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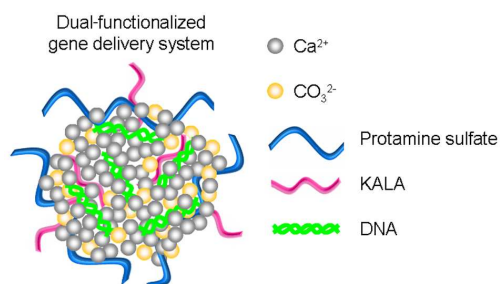
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Table of Contents Entry

Dual-functionalized KALA/PS/CaCO₃/DNA nanoparticles containing a cell penetrating peptide (KALA) and protamine sulfate (PS) could effectively mediate gene transfection at a low DNA concentration.



ABSTRACT

A cell penetrating peptide (KALA) and protamine sulfate (PS) were introduced to a nano-structured calcium carbonate based gene delivery system to obtain dual-functionalized KALA/PS/CaCO₃/DNA nanoparticles. The dual-functionalized nanoparticles were prepared by a facile co-precipitation method, during which all functional components were introduced to the nanoparticles simultaneously. For comparison, mono-functionalized gene delivery systems were also prepared to study the individual effects of PS and KALA. The size and zeta potential measurements indicated KALA/PS/CaCO₃/DNA nanoparticles exhibited a decreased size and an increased zeta potential. *In vitro* gene transfections mediated by different nanoparticles were carried out in 293T cells and HeLa cells. As compared with unmodified and mono-functionalized nanoparticles, dual-functionalized nanoparticles exhibited significantly improved gene delivery efficiency due to the enhanced cellular uptake and nuclear localization, especially at a low DNA concentration. The dual-functionalized nanoparticles have promising applications in gene therapy since the therapeutic nucleic acid reaching target cells is usually limited in the practice application.

Keywords: gene expression; nanoparticles; calcium; proteins; peptides

Introduction

In gene therapy, gene vectors which deliver the nucleic acid based drugs into target cells are of critical importance. Compared with viral vectors, non-viral vectors have attracted increasing research interest because of their advantages including high safety, low immunogenicity, and ease of large-scale production.^{1,2}

Although non-viral vectors are safer than viral vectors, the biocompatibility of widely investigated non-viral vectors based on cationic polymers and cationic liposomes is still not satisfactory and their toxicity is a major concern.³ To address this issue, different non-viral gene delivery methods were developed, among which the technique of co-precipitation of Ca^{2+} with DNA in the presence of inorganic anions present in bodies, such as PO_4^{3-} and CO_3^{2-} , has become an attractive option because of the ideal biocompatibility and biodegradability of calcium phosphate and calcium carbonate.^{3,4} However, during the co-precipitation, the uncontrollable growth of the co-precipitates leads to decreased transfection efficacy due to the low cellular internalization caused by the increased size of co-precipitates.^{5,6} To improve the thermodynamic stability of co-precipitated nanoparticles and to enhance the delivery efficiency have been the focus of intense research efforts.⁵⁻¹⁰ For example, the careful optimization of transfection experiment parameters, such as the initial concentrations of ions, temperature and the mixing mode, could minimize the variability in transfections.^{5,6} Introduction of additional components including ions⁷ and polymers⁸⁻¹⁰ to the co-precipitation systems led to controlled particle sizes of co-precipitated nanoparticles and thus achieved enhanced transfection efficiency. For example, in our previous work, alginate was used to suppress the crystallization and improve the colloidal stability of CaCO_3 based nanoparticles for gene delivery.¹⁰

In gene transfection mediated by non-viral vectors, a critical issue is to improve the transfection efficiency since the low delivery efficiency is one of the main obstacles in non-viral vector mediated gene transfer. By introduction of specific functional components which are favorable for overcoming the delivery barriers, the gene delivery efficiency can be improved through enhancing the cell uptake, endosomal escaping and nuclear translocation.¹¹ Although individual functional component results in an improved gene expression level, the combination of different components may not have additive or synergistic effects on the improvement of delivery efficiency, especially when one of the functional components can significantly enhance the delivery efficiency individually.

The purpose of our study is to investigate the additive or synergistic effect of different functional components on the improvement of gene delivery efficiency. Two functional components, protamine sulfate and KALA which result in enhanced gene delivery efficiency individually,^{12,13} were introduced to the CaCO₃ based gene delivery system simultaneously. Compared with mono-functionalized nanoparticles, dual-functionalized nanoparticles exhibited significantly improved gene delivery efficiency, especially at a low DNA amount for transfection.

Protamine is a biocompatible cationic and naturally occurring polypeptide with an arginine-rich sequence, which can provide unique membrane translocating and nuclear-localizing activities. Protamine sulfate is a FDA-approved compound with a documented safety profile used as heparin antidote and vaccine stabilizers.^{14,15} In gene delivery, protamine and its salts are often used to combine with liposomal vectors,

polymer vectors and viral vectors to achieve enhanced delivery efficiency.¹⁶⁻²¹ In our previous study, we introduced protamine sulfate (PS) to CaCO₃/DNA nanoparticles to achieve improved gene delivery efficacy.¹² In the PS modified nanostructured CaCO₃ based gene delivery systems, PS had favorable effects on overcoming delivery barrier due to its high arginine content and preventing the overgrowth and aggregation of nanoparticles.

As an endosomolytic and fusogenic peptide, KALA can destabilize lipid membranes and facilitate cellular uptake. KALA and its derivatives were not only used as gene vectors to condense pDNA^{22,23} and siRNA,²⁴ but also introduced into gene delivery systems as additives to overcome delivery barriers and to improve the transfection efficiency.²⁵⁻³⁰ Previous studies on KALA modified nanostructured CaCO₃ based delivery systems indicated the presence of KALA resulted in improved delivery efficiency for both gene and drug deliveries.¹³

In this study, we simultaneously introduced PS and KALA to the CaCO₃ based gene delivery system. *In vitro* study showed that the dual-functionalization resulted in additive effects on the gene transfection, especially when the concentration of DNA was low. Since the concentration of therapeutic DNA plasmid in target sites is very limited for *in vivo* gene delivery, the dual-functionalized system we prepared has great promising in gene therapy.

Experimental

Materials

Anhydrous calcium chloride and anhydrous sodium carbonate of analytical grade

were supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and used as received. Protamine sulphate (PS) was obtained from BoMei Biotechnology Co. Ltd. (Hefei, China). KALA (WEAKLAKALAKALAKHLAKALAKALKACEA) was obtained from GL Biochem (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Amresco. Dimethylsulfoxide (DMSO) was from Sigma. Molecular probes (Hoechst 33258 and YOYO-1 iodide) and Lipofectamine 2000 were purchased from Invitrogen.

Human embryonic kidney cell line 293T and human cervical carcinoma cell line HeLa were obtained from China Center for Typical Culture Collection (Wuhan, China). The medium for cell culture was Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), $2 \text{ mg}\cdot\text{ml}^{-1}$ NaHCO_3 , and $100 \text{ U}\cdot\text{ml}^{-1}$ penicillin/streptomycin. Cells were incubated at $37 \text{ }^\circ\text{C}$ in humidified air/5% CO_2 .

The reporter plasmid, pGL3-Luc, was purchased from Promega. The plasmid was amplified in *Escherichia coli*, extracted and purified by QIAfilter Plasmid Mega Kit (QIAGEN), suspended in water, and stored at $-20 \text{ }^\circ\text{C}$.

Preparation of KALA/PS/ CaCO_3 /DNA nanoparticles

Plasmid solution with $1 \text{ }\mu\text{g}$ of plasmid DNA was diluted to $10 \text{ }\mu\text{l}$, and then $16 \text{ }\mu\text{l}$ of CaCl_2 solution (0.5 M) was added dropwise. The solution was diluted with deionized water to make solution **A** with a total volume of $60 \text{ }\mu\text{l}$. Protamine sulphate ($0.15 \text{ }\mu\text{g}$) and KALA with a particular amount ($0.1, 0.2, 0.5, 1, 2$ and $5 \text{ }\mu\text{g}$, respectively) and $16 \text{ }\mu\text{l}$ of Na_2CO_3 solution (0.01 M) were mixed and then diluted with deionized water to

make solution **B** with a total volume of 40 μl . Then solution **A** was added into solution **B** dropwise and mixed gently to obtain 100 μl of KALA/PS/CaCO₃/DNA nanoparticles containing solution with 1 μg of DNA.

For comparison, CaCO₃/DNA nanoparticles were prepared by adding 60 μl of solution containing DNA (1 μg) and CaCl₂ (8 μmol) to 40 μl of solution containing Na₂CO₃ (0.16 μmol), and mixing gently. PS/CaCO₃/DNA nanoparticles were prepared by adding 60 μl of solution containing DNA (1 μg) and CaCl₂ (8 μmol) to 40 μl of solution containing PS (0.15 μg) and Na₂CO₃ (0.16 μmol), and mixing gently. KALA/CaCO₃/DNA nanoparticles were prepared by adding 60 μl of solution containing DNA (1 μg) and CaCl₂ (8 μmol) to 40 μl of solution containing KALA (0.5 μg) and Na₂CO₃ (0.16 μmol), and mixing gently.

To determine the encapsulation efficiency of DNA, the nanoparticles containing solution was centrifuged at 4 °C for 1 h at 18000 rpm. After centrifugation, the amount of unprecipitated free DNA remaining in the supernatant of solution was determined by the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes) according to the manufacturer's protocol using a spectrofluorophotometer (RF-5301 PC, Shimadzu). DNA encapsulation efficiency was calculated as follows.

$$\text{Encapsulation efficiency} = (W_T - W_F) / W_T \times 100\%,$$

where W_T is the total weight of DNA fed and W_F is the weight of unencapsulated free DNA.

Particle size and ζ potential measurements

The size and ζ -potential of the nanoparticles were measured by a zetasizer (Nano ZS,

Malvern Instruments). Prior to measurements, 50 μl of nanoparticles containing solution (containing 0.5 μg of DNA) was diluted to 1 ml. Data were given as mean \pm standard deviation (SD) based on 3 independent measurements.

***In vitro* transfection of luciferase plasmid**

The cells in 1 ml of complete medium with 10% fetal bovine serum (FBS) were directly seeded in the well of a 24-well plate (5×10^4 cells per well) and incubated for 24 h. Then a particular amount of freshly prepared nanoparticle containing solution (20 μl with 0.2 μg of DNA, 50 μl with 0.5 μg of DNA, and 100 μl with 1 μg of DNA, respectively) was added to each well, and the cells were co-incubated with the nanoparticles at 37 $^{\circ}\text{C}$ for 48 h. After that, the medium was removed and the cells were rinsed gently by phosphate buffered saline (PBS, 0.1 M, pH 7.4). After thorough lysis of the cells with reporter lysis buffer (Promega) (200 μl per well), the luciferase activity was determined by detecting the light emission from 20 μl of cell lysate incubated with 100 μl of luciferin substrate (Promega) in a luminometer (Lumat LB9507, Berthold). The protein content of the cell lysate was determined by a BCA protein assay kit (Pierce). The optical density (OD) value was determined at 570 nm using a microplate reader (Bio-rad 550). The data were given as mean \pm standard deviation (SD) based on 3 independent measurements. The statistical significance between two sets of data was calculated using Student's *t*-test. A *p* value < 0.05 was considered statistically significant.

For comparison, the expression of luciferase mediated by Lipofectamine 2000/DNA complexes was also measured. To prepare the Lipofectamine 2000/DNA complexes, 1 μl Lipofectamine 2000 in 50 μl deionized water was added to a plasmid DNA solution

(1 μg DNA in 50 μl deionized water) and mixed gently. Then the mixture was incubated at room temperature for 30 min to obtain Lipofectamine 2000/DNA complexes.

Cell viability measurement

After the HeLa cells were co-incubated with nanoparticles for 48 h, the medium was removed, and then fresh medium (1 ml) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (60 μl , 5 $\text{mg}\cdot\text{ml}^{-1}$) were added to each well, followed by incubation at 37 $^{\circ}\text{C}$ for 4 h. Then the supernatant was carefully removed, and 850 μl of DMSO was added to each well to dissolve the formazan crystals produced by viable cells. The absorbance of the solution was measured using microplate reader (Bio-Rad 550) at 570 nm to determine the OD value. The data were given as mean \pm standard deviation (SD) based on 3 independent measurements.

Confocal microscopy observation

For confocal microscopy observation, the DNA containing nanoparticles were prepared with YOYO-1 labeled pGL3-Luc. HeLa cells in 2 ml of complete medium containing 10% FBS were seeded directly in the well of a 6-well plate (10^5 cells per well) and incubated for 24 h. Then the nanoparticle containing solution (100 μl with 1 μg of DNA) was added to each well, and the cells were incubated at 37 $^{\circ}\text{C}$ for 4 h and 24 h, respectively. After that, the medium was removed, and the cells were washed three times by phosphate buffered saline (PBS, 0.1 M, pH 7.4). The cell nuclei were stained with Hoechst 33258 solution (40 μl Hoechst 33258 in 400 μl DMEM) for 20 min at 37 $^{\circ}\text{C}$. Subsequently, the cells were washed with PBS three times and then

incubated with 200 μL of PBS. The cells were visualized on a confocal laser scanning microscope (Nikon C1-si TE2000) under magnification of 1000.

Results and discussion

Preparation and characterizations of dual-functionalized nanoparticles

In this study, dual-functionalized KALA/protamine sulfate/calcium carbonate/DNA (KALA/PS/CaCO₃/DNA) nanoparticles were prepared by a facile co-precipitation method. KALA/PS/CaCO₃/DNA nanoparticles were obtained by mixing the solution containing DNA and CaCl₂ with the solution containing KALA, PS and Na₂CO₃. Due to the electrostatic interaction between positively charged PS and KALA and negatively charged DNA, as well as the interaction between DNA and Ca²⁺ ions, KALA/PS/CaCO₃/DNA nanoparticles could be formed during co-precipitation.

In our previous investigation, the effect of PS content on the properties of mono-functionalized PS/CaCO₃/DNA nanoparticles was studied.¹² Generally the size of PS/CaCO₃/DNA nanoparticles was smaller than that of CaCO₃/DNA nanoparticles. This was due to the reason that PS could retard the growth of CaCO₃ based co-precipitates and the complexation of PS and DNA resulted in a more compact structure. In addition, the zeta potential increased with increasing PS content. Compared with CaCO₃/DNA nanoparticles, PS/CaCO₃/DNA nanoparticles exhibited enhanced transfection efficacy. The gene expression increased with increasing PS and the maximum expression level was achieved at a PS/DNA weight ratio of 0.15/1 for HeLa cells.

Since the $\text{Ca}^{2+}/\text{CO}_3^{2-}$ molar ratio of 50/1 and the PS/DNA weight ratio of 0.15/1 were suitable for preparing PS/ CaCO_3 /DNA co-precipitates with a good performance in gene delivery based on our previous studies,^{10,12,13} in this study we fixed the $\text{Ca}^{2+}/\text{CO}_3^{2-}$ molar ratio at 50/1 and the PS/DNA weight ratio at 0.15/1, and adjusted the content of KALA in the dual-functionalized KALA/PS/ CaCO_3 /DNA nanoparticles to study the effect of functional components on the gene delivery efficiency.

As shown in Table 1, the addition of PS and KALA resulted in improved encapsulation efficiency of DNA. Both PS/ CaCO_3 /DNA and KALA/PS/ CaCO_3 /DNA nanoparticles exhibited an apparently increased DNA encapsulation efficiency, which were higher than 90%.

The size and zeta potential of the nanoparticles in deionized water were measured by a zetasizer. As can be seen from the Table 2, the sizes of PS containing nanoparticles were smaller than that of CaCO_3 /DNA nanoparticles. This was due to the facts that PS could retard the growth of CaCO_3 based co-precipitates. In addition, the complexation of the positively charged PS with the negatively charged DNA may result in a more compact structure, leading to the decreased particle size. As compared with CaCO_3 /DNA nanoparticles, the size of KALA/ CaCO_3 /DNA nanoparticles slightly decreased and the size change was not significant. This may due to the relatively lower molecular weight of KALA as compared with PS. As a result, KALA could not effectively retard the growth of co-precipitates and could not well complex with DNA, leading the limited decrease in the particle size.

Except for CaCO_3 /DNA nanoparticles, the zeta potential values of other nanoparticles

were positive because of the addition of positively charged PS and KALA. For KALA/PS/CaCO₃/DNA dual-functionalized nanoparticles, the zeta potential increased monotonically with increasing KALA content. It should be noted that the nanoparticles with positive zeta potentials in deionized water exhibited negative zeta potentials in 10% FBS containing cell culture medium (data not shown). This may be due to the fact that the negatively charged serum could interact with the nanoparticles with positively charged PS/KALA and Ca²⁺ ions to form stabilized nanoparticles.

Luciferase expression mediated by different nanoparticles

To evaluate the transfection efficiency of the dual-functionalized nanoparticles, the *in vitro* transfections mediated by KALA/PS/CaCO₃/DNA nanoparticles in complete media with 10% FBS were evaluated as compared with other nanoparticles. Based on our experiments, we found that the DNA amount conventionally used, 1 µg of DNA for the transfection of the cells in each well of a 24-well plate, was too high for the current systems and the gene expression levels in 293T cells (>~10¹¹ RLU/mg protein, data not shown) mediated by dual-functionalized nanoparticles were higher than the detection limitation of the luminometer. To precisely identify the effects of PS and KALA on the transfection efficiency of the dual-functionalized nanoparticles, in this study, we reduced the DNA amount to 0.5 µg for the transfection of 293T cells in each well of a 24-well plate. As depicted in the Figure 1, unmodified CaCO₃/DNA nanoparticles exhibited the lowest gene expression. As expected, compared with CaCO₃/DNA nanoparticles, both PS/CaCO₃/DNA and KALA/CaCO₃/DNA mono-functionalized nanoparticles exhibited enhanced transfection efficiencies. The presence of PS and KALA simultaneously resulted in further improved gene expression levels of the dual-functionalized nanoparticles, which were higher than

that of Lipofectamine 2000/DNA complexes prepared by the commercially available transfection reagent. As expected, the transfection efficacy of KALA/PS/CaCO₃/DNA nanoparticles in 293T cells increased with increasing KALA amount in the relatively low KALA content range, and the maximum gene expression was achieved at the KALA/DNA weight ratio of 1/2.

In the dual-functionalized nanoparticles, both PS and KALA have favorable effects on gene transfection. As a cationic peptide with high arginine content, PS is favorable for overcoming nucleus barrier in gene therapy and can deliver genome into nucleus.¹⁴ According to previous studies, the addition of PS resulted in improved the gene transfection efficiency.¹⁵⁻²⁰ KALA is a cationic amphiphilic cell penetrating peptide with a helical structure, which can interact with lipid membranes. The membrane-active property of KALA facilitates the cellular entry of cargo DNA and promotes the endosomal escape. According to previous studies, the presence of KALA and its derivatives in composite gene delivery systems results in marked increase in transfection efficiency.²⁵⁻²⁷

As we know, generally the small size and the positive charge of nanoparticles are favorable for cell internalization. In the current study, as compared with unmodified CaCO₃/DNA nanoparticles, the increased zeta potential and decreased particle size of PS and/or KALA modified nanoparticles were also favorable for the improvement of delivery efficiency. Besides, the improved gene expression of functionalized nanoparticles was consistent with the increased DNA encapsulation efficiency for PS and/or KALA modified nanoparticles.

It should be noted that the gene expression level mediated by dual-functionalized KALA/PS/CaCO₃/DNA nanoparticles did not monotonically increase with the KALA amount. After achieving the maximum expression level at KALA/DNA weight ratio of 1/2, a further increase in the KALA amount did not result in the enhanced gene expression level. The possible reason for this phenomenon is that the excess KALA could not bind with DNA efficiently and thus could not result in further enhanced cellular entry and endosomal escape.

In this study, the gene delivery efficiency of the dual-functionalized nanoparticles was also evaluated in HeLa cells. As shown in Figure 2, similar trend could be found for HeLa cells, i.e., as compared with mono-functionalized nanoparticles, dual-functionalized nanoparticles exhibited enhanced gene delivery efficiency and the maximum gene expression was achieved when the KALA/DNA weight ratio was 1/2. As compared with 293T cells, the luciferase expression levels in HeLa cells being transfected by the same agents were lower, which was due to the specifications of the particular cell lines. As is well known, 293T cells are relatively easy to be transfected.

To evaluate the exact role of PS and KALA modifications on the gene delivery efficiency, we further investigated the effect of the DNA amount on the transfection in HeLa cells. From Figure 3, an interest phenomenon could be observed, i.e., the dual-functionalized nanoparticles could mediate an efficient gene expression even at a very low DNA amount (0.2 µg for the cells in each well of a 24-well plate). In other words, when the DNA amount was relatively high (1 µg for the cells in each well of a 24-well plate), the PS and KALA modification on the improvement in gene delivery efficiency was not so significant. However, the decrease in DNA amount did not

result in obvious decrease in gene expression for the dual-functionalized nanoparticles. As a result, when the DNA amount in each well decreased to 0.2 μg , the significant enhanced gene delivery efficiency could be achieved by dual-functionalization of PS and KALA. In contrast, for unmodified nanoparticles (CaCO_3/DNA) and mono-functionalized nanoparticles (PS/ CaCO_3/DNA and KALA/ CaCO_3/DNA), the lower DNA amount resulted in obviously decreased gene expressions. As we know, most commonly the concentration of the therapeutic DNA reaching the target cells is limited due to the extracellular barriers in the practice application. The unique property of current system is of importance for the *in vivo* application of gene therapy.

Confocal microscopy observation

To investigate the transfection details, we used confocal microscopy to visualize the HeLa cells after being transfected by different nanoparticles for 4 h and 24 h, respectively. As can be seen in Figure 4, compared with the cells treated by unmodified CaCO_3/DNA nanoparticles, the cell transfected by PS and/or KALA modified nanoparticles showed significantly enhanced green fluorescence (YOYO-1 labeled pGL3-Luc) intensities at both 4 h and 24 h. After transfection for 4 h, the cells transfected by CaCO_3/DNA nanoparticles had the lowest green fluorescence intensity. Very limited fluorescent particles could be observed. Green fluorescence with enhanced intensities could be observed for the cells transfected by mono-functionalized nanoparticles (PS/ CaCO_3/DNA and KALA/ CaCO_3/DNA). The highest fluorescence intensity could be detected in the cells treated by dual-functionalized nanoparticles (KALA/PS/ CaCO_3/DNA). Moreover, many fluorescent particles in the cell nuclei could be observed after the treatments by the dual-functionalized nanoparticles. After transfection for 24 h, the green fluorescence

intensity increased in the cells treated by all agents due to the prolonged transfection time. The comparison among different samples showed the same trend, i.e. the fluorescence intensity increased in the order of $\text{CaCO}_3/\text{DNA} < \text{KALA}/\text{CaCO}_3/\text{DNA} < \text{PS}/\text{CaCO}_3/\text{DNA} < \text{KALA}/\text{PS}/\text{CaCO}_3/\text{DNA}$. Clearly, among different nanoparticles, the dual-functionalized nanoparticles exhibited the highest gene delivery efficiency with enhanced cell uptake and improved nuclear translocation. The confocal observation was in good agreement with the results of luciferase assay.

***In vitro* cytotoxicity evaluation**

An ideal vector for gene delivery should have a low level of cytotoxicity as well as the high transfection efficiency. In the current study, we evaluated the *in vitro* cytotoxicity of different nanoparticles after mediating gene transfection by MTT assay. From Figure 5, HeLa cell viabilities were higher than 80% after being transfected by all nanoparticles for 48 h, indicating that all these nanoparticles did not have apparent cytotoxicity.

Conclusions

A dual-functionalized gene delivery system, KALA/PS/CaCO₃/DNA nanoparticles, was prepared by a facile co-precipitation method in an aqueous medium. The presence of KALA and PS resulted in a decreased size and an increased ζ -potential for KALA/PS/CaCO₃/DNA nanoparticles. As compared with unmodified CaCO₃/DNA nanoparticles and mono-functionalized PS/CaCO₃/DNA and KALA/CaCO₃/DNA nanoparticles, dual-functionalized KALA/PS/CaCO₃/DNA nanoparticles exhibited significantly improved gene delivery efficiency due to the enhanced cellular uptake and nuclear localization. More importantly, at a lower DNA amount, the enhancement

of delivery efficiency caused by the dual-functionalization of PS and KALA was more significant. Since the concentration of the therapeutic DNA reaching the target cells is limited in the practice application, the dual-functionalized gene delivery system has promising application in gene therapy due to its unique property.

Acknowledgments

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Table 1. DNA encapsulation efficiency of nanoparticles with different compositions.

Sample	KALA/DNA wt/wt	Encapsulation efficiency (%)
CaCO ₃ /DNA	-	62
PS/CaCO ₃ /DNA	-	95
KALA/CaCO ₃ /DNA	1/2	67
	1/10	96
	1/5	98
	1/2	98
KALA/PS/CaCO ₃ /DNA	1/1	99
	2/1	99
	5/1	99

The PS/DNA weight ratio was 0.15/1 for all PS containing nanoparticles.

Table 2. Size and zeta potential of nanoparticles with different compositions.

Sample	KALA/DNA wt/wt	Size (nm)	ζ -potential (mV)
CaCO ₃ /DNA	-	323±24	-0.9±0.2
PS/CaCO ₃ /DNA	-	231±9	2.2±0.7
KALA/CaCO ₃ /DNA	1/2	318±5	0.2±0.4
	1/10	228±17	2.2±0.6
	1/5	238±41	2.4±0.4
	1/2	196±12	2.8±1.2
	1/1	218±21	3.6±1.1
	2/1	218±9	5.0±1.6
	5/1	223±4	7.4±2.3

The PS/DNA weight ratio was 0.15/1 for all PS containing nanoparticles.

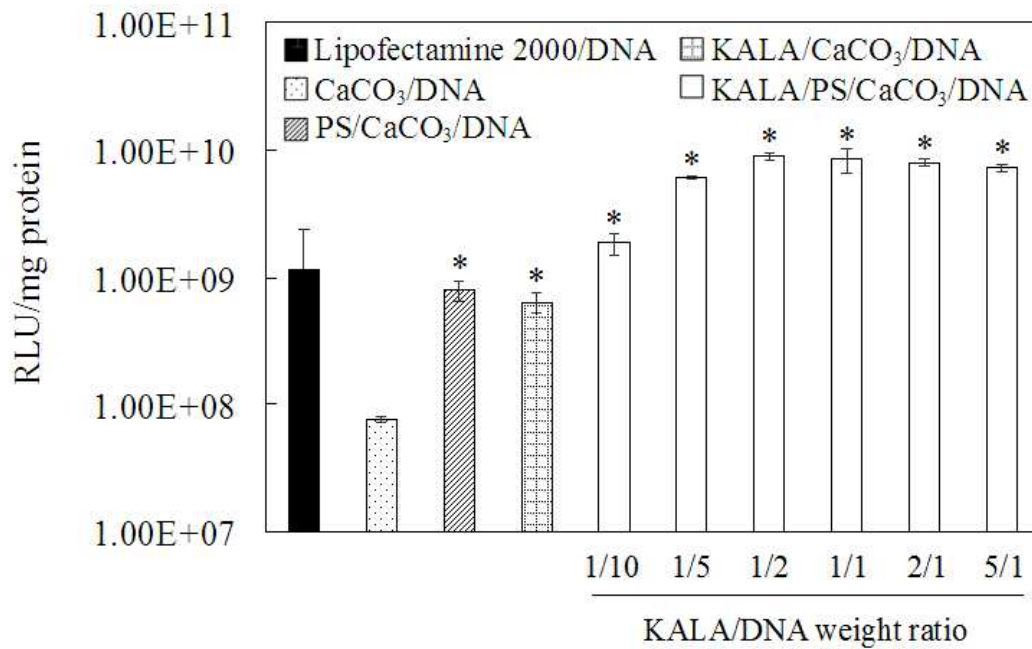


Figure 1. Luciferase expression in 293T cells after transfection for 48 h mediated by different nanoparticles. The PS/DNA weight ratio was 0.15/1 for PS containing nanoparticles. The DNA amount for the transfection of the cells in each well of a 24-well plate was 0.5 μ g. (* $p < 0.05$ as compared with CaCO₃/DNA.)

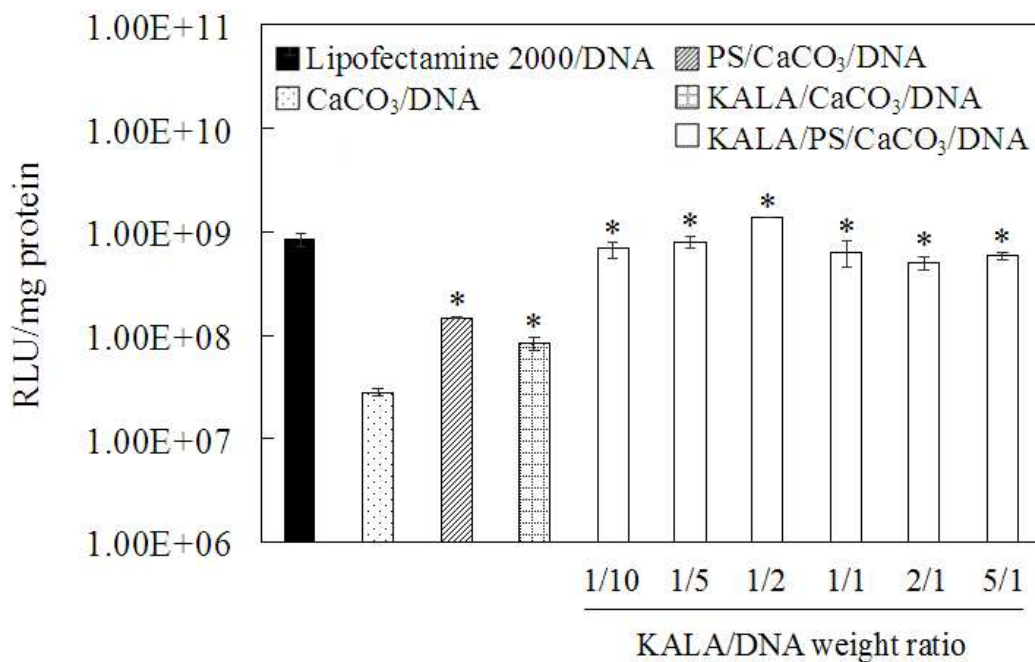


Figure 2. Luciferase expression in HeLa cells after transfection for 48 h mediated by different nanoparticles. The PS/DNA weight ratio was 0.15/1 for PS containing nanoparticles. The DNA amount for the transfection of the cells in each well of a 24-well plate was 0.5 μ g. (* $p < 0.05$ as compared with CaCO₃/DNA.)

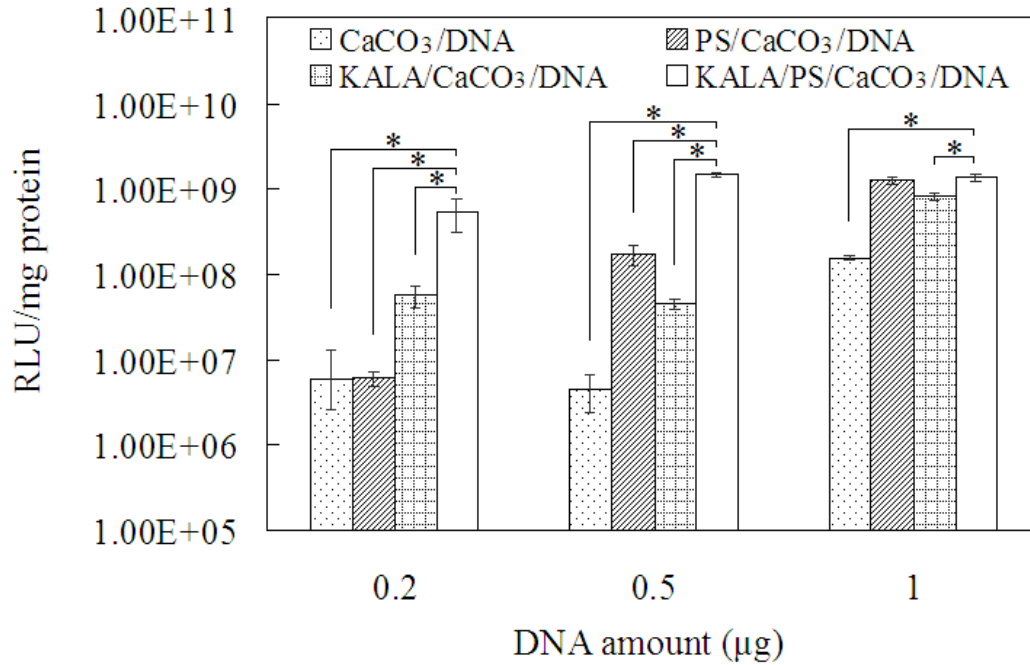


Figure 3. Luciferase expression in HeLa cells after transfection for 48 h mediated by different nanoparticles with different DNA amounts. The PS/DNA weight ratio was 0.15/1 for all PS containing nanoparticles, and the KALA/DNA weight ratio was 1/2 for all KALA containing nanoparticles. The DNA amounts for the transfection of the cells in each well of a 24-well plate were 0.2, 0.5, and 1 µg, respectively. (* $p < 0.05$.)

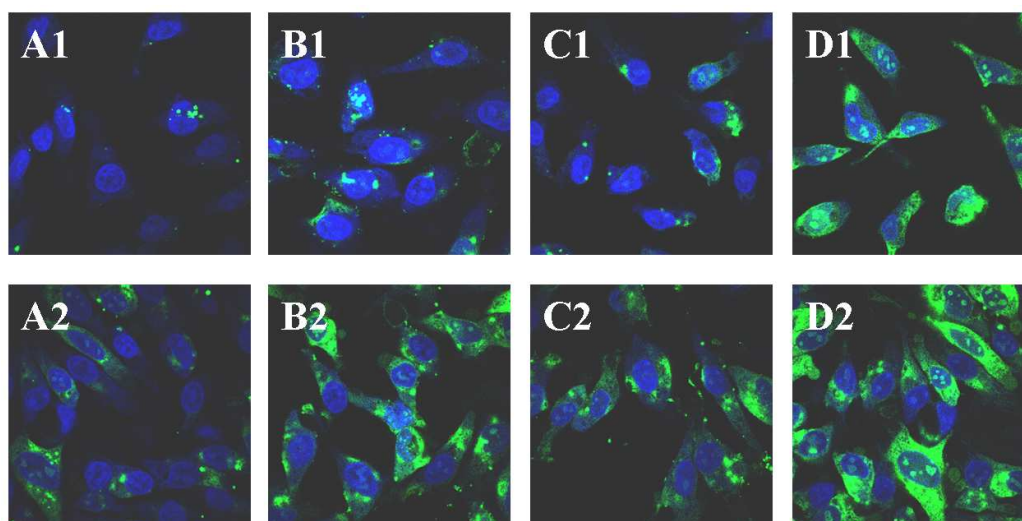


Figure 4. Confocal images of HeLa cells after being transfected by (A) CaCO₃/DNA, (B) PS/CaCO₃/DNA, (C) KALA/CaCO₃/DNA and (D) KALA/PS/CaCO₃/DNA nanoparticles for different times (4 h for A1, B1, C1, D1 and 24 h for A2, B2, C2, D2). Cell nuclei (blue) were stained with Hoechst 33258, and DNA was labeled by YOYO-1. The images were obtained under magnification of 1000. The PS/DNA weight ratio was 0.15/1 for PS containing nanoparticles, and the KALA/DNA weight ratio was 1/2 for KALA containing nanoparticles. The DNA amount for the transfection of the cells in each well of a 6-well plate was 1 μ g.

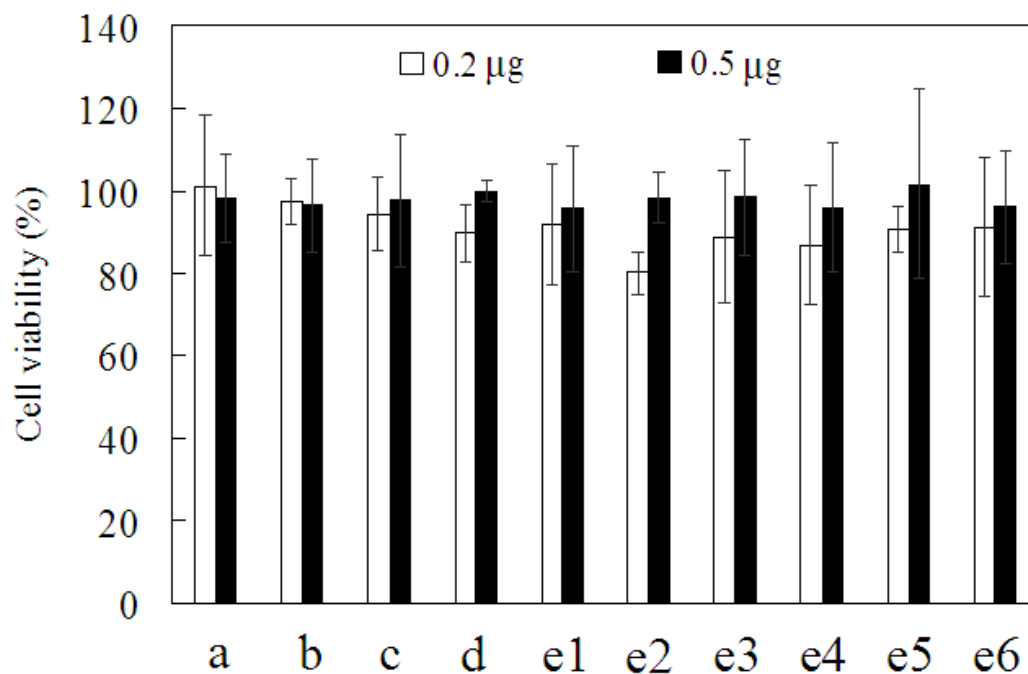


Figure 5. Cell viability of HeLa cells after transfection for 48 h mediated by different nanoparticles: (a) Lipofectamine 2000/DNA, (b) CaCO₃/DNA, (c) PS/CaCO₃/DNA, (d) KALA/CaCO₃/DNA, (e1-6) KALA/PS/CaCO₃/DNA with the KALA/DNA weight ratio of 1/10, 1/5, 1/2, 1/1, 2/1, and 5/1, respectively. The PS/DNA weight ratio was 0.15/1 for PS containing nanoparticles. The DNA amounts for the transfection of the cells in each well of a 24-well plate were 0.2 and 0.5 μg, respectively.