the particle size and zeta potential of ACSNs (N/P ratio 5). The results showed that the zeta potential decreased with pH increasing (Fig. 2B), which suggested that the protonation of the free amino groups in chitosan backbone could reduce with pH increasing. The zeta potential decreased to near neutral when the pH of the medium was 7.2. The particle size showed slightly decrease when pH altered from 6.8 to 7.0, however, when pH got up over 7.2, the particle size increased sharply, which meant that the pH of transfection medium could affect the transfection efficiency of ACSNs.

Fig. 3a showed that the highest expression efficiency was obtained at pH 7.2, which showed a similar result with CSNs that the highest expression efficiency occurred when the surface charge of CSNs was neutral or slight negative (Zhao et al., 2006). When pH altered from 6.8 to 7.2, the protonization of free amino groups in chitosan backbone reduced and the positive surface charge of ACSNs decreased accordingly. Then, the non-ionic interactions between carbohydrate backbone of chitosan and cell membrane could play a more important role than the ionic interactions during the process of cell uptake (Venkatesh and Smith, 1998). Although, the ACSNs in higher pH were apt to cell uptake, the greatly reducing in expression efficiency when pH increased from 7.2 to 7.6 suggested that exorbitant pH, on the contrary, led to the free plasmid dissociated from the complexes, which could be because the free amino groups in the chitosan backbone could not be protonized, and the positive charge of arginine groups could not be enough for condensing DNA. In addition, the release of plasmid from complexes could be attributed to the low amount of cell uptake (Ishii et al., 2001).

In order to know the influence of N/P ratios, transfection was carried out with ACSNs prepared at different N/P ratios against HEK 293 and COS-7 cells (Fig. 3b). The results showed that transfection efficiency increased with the N/P ratios from 1 to 5. The transfection efficiency reached the highest level with the N/P ratio 5. It has been reported that CSNs with the N/P ratio 3 showed the highest transfection activity in 10% serum and the positive correlation between transfection activity and cell uptake (Ishii et al., 2001). So, the result could be attributed to the cell uptake saturation at N/P ratio 5.

Effect of plasmid dosage on the transfection efficiency was shown in Fig. 3c. The highest transfection efficiency was obtained at 4 μg/well for HEK 293 cells and 6 μg/well for COS-7 cells. When the dosage increased to 8 μg/well, the transfection efficiency decreased, which could be due to the aggregation of nanoparticles absorbed in cell membrane with amount of plasmid increasing, which led to the difficulty of cell uptake.

To determine whether arginine conjugated to the chitosan backbone could improve its transfection efficiency, we compared the transfection efficiency of ACSNs with that of CSNs and naked DNA. Transfection experiment was performed against HEK 293 cells and COS-7 cells with N/P ratio 5 and dosage 4 μg/well for HEK 293 cells and 6 μg/well for COS-7 cells in DMEM (pH 7.2) containing 10% FBS. As positive control, transfection of Lipofectamine™ 2000 with plasmid dosage 0.8 μg/well was performed in DMEM (pH 7.4) without serum according to the manufacturer's procedures.

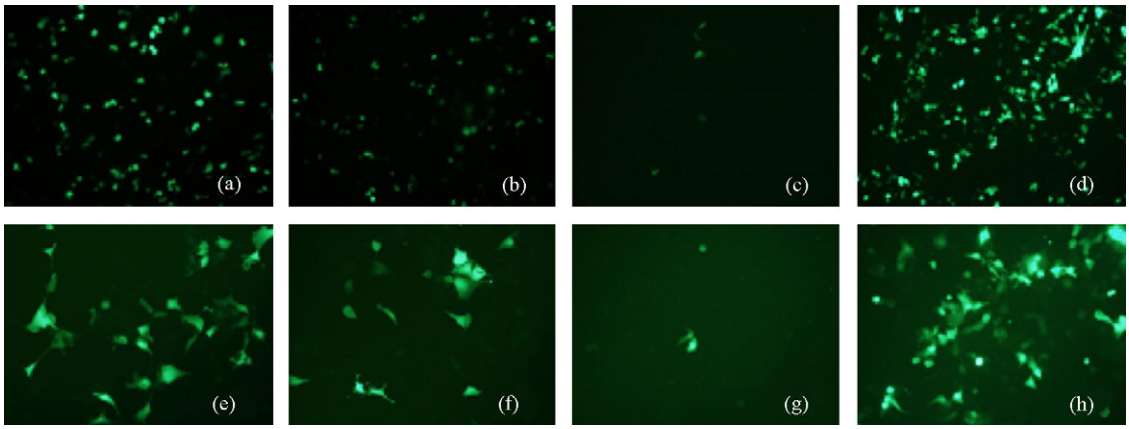

Fig. 4. Images of HEK 293 (Line 1, 4 μg/well) cells and COS-7 (Line 2, 6 μg/well) transfected with ACSNs, CSNs, naked DNA and Lipofectamine™ 2000 as observed under fluorescent microscope (20× magnification). ACSNs (a and e); CSNs (b and f); naked DNA (c and g) and Lipofectamine™ 2000 (d and h).

Fig. 3d and Fig. 4 showed that the transfection efficiency of ACSNs (Fig. 4a,e) could not reach the same high level as Lipofectamine™ 2000 (Fig. 4d,h), but it was much higher than that of CSNs (Fig. 4b,f). Under the optimal condition, the mean fluorescence intensity from ACSNs could reach to 2168.37 in COS-7 cells and over 1550.74 in HEK 293 cells. To CSNs group, the mean fluorescence intensity was only 387.68 for COS-7 cells and 271.05 for HEK 293 cells. In addition, the transfection efficiency of CSNs under its optimal transfection condition with N/P ratio 3, pH 7.0, plasmid dosage 6 μg/well and 10% FBS (Ishii et al., 2001), the mean fluorescence intensity was also lower than that of ACSNs (less than 1500 in COS-7 cells and less than 900 in HEK 293 cells). These results showed that the pH of the transfection medium and zeta potential indeed produced significant influence on cell uptake, but they could not explain the increased gene expression level of ACSNs compared with CSNs. Therefore, the increase of gene expression could be attributed to the nuclear localizing efficiency after entry into the cytosol of the arginine residues. As a control, naked (non-condensed) plasmid DNA showed only neglectable fluorescence intensity (relative to background level) in the cytoplasm of HEK 293 and COS-7 cells (Fig. 3d and Fig. 4c,g), which demonstrated that plasmid DNA without any carrier had very low transfection efficiency as reported in our previous work (Gao et al., 2007).

3.3. Cell viability

The cytotoxicity of free polymer (Cs and Arg-Cs) and polyplex (CSNs or ACSNs) were estimated by MTT assay. Arg-Cs showed slightly increased toxicity compared with native chitosan in our preliminary experiment (data not shown), which could be the result from the increase of charge density and molecular weight because the toxicity of cationic polymers could be a function of the interaction of the polymers to cell membranes (Fischer et al., 2003). When complexed with DNA, the polyplexes showed slight reduction of cytotoxicity (Fig. 5), which indicated that (+) charge of Arg-Cs counterbalanced by (−) charge of DNA minimized the direct contact of polycation with cell membrane. The average cell viability of ACSNs was around 90%, which is much higher than that of Lipofectamine™ 2000 (Fig. 5). The results meant that ACSNs should be a safer carrier.

4. Conclusion

Arg-Cs/DNA self-assemble nanoparticles (ACSNs) showed that the particle size and zeta potential were 200–400 nm and 0.23–12.25 mV, respectively. The *in vitro* transfection efficiency of ACSNs showed dependence on pH of transfection medium, and the highest expression efficiency was obtained at pH 7.2. The transfection efficiency increased with the N/P ratios from 1 to 5, and reached the highest level with the N/P ratio 5. Effect of plasmid dosage on the transfection efficiency showed the highest transfection efficiency was obtained at 4 μg/well for HEK 293 cells and 6 μg/well for COS-7 cells. The transfection efficiency of ACSNs was much higher than that of CSNs. The average cell viability of ACSNs was over 90%. These results suggested that ACSNs could be a safe and effective non-viral vector for gene delivery. Further studies would focus on evaluating in vivo transfection efficiency of ACSNs.

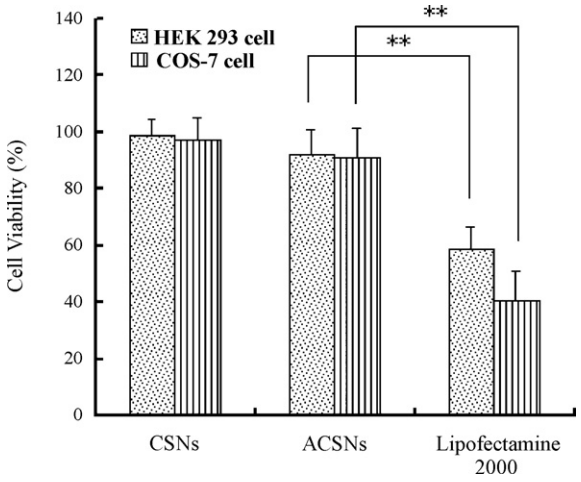

Fig. 5. Cell viability of ACSNs, CSNs and Lipofectamine™ 2000 in HEK 293 and COS-7 cell lines. $^{**}p < 0.01$ compared with Lipofectamine™ 2000.

Acknowledgements

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