Components Simulation of Viral Envelope via Amino Acid Modified Chitosans for Efficient Nucleic Acid Delivery: In Vitro and In Vivo Study

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Novel nonviral gene vectors of alkaline amino acids such as arginine- (Arg), histidine- (His), and lysine- (Lys) modified chitosans (AAA-CSs) are developed to simulate the components of viral envelopes to enhance transfection efficiency. The structures of the modified chitosans are characterized using ^{1}H NMR spectroscopy. Acid-base titration results indicate that the modified chitosans exhibit strong buffering capacity. The morphology of the AAA-CSs/pDNA complexes is observed by use of transmission electron microscopy and atomic force microscopy. The complexes are spherical nanoparticles with a mean size around 100 nm. Zeta potential tests reveal that the complexes are positively charged and their zeta potentials vary from +0.1 to +19.5 mV. The MTT assay and agarose gel electrophoresis demonstrate that the AAA-CSs are non-cytotoxic and have excellent DNA condensation and protection abilities. Cellular uptake investigation of the AAA-CSs/pDNA complexes demonstrates that Arg-CS and His-CS have better cellular internalization property than the unmodified chitosan. The in vitro gene transfection is evaluated in HEK293 and NIH3T3 cell lines and in vivo transfection is carried out in tibialis anterior muscles. The results reveal that the arginine-modified chitosan could significantly enhance gene-transfection efficiency both in vitro and in vivo.

1. Introduction

Gene therapy is considered as a promising treatment for formidable diseases. It works by introducing genetic materials into cells via viral or nonviral vectors to activate normal function or correct abnormal function of the cells.[1–4] Although viral vectors have exhibited high transfection efficiencies, safety issues of insertional mutagenesis, and immunogenic and inflammatory responses are concerning and can result in clinical death.[5,6] Simultaneously, manipulable capacities such as the targeting ability and controllability of size and morphology have also limited the applications of viral gene vectors.[7] Recently, nonviral gene vectors such as cationic liposomes and polymers[8,9] have attracted much interest for their advantages in safety,[10] ability to deliver large genetic materials,[11] potential cell-targeting properties,[12] large-scale production, and stability in storage.[13] However, low transfection efficiency is a challenge for nonviral gene vectors.[14,15]

As the major components of living organisms such as bacteria, viruses, and cells are produced by intermolecular interactions directed by the self-assembly of peptides, carbohydrates, and nucleic acids,[16,17] various strategies have been developed to design nonviral gene vectors via the self-assembly of natural molecules into nanostructured materials with the essential features of natural materials.[18–20] To mimic the components of viral envelopes is thought to be a good choice to construct nonviral gene vectors as the main components of viral envelopes, which are composed of peptides and polysaccharides, are favorable for gene transfection in viral vectors.

As a natural polysaccharide, chitosan (CS) has been widely investigated as a nonviral gene vector due to its cationic nature, excellent biocompatibility, and biodegradability.[21,22] Chitosan is a linear copolymer of β-1,4 linked *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN). At the physiological pH value of 7.4, chitosan is positively charged because the pKa value of the GlcN residue in chitosan is 6.2 to 6.6. This charge makes chitosan condense with negatively charged DNA to form nanoparticles which protect DNA from DNase degradation.[23,24] However, its poor water solubility impedes the application of chitosan as a promising nonviral gene vector. Modifications of chitosan aimed at increasing the water solubility have been reported, such as graft water-soluble poly(ethylene glycol) (PEG)[25,26] and positively charged poly(ethylene imine) (PEI).[27,28] Though the water solubility of PEG- or PEI-modified chitosans was significantly improved, other disadvantages were found, for example, the cellular internalization of PEG-modified chitosan was retarded due to the intrinsic cell-membrane resistance of PEG, and the PEI modified chitosan was somewhat cytotoxic due to the high cytotoxicity of PEI.

Peptides, which are the main components of viral envelopes, have excellent gene-transfection properties[29,30] due to their specific interactions with cell and organelle membranes.

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Alkaline amino acids such as arginine, histidine, and lysine in the peptides could not only condense DNA effectively[31,32] but also interact with the membranes of cells to promote transfection efficiency. Arginine-enriched peptides could interact simultaneously with the phosphate moieties of the multiple lipid headgroups in cell membranes to form bidentate hydrogen bonds, which thus enhance their cell-penetrating ability for internalization.[33] Several in vitro studies on an arginine-modified polysaccharide also demonstrated its good cytocompatibility and gene-transfection ability.[34–37] The pKa value of the imidazole group in histidine is 6.0, so the imidazole groups are protonated in the acidic environment of the endosome or lysosome and can disrupt the membrane via osmotic swelling to cause DNA escape; thus the histidine residue in peptides has been suggested to have a "proton-sponge" effect.[38] Lysine residue in peptides has also been reported to be able to enhance osmolarity to let DNA escape by endosomal rupture.[39] However, the cellular uptake tracking and the in vivo gene-transfection efficiency of the alkaline amino acid modified vectors are unclear and rarely reported.

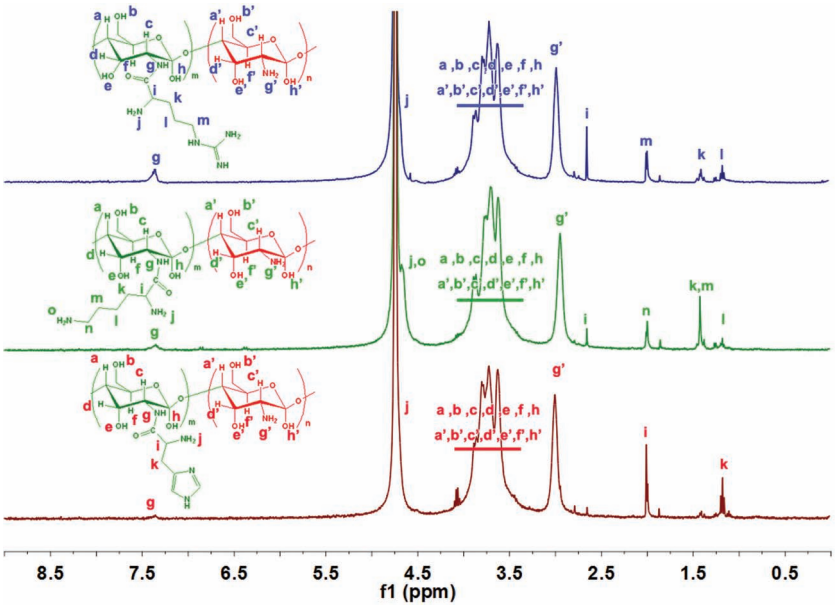

Figure 1. ^{1}H NMR spectra of arginine-, lysine-, and histidine-modified chitosans in DMSO-d_6 as solvent.

In our previous work, cationic lipids with arginine, histidine, and lysine as hydrophilic heads,[40] poly(L-lysine) dendrimers,[41] arginine-modified peptide dendrimers,[42] and viral envelopes mimicking nanoarchitectures via the self-assembly of peptides[18] were studied as nonviral gene vectors. The vectors displayed high transfection efficiencies in vitro in diverse cell lines, however, like most cationic polymers, they were somewhat cytotoxic and showed poor transfection activity in vivo. Herein, we design new alkaline amino acid modified chitosans (AAA-CSs) as nonviral gene vectors to simulate the components of viral envelopes. The AAA-CSs are expected to combine the advantages of both alkaline amino acids and chitosan in gene transfection to overcome the problems faced by nonviral gene vectors. Arginine-, histidine-, and lysine-modified chitosans are synthesized and characterized by ^{1}H NMR spectroscopy. The morphologies and properties of the AAA-CSs/pDNA complexes are studied in detail. The cellular internalization and intracellular disassociation of the complexes are investigated. The in vitro transfection of the complexes are carried out in HEK293 and NIH3T3 cell lines and the in vivo transfection efficiency is tested via intramuscular injection.

2. Results and Discussion

2.1. Synthesis and Characterization of AAA-CSs

Three alkaline amino acids of L-arginine, L-histidine, and L-lysine were immobilized on the pendant amino groups of chitosan. Unmodified chitosan did not dissolve in water and dimethylsulfoxide (DMSO), but after modification, the AAA-CSs dissolved in both water and DMSO. The water solubility of chitosan was improved greatly by modification.

^{1}H NMR spectra of the three modified chitosans are presented in **Figure 1**. As chitosan did not dissolve in DMSO-d_6, the spectrum of chitosan is not presented for comparison. The chemical environment of the protons in the backbones is similar, thus most of the chemical shifts of signals arising from the protons in backbones are aggregated together. The signals appearing in high field were attributed to the immobilized amino acids. As shown in Figure 1, the peaks from 4.5 to 5.5 ppm were from the protons (α-H) on the amide link, and the peaks from 3.0 to 4.0 ppm were attributed to the characteristic signals from the chitosan skeleton. The graft percentages of the amino acids on chitosans were approximately calculated as 32.08% in Arg-CS, 33.17% in His-CS, and 31.04% in Lys-CS from the ^{1}H NMR spectra. According to the graft rates and the molecular weight of unmodified chitosan, the estimated molecular weights of Arg-CS, His-CS, and Lys-CS are 26.9, 26.3, and 25.6 kDa, respectively.

2.2. Buffering Capacity

The buffering capacities of the CS and the AAA-CSs were tested by acid-base titration. As shown in Figure S1 (see Supporting Information), both CS and the AAA-CSs showed optimal buffering capacity in the pH range of 5.0–7.0. The highest buffering capacity of Arg-CS appeared at pH = 6.4 and the value was about 0.032, while those of His-CS and Lys-CS were at pH = 6.4 and 6.6, and the values were both around 0.02. The buffering capacity value of chitosan was about 0.008. By comparing the highest buffering capabilities of the AAA-CSs and CS, it could be concluded that the buffering capacity of CS was enhanced greatly after the modification. Among the three AAA-CSs,

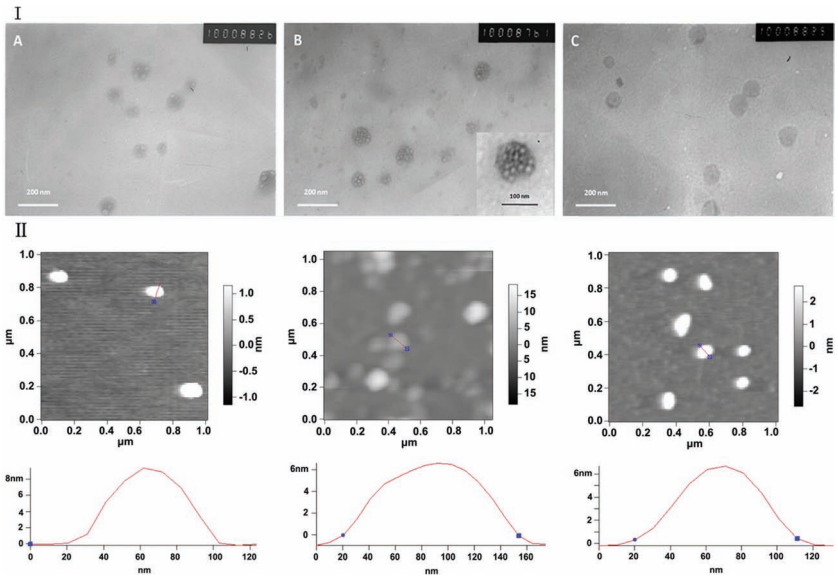

Figure 2. Morphologies of AAA-CSs/pDNA complexes. I: TEM images of AAA-CSs/pDNA complexes, A) Arg-CS; B) His-CS; C) Lys-CS; II: AFM photographs of AAA-CSs/pDNA complexes, A) Arg-CS; B) His-CS; C) Lys-CS.

Arg-CS exhibited the best buffering capacity, which was about fourfold higher than that of CS. It was reported that the buffering capacity of the polymers would increase by the modification of amino acids.[43–45] As the buffering capacity of amino acid modified chitosans is ascribed to the combination of NH_2 groups in the chitosan chains and α-amine groups as well as in the side groups of amino acids, the best buffering capacity of the arginine-modified chitosan was due to the highest pK value of the side guanidine group. Moreover, the high buffering capacity of gene vectors could cause the swelling of endocytic vesicles to enhance endosomal escape ability,[46] which is important for the release of condensed pDNA to result in high transfection efficiency.

2.3. The Morphology of AAA-CSs/pDNA Complexes

The morphologies of AAA-CSs/pDNA complexes were observed by transmission electron microscopy (TEM) and atomic force microscopy (AFM; **Figure 2**). The AAA-CSs were dissolved in phosphate buffer solution (PBS) to condense pDNA. All the three complexes were nanoparticles sized around 100 nm diamter. Interestingly, it was clear in TEM photographs that the complex nanoparticles were composed of smaller nanoparticles of around 10 nm diameter (insert photograph in Figure 2 IB), which are nearly the same size as a randomly coiled pEGFP-C1 polymer chain. As the pEGFP-C1 is a plasmid of 5.3 kb, it is a polymer with high molecular weight. The molecular weight of chitosan is only 20 kDa, which is much smaller than that of pEGFP-C1. After the condensation, a pEGFP-C1 chain could condense more than one chitosan chain, which thus formed a small complex nanoparticle with pDNA as core and chitosan chains as shell. The small nanoparticles aggregated into bigger nanoparticles sized around 100 nm. All the three pDNA condensed nanoparticles were characterized using AFM (Figure 2II). The AFM photographs reveal that all the nanoparticles were well dispersed. The heights of the nanoparticles ranged from 6 to 8 nm, and the sizes of most nanoparticles were around 100 nm, in accordance with the TEM results.

2.4. Zeta Potentials of AAA-CSs/pDNA Complexes

Zeta potential is an important parameter to characterize the surface charge of nanoparticles. In gene-delivery systems, the surface charge of gene vectors and complexes is known to be a major factor influencing gene-transfection efficiency.[47] As the modified chitosans were soluble in water, they could form nanoscale random coils for zeta potential tests. The zeta potentials of AAA-CSs and AAA-CSs/pDNA nanoparticles are shown in Supporting Information Table S1. All AAA-CSs were positively charged, and their zeta potentials ranged from +16 to +22 mV. After the AAA-CSs were condensed with plasmids (N/P ratio = 45), all the zeta potentials of the three complexes were reduced. Among all the complexes, the zeta potential of Arg-CS/pDNA nanoparticles was changed most significantly, varying from +21.7 mV (without plasmid) to +0.1 mV (with plasmid).

2.5. Agarose Gel Electrophoresis of AAA-CSs/pDNA Complexes

The condensation ability of AAA-CSs was further assessed by agarose gel-retardation assays. All samples showed good DNA binding ability (**Figure 3**I); the DNA mobility in AAA-CSs/pDNA complexes was completely retarded at the N/P ratio of 8 (Figure 3Ib–d), whereas that of CS was found at the N/P ratio of 2 (Figure 3Ia). Some papers reported that the high DNA binding ability would decrease the gene transfection because of the poor release efficacy of DNA;[48] this implies that the AAA-CSs should have better DNA release efficacy, and might show higher transfection efficiency than CS.

The DNA condensed complexes were exposed to nucleases to assess their ability to protect the AAA-CSs. Two concentrations of 1U and 2U DNase I were used. The plasmids were condensed with CS and AAA-CSs at the N/P ratio of 25. In the 1U DNase I series (Figure 3IIa), both CS and AAA-CSs could protect DNA effectively, as observed by the presence of DNA bands in the lanes with nuclease. In the 2U DNase I series (Figure 3IIb), the DNA bands of all the three AAA-CSs appeared in lane A(+), B(+) and C(+), while that of chitosan in lane D(+) disappeared. This observation demonstrates that the plasmids in lane D(+) were digested by 2U DNase I, which means that the plasmid protection ability of CS was weaker than that of AAA-CSs. The results in Figure 3II indicate that the CS could only protect plasmids from enzymatic digestion in 1U DNase I, while the AAA-CSs could protect plasmids from enzymatic digestion not only in 1U DNase I but also in 2U DNase I. The AAA-CSs

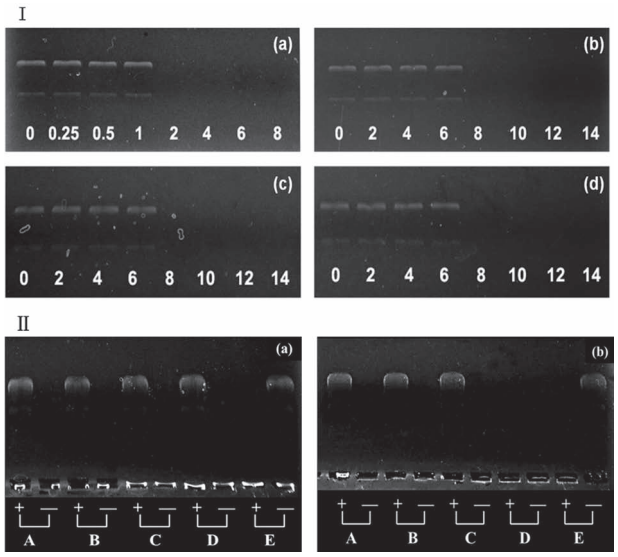

Figure 3. Agarose gel electrophoresis of AAA-CSs/pDNA complexes. I: Retardation assay of complexes with different N/P ratios, a) CS/pDNA complexes; b) Arg-CS/pDNA complexes; c) His-CS/pDNA complexes; d) Lys-CS/pDNA complexes, the numbers in the photos were the N/P ratio values. II: Protection of DNA with (+) or without (–) DNase I digestion, a) 1U DNase I; b) 2 U DNase I. A: Arg-CS/pDNA complexes; B: His-CS/pDNA complexes; C: Lys-CS/pDNA complexes; D: CS/pDNA complexes; E: Naked pDNA. All complexes in (II) were prepared at the N/P ratio of 25.

could provide better protection to plasmids in nucleases than could the CS. The clear bands of DNA observed in Figure 3II suggest that the condensation of plasmids in AAA-CSs does not affect the integrity of DNA and the plasmids could be released from the complexes.

2.6. Cytotoxicity and Cellular Uptake of AAA-CSs/pDNA Complexes

Cytotoxicity is an unavoidable problem for most cationic polymeric gene vectors such as PEI. It was reported that the cytotoxicity of cationic polymers could cause severe side effects on cell membranes and extracellular matrix proteins.[49] The MTT assay was used to investigate the cytotoxicity of AAA-CSs and AAA-CSs/pDNA complexes on the HEK293 cell line. The cell viability of each group was above 95% when the concentrations ranged from 50 to 200 μg mL^{-1} (**Figure 4**I), which demonstrates that both the AAA-CSs and AAA-CSs/pDNA complexes are nontoxic. There was no difference in cell viability between the AAA-CSs and chitosan groups, which implies that modification did not destroy the excellent biocompatibility of chitosan. In some amino acid modified chitosan groups, the cell proliferation was higher than that of CS group, which was due to the nutrient effects of the immobilized amino acids. As many cationic polymer vectors showed some cytotoxicity,[50] the excellent biocompatibility of the AAA-CSs was favorable for gene delivery.

TOTO-3 was used to label pDNA to investigate the cellular uptake of AAA-CSs/pDNA complexes. Chitosan and PEI 25k were used as controls. The confocal laser scanning microscopy (CLSM) images are shown in Figure 4II. It was clear that the amount of plasmid in the cells increased with increasing co-culture time. Few plasmids were found in HEK293 cells after 1 h incubation and most of the labeled plasmids were not in the cells. After incubation for 2 h, some plasmids entered into cytoplasm and more plasmids were found in the cells after incubation for 4 h. More plasmids condensed with AAA-CSs and PEI 25k were observed in the cells. These results imply that the plasmids, when combined with AAA-CSs and PEI 25k, could enter the cytoplasm efficiently.

Quantitative cellular uptake of the amino acid modified chitosans was tested by flow cytometry analysis. As shown in Supporting Information Figure S2, the proportions of the cells with fluorescence in Arg-CS group and His-CS group were 12.2% and 12.4%, and those of CS and PEI 25k were 1.7% and 12.2%. These results demonstrate that the Arg-CS/pDNA and His-CS/pDNA complexes were more easily internalized by the cells than the unmodified chitosan. The cellular uptake of these two complexes was comparable to that of PEI 25k, which was much higher than that of chitosan, which means that both Arg-CS and His-CS are beneficial for the cellular internalization of complexes and favorable for the promotion of transfection efficiency.

2.7. In Vitro Transfection

The in vitro gene transfection of AAA-CSs/pDNA complexes was performed on HEK293 cells with pEGFP-C1 and pGL3-Luc as reporter genes. The golden standard nonviral vector PEI 25k was used as a positive control. **Figure 5**I displays typical fluorescence images of the transfected HEK293 cells. The AAA-CSs exhibited more notable green fluorescence than chitosan. The transfection efficiency of the AAA-CSs was closely related to the charge ratios of the complexes and the type of alkaline amino acids immobilized on chitosan. For the AAA-CSs/pEGFP-C1 complexes, the gene expression increased with increasing N/P ratios. When the N/P ratio was higher than 45, the expression remained at nearly the same level. The Arg-CS/pEGFP-C1 and His-CS/pEGFP-C1 complexes exhibited better transfection than did the Lys-CS/pEGFP-C1 and CS/pEGFP-C1 complexes. The quantitative results of the pEGFP-C1 transfection were tested by flow cytometry analysis (Figure S3). The mean GFP fluorescent intensities of the Arg-CS, His-CS, and Lys-CS groups with an N/P ratio of 45 were 61 995, 67 888, and 31 922, while those of CS and PEI 25k were 12 650 and 65 308. These results demonstrate that the mean GFP fluorescent intensity of the Arg-CS and His-CS was comparable to that of PEI 25k, and was much higher than those of CS and Lys-CS.

The pGL3-Luc gene transfection of the AAA-CSs was evaluated by means of a luciferase assay. As shown in Figure 5II, the pGL3-Luc transfection efficiency of chitosan was only 6812 RLU mg^{-1} protein (RLU=relative light units), while that of Arg-CS and His-CS was more than 6.0×10^6 RLU mg^{-1} protein. The transfection activity of chitosan was enhanced greatly by the modification of arginine and histidine. Arg-CS showed the highest transfection activity of the three AAA-CSs; it was 1000-fold higher than that of chitosan. By comparing to the 9.0×10^6 RLU mg^{-1} protein of PEI 25k, we could conclude that the transfection efficacy of Arg-CS or His-CS was comparable to

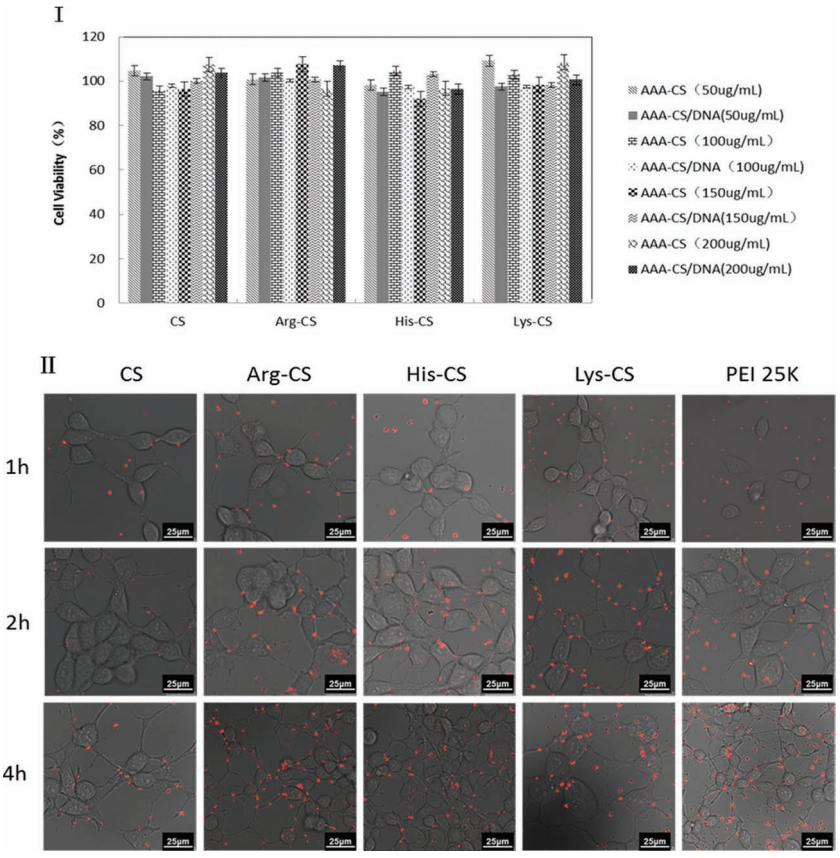

Figure 4. Cytoxicity and cellular uptake of AAA-CSs/pDNA complexes in HEK293 cells. I: Cell viabilities with different concentrations of AAA-CSs and AAA-CSs/pDNA complexes at the N/P ratio of 45 for 24 h, the data are expressed as mean values (with standard deviation) of four experiments. II: CLSM images of AAA-CSs/pDNA complexes (N/P = 45) with time (scale bar: 25 μm).

that of PEI 25k. These results are consistent with the pEGFP-C1 transfection results in Supporting Information Figure S3.

In order to evaluate the transfection efficiency of alkaline amino acid modified chitosan in other cell lines, Arg-CS was selected to transfect NIH3T3 cells as it showed the highest transfection efficiency in HEK293 cells. Supporting Information Figure S4 shows typical fluorescence images of the transfected NIH3T3 cells. The Arg-CS group exhibited stronger green fluorescence than the chitosan group and its transfection effect was comparable to PEI 25k. The pGL3-Luc gene transfection result is shown in Supporting Information Figure S5. The pGL3-Luc transfection efficiency of chitosan was 1012 RLU mg^{-1} protein, while that of Arg-CS was 2.6×10^6 RLU mg^{-1} protein. The transfection activity in NIH3T3 cell line was also enhanced greatly by the modification of arginine, which was in accordance with its transfection results in the HEK293 cell line. These results support our assertion that the modified chitosans are high-performance nonviral gene vectors.

2.8. Intracellular Tracking of Arg-CS/pDNA Complexes

In order to discover the details of intracellular tracking of the AAA-CSs/pGL3-Luc complex, fluorescent dyes fluorescein isothiocyanate (FITC) and TOTO-3 were used to label Arg-CS and pDNA. The CLSM images are shown in **Figure 6**. The nuclei of cells were stained with Hoechst 33258. The images show that most of the Arg-CS/pGL3-Luc complexes could be internalized into the cells. The complexes are dispersed in both cytoplasm and nuclei. As the nuclei of HEK293 cells are very large, the cytoplasm is not obvious in the photographs. Both strong red and green fluorescence are observed in the nuclei, which implies that the complexes are dissociated to release the condensed DNA and both Arg-CS and pGL3-Luc penetrated into the nuclei. The existence of Arg-CS in the nuclei was probably due to the enzymatic degradation of chitosan catalyzed by lysozyme in the cells.[51] The enzyme degraded the polysaccharide by hydrolyzing the glycosidic bonds. As chitosan contains a hexameric binding site, hexasaccharide sequences containing three, four, or more acetylated units contribute mainly to the initial degradation rate of chitosan.[52] The specific function of promoting internalization as shown in Figure 4, the easy dissociation of the complexes, and the enzyme-catalyzed degradation of chitosan in cells could explain why the Arg-CS exhibited high gene-transfection activity.

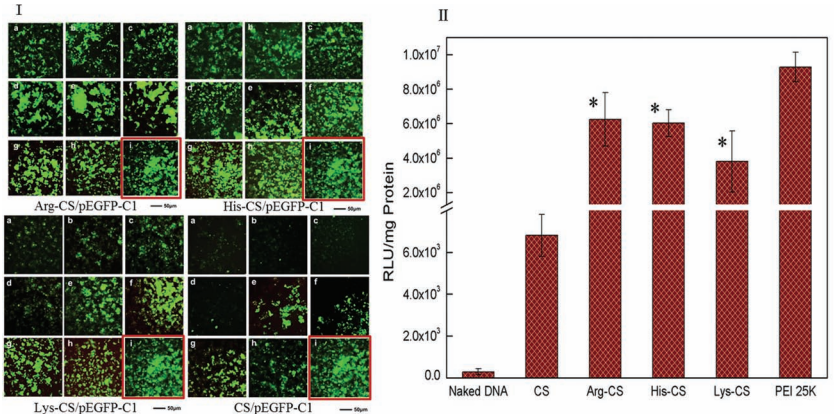

Figure 5. In vitro transfection of AAA-CSs/pDNA complexes (containing 0.8 μg pDNA) in HEK293 cells. I: Typical fluorescence images of AAA-CSs/pEGFP-C1 complexes with different N/P ratios of a) 20, b) 25, c) 30, d) 35, e) 40, f) 45, g) 50, h) 55. The red frame was PEI 25k with N/P ratio of 10. II: Transfection efficiency of AAA-CSs/pGL3-Luc complexes at the N/P ratio of 45, the data are expressed as mean values (with standard deviation) of six experiments, *: significant difference from CS ($p < 0.05$).

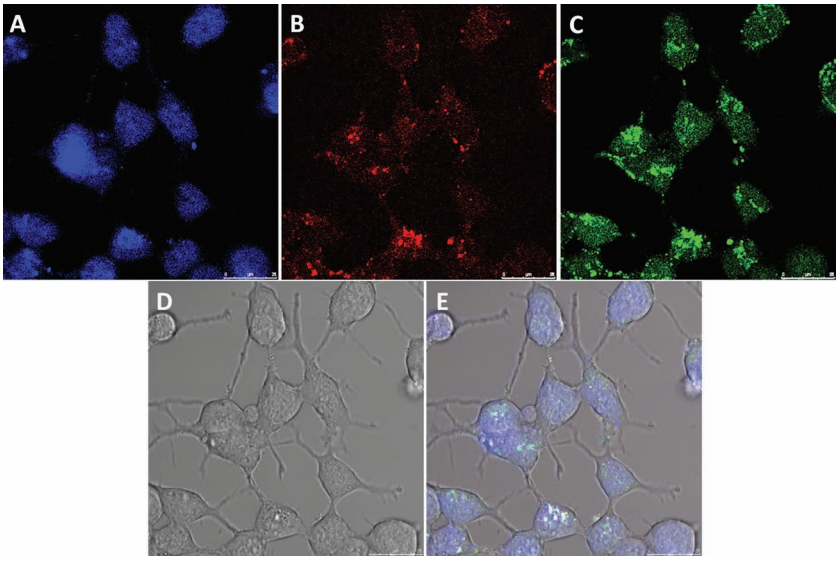

Figure 6. CLSM images of HEK293 cells transfected with Arg-CS/pGL3-Luc complexes (containing 1.6 μg pDNA) with FITC-labeled Arg-CS and TOTO-3-labeled plasmids at the N/P ratio of 45 for 4 h; scale bar represents 25 μm. A) Hoechst 33258 stained nuclei; B) TOTO-3-labeled plasmid; C) FITC-labeled Arg-CS; D) bright field; E) overlapped image.

2.9. In Vivo Transfection via Intramuscular Injections

Intramuscular administration for gene therapy is a promising strategy to express therapeutic genes, especially in the treatment of ischemic diseases.[53,54] In order to study the feasibility of the in vivo transfection application of AAA-CSs, preliminary studies of pCMV-β gal and pGL3-Luc gene transfection of Arg-CS were carried out via intramuscular injection. The β-galactosidase and luciferase assays were used to test the transfection efficiency, while PEI 25k was used as control.

The results of β-galactosidase expression in vivo are shown in **Figure 7**I. The muscles were examined histologically for β-galactosidase expression after injecting for three days. A large number of β-galactosidase-positive myofibers were obtained and the tissues were intensely blue in the Arg-CS group (Figure 7Ia), while no obvious blue myofibers were found in the PEI 25k group (Figure 7Ib). The histological analysis demonstrates that the areas of positive β-galactosidase stained muscle treated with Arg-CS in mice are considerably larger than those treated with PEI 25k. This finding suggests that Arg-CS could promote the expression of pDNA in muscles.

The results of the luciferase assay are shown in Figure 7II; the transfection efficiency of PEI 25k was only 573 RLU mg^{-1} protein, while that of Arg-CS was about 8.5×10^4 RLU mg^{-1} protein. The transfection efficacy of Arg-CS/pGL3-Luc complexes was almost 150-fold higher than that of PEI 25k. These results reflect that Arg-CS exhibited efficient gene transfection via intramuscular injections, which implies that Arg-CS may be a good choice as a nonviral gene vector for intramuscular injection.

3. Conclusion

In order to enhance the gene-transfection efficiency by simulating the components of viral envelopes, alkaline amino acids of arginine, histidine, and lysine were immobilized on chitosans. The AAA-CSs exhibited high buffering capability, good stability against DNase I degradation, excellent biocompatibility, and efficient cellular internalization. The in vitro gene-transfection results showed that the transfection efficiency of AAA-CSs was at least 500-fold higher than that of chitosan. Arginine-modified chitosan exhibited the highest transfection activity, which was comparable to that of PEI 25k in vitro. The in vivo experimental results revealed that the transfection ability of Arg-CS was much better than that of PEI 25k in the tibialis anterior muscles; this suggests that the arginine-modified chitosan could enhance gene-transfection efficiently

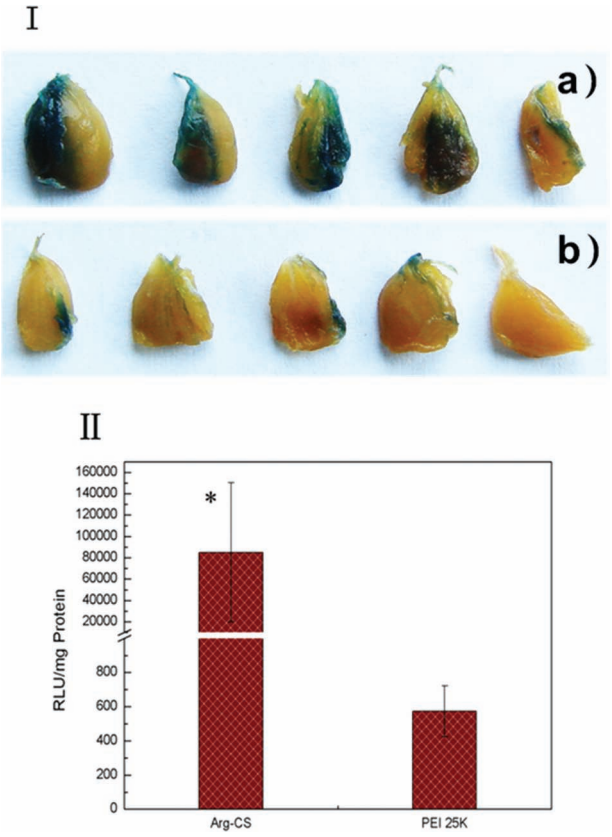

Figure 7. Gene-transfection efficiency of Arg-CS/pDNA complexes (containing 10 μg pDNA) at the N/P ratio of 45 in the tibialis anterior muscles. I: Gene-transfection efficiency of Arg-CS/pCMV-β-gal complexes, a) Arg-CS; b) PEI 25k with N/P ratio of 10. II: Gene-transfection efficiency of Arg-CS/pGL3-Luc complexes, data are expressed as mean values (with standard deviation) of six experiments. *: significant difference from PEI 25k group ($p < 0.05$).

both in vitro and in vivo, which would make it a potentially promising nonviral gene vector.

4. Experimental Section

Materials: The protected amino acids Boc-Arg(Pbf)-OH, Boc-Lys(Boc)-OH, and Boc-His(Boc)-OH were purchased from Gil Biochem. Co. Ltd. (Shanghai, China) and chitosan (M_n = 20 kDa, deacetylation degree of 90%) was purchased from Haihui Biochemical Co. Ltd. (Qingdao, China). Plasmids encoding for green fluorescent protein (pEGFP-C1), β-galactosidase (pCMV-β gal), and luciferase (pGL3-Luc) were purified with the EndoFree Plasmid Kit from Qiagen (Germany). HEK293 and NIH3T3 cell lines were purchased from Institute of Biochemistry and Cell Biology, SIBS, CAS. Dulbecco's Modified Eagles Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (USA). Fluorescein isothiocyanate (FITC) and Hoechst 33258 were purchased from Sigma-Aldrich (USA) and fluorescent dye TOTO-3 was obtained from Invitrogen (CA). DNase I was purchased from Fermentas (USA). Cell lysate and the luciferase reporter gene assay kit were purchased from Promega (USA). β-Galactosidase reporter gene assay kit was purchased from Beyotime Institute of Biotechnology (China). A BCA protein assay kit was purchased from Pierce (USA). All other chemicals were purchased from Sigma-Aldrich (USA) and used without further purification.

Synthesis of AAA-CSs: Boc-Arg(Pbf)-OH (1.05 g, 2mmol), Boc-Lys(Boc)-OH, and Boc-His(Boc)-OH were dissolved in *N*,*N*-dimethylformamide (DMF) and the free carboxyl groups were activated by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl; 1.5 eq. with amino acid) and *N*-hydroxysuccinimide (NHS; 1.5 eq. with amino acid) in N_2 atmosphere for 2 h. After the chitosan was dissolved in DMF (with 0.1 M hydrochloric acid) and stirred at 50 °C for 2 h, the protected amino acid solutions were added separately into chitosan solution at room temperature. The reaction was terminated after stirring for 2 days. The compounds (Arg(Boc,Pbf)-CS, Lys(Boc,Boc)-CS, His(Boc,Boc)-CS) were obtained by reduced-pressure distillation and purified by precipitation in distilled water, methanol, and methylene dichloride (yield = 80%).

The three amino acid modified chitosans were deprotected with trifluoroacetic acid (TFA, 10 eq., according to the number of protecting groups) in N_2 atmosphere and the products were dialyzed for 3 days. The products were freeze-dried. The modified chitosans were dissolved in DMSO-d_6 for the ^{1}H NMR (BRUKER av-400, USA) test.

Buffering Capacity: The buffering capacity of AAA-CSs was determined by acid-base titration assay.[55] Briefly, each AAA-CS sample was dissolved in double-distilled water. The pH values of the AAA-CSs solution were adjusted to 12 with 0.2 M NaOH. The concentration of the solution was controlled to 2 mg mL^{-1}. The solution was titrated with 0.1418 M HCl solution to pH = 2.0. The pH values were determined using a microprocessor pH meter (Thermo Orion Star LogR, USA).

Preparation of AAA-CSs/pDNA Complexes: The three AAA-CSs were dissolved in PBS solution (1 mmol, pH 7.4). The concentration was adjusted to 1 mg mL^{-1}. Green fluorescent protein (GFP) and luciferase-encoding plasmid DNAs were used to prepare complexes in MilliQ water in a concentration of 0.8 mg mL^{-1}. AAA-CSs/pDNA complexes were prepared by adding AAA-CSs solution to equal volume of DNA solution (containing 400 ng DNA) with different N/P ratios. The morphologies of AAA-CSs and AAA-CSs/pDNA complexes (N/P = 45) were observed by TEM (JEOL JEM-100CX, Japan) and AFM (MFP-3D-BIO, USA), and the zeta potentials of the nanoparticles were measured using a Zeta sizer Nano ZS (Malvern, UK) at 25 °C.

Gel Retardation Assay: DNA condensation ability of the AAA-CSs was assessed by using the electrophoresis method. The AAA-CSs/pDNA complexes were vortexed gently and incubated at 37 °C for 30 min before use. The complexes were loaded onto 1% (w/v) agarose gel with tris-acetate (TAE) running buffering at 80 V for about 70 min. DNA retardation was then visualized by a UV illuminator (Bio-Rad ChemiDoc XRS+, USA) with GelView staining and the condensed DNA bands were analyzed with Image Lab software.

Protection Against DNase I Degradation: Naked plasmid, CS/pDNA complexes, and AAA-CSs/pDNA complexes with the N/P ratio of 25 were treated with 1U and 2U DNase I separately for 10 min at 37 °C, followed by heat inactivation (70 °C for 10 min). 0.5 μL of ethylenediaminetetraacetic acid (EDTA; 0.5 M), 5 μL of sodium dodecyl sulfate (SDS; 10%), and 4.5 μL of heparin sodium were added and incubated at 37 °C for 1–2 h to release DNA. The complexes were loaded on 1% (w/v) agarose gel with TAE running buffering at 80 V for about 70 min. DNA retardation was then visualized using a UV illuminator (Bio-Rad ChemiDoc XRS+, USA) with GelView staining.

Cell Viability Assay of AAA-CSs and AAA-CSs/pDNA: The evaluation of the cytotoxicity of AAA-CSs as well as AAA-CSs/pDNA complexes was performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HEK293 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated FBS and antibiotics (100 U mL penicillin G and 100 mg mL^{-1} streptomycin). The cells were cultured on 96-well plates at a density of 4×10^4 cells cm^{-2} and were incubated at 37 °C under an atmosphere of 5% CO_2 in air and 100% relative humidity. After incubating for 24 h, the cells were treated with AAA-CSs or AAA-CSs/pDNA complexes at the N/P ratio of 45. For both AAA-CSs and AAA-CSs/pDNA complexes samples, the corresponding AAA-CSs concentrations were 50, 100, 150, and 200 μg mL^{-1}. After incubating for 24 h, an MTT test was carried out to quantify the viability of the cells. Briefly, 20 μL of MTT solution dissolved in PBS (5 mg mL^{-1}) was added to each well and further incubated for 4 h. The medium was then replaced with 150 μL DMSO to dissolve the formazan crystals. After gently agitating for 5 min, the absorbance at 570 nm was measured using a Thermo Varioskan Flash microplate reader. The cells in the culture plate were used as control. The relative cell viability was measured as: Cell viability (%) = (OD sample)/(OD control)×100%

Cellular Uptake: The pDNA was labeled with TOTO-3 following the manufacturer's instructions. AAA-CSs/pDNA complexes were prepared by adding AAA-CSs solutions to equal volume of DNA solution at the N/P ratio of 45. The complexes were vortexed gently and incubated at 37 °C for 30 min before used. HEK293 cells at a concentration of 6×10^4 per well were seeded and grew for 24 h. Then the complexes were incubated with the cells for 1, 2, and 4 h at 37 °C in DMEM without FBS. After washing twice with PBS, the cells were directly visualized using CLSM (Leica TCS SP5, Germany). For excitation of TOTO-3 fluorescence, an argon laser with an excitation wavelength of 488 nm was used. The fluorescence intensity of TOTO-3 was also quantified using flow cytometry (FACSAria, BD, USA).

In Vitro Transfection: To evaluate the efficiency of the AAA-CSs to induce gene expression in HEK293 and NIH3T3 cells, the cells were analyzed for green fluorescent protein (pEGFP-C1) and luciferase (pGL3-Luc) expression. Briefly, AAA-CSs/pDNA complexes with N/P ratios ranging from 20 to 55 were prepared as described above. HEK293 and NIH3T3 cells were seeded on 24-well plates with an initial density of 8×10^4 cells per well and incubated for 24 h. The AAA-CSs/pDNA complexes (containing 0.8 μg pDNA) were added to each well and incubated at 37 °C for 4 h in DMEM without FBS. The complexes were removed and the cells were incubated in fresh DMEM at 37 °C for another 48 h. The cells were analyzed for green fluorescent protein expression with a fluorescence microscope (Leica, Germany). The fluorescence intensity of GFP was also quantified by flow cytometry (FACSAria, BD, USA).

To assay the expression of luciferase, the medium was removed and the cells were rinsed gently with cold PBS and lysed using luciferase lysis buffer at the concentration of 200 μL well^{-1}. The cell suspension was subjected to freezing (–80 °C, 30 min) and thawing, and then centrifuged at 12 000 rpm for 3 min. Luciferase activity was measured by detecting the light emission from an aliquot of cell lysate incubated with 100 μL of luciferase substrate in a Thermo Varioskan Flash microplate reader. The relative light units (RLU) were normalized to protein concentrations in the cell extracts, which were measured using a bicinchoninic acid (BCA) protein assay kit. The PEI 25k with N/P ratio of 10 was used as

control. All the experiments were carried out in triplicate to ascertain reproducibility.

Intracellular Tracking of the Complex Nanoparticles: HEK293 cells at a concentration of 6×10^4 per well were seeded in glass-bottomed plate and grown for 24 h. The pDNA was labeled with TOTO-3 following the manufacturer's instructions, while Arg-CS was labeled with FITC according to the method previously reported. Arg-CS/pDNA complexes at the N/P ratio of 45 were prepared as described above. The complexes (containing 1.6 μg pDNA) were incubated with the cells in each plate for 4 h at 37 °C in DMEM without FBS. The medium was abandoned and the cells were stained with Hoechst 33258 for nucleus staining. After washing twice with PBS, the cells were directly visualized using CLSM (Leica TCS SP5, Germany). The micrographs were obtained at the magnification of 400×. For excitation of Hoechst 33258, TOTO-3, and FITC fluorescence, argon lasers with excitation wavelengths of 405, 488, and 630 nm were used.

Intramuscular Injections: Permission for the animal experimention was obtained from the ethics committee of Sichuan Univeristy. To evaluate the gene-transfection ability of Arg-CS in muscle, 12 six-week-old male Babl/c mice (Jianyang Experimental Animal Centre, Chengdu) were used. 50 μL Arg-CS/pDNA solution containing 10 μg plasmid DNA (pGL3-Luc) at the N/P ratio of 45 was injected directly into the tibialis anterior muscles. The muscles were harvested 72 h after injection, and the luciferase activity was assayed. The PEI 25k with N/P ratio of 10 was used as the control. The expression of β-galactosidase was evaluated using the same method and the β-galactosidase was assayed using the β-galactosidase reporter gene assay kit and photographed with a camera.

Statistical Analysis: All data were expressed as means ± SD of a representative of several similar experiments. Statistical difference between treatment groups and control group in each study was evaluated via one-way analysis of variance (ANOVA), and a value of $P < 0.05$ was considered significant (computed by SPSS version 13.0 Software).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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