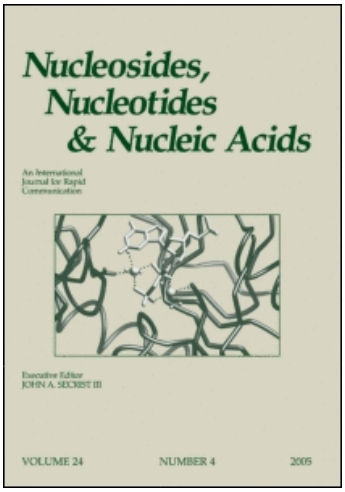


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# METHOD TO IMPROVE DNA CONDENSATION EFFICIENCY BY ALKALI TREATMENT

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□ *The improvement of DNA's bioactivities by altering their structure is meaningful for their biological applications, ranging from DNA condensation study to gene therapeutic research. In this study, we treated the plasmid DNA with alkali and investigated the structure and the condensation efficiency of the alkali-treated DNA. We noticed that the alkali treatment could significantly increase the DNA condensation efficiency with spermidine and polyethylenimine (PEI). In addition, due to the improved interactions between the alkali-treated DNA and PEI, gene transfection experiments could be performed in the presence of less PEI. This research can contribute to the creation of a universal method to enhance the interaction between DNA and gene delivery vectors by alkali treatment, and should have significant potential in the field of gene therapy.*

**Keywords** DNA condensation; alkali treatment; gene transfection



## INTRODUCTION

During the past decades, gene delivery has been a hot topic in the field of biology because of its gene therapeutic applications.[1] As a requisite for gene delivery, DNA condensation has received much attention from scientists in many fields, ranging from DNA condensation study to gene delivery vector designing. During the DNA delivery process, the enzymes from cells, such as enzymes in lysosomes, can destroy exterior DNA. Herein, DNA must be well packaged to avoid enzyme damage by gene delivery vectors. The process dramatically decreases the volume of DNA to an orderly collapsed state; this is defined as DNA condensation.[2]

DNA condensation can be induced by nearly all kinds of gene delivery systems ranging from viral gene delivery systems[3,4] to nonviral gene

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delivery systems, such as cationic lipids,[5] gemini surfactants,[6] and cationic polymers.[7] The interactions between DNA and gene delivery vectors establish some correlations between the DNA condensation process and gene transfection efficiency,[8] for instance, the dimension of complexes, the ratio of amino group in polymer to the phosphate group in DNA, and the charge of polymer influence gene transfection efficiency.[9,10] Much effort has been expanded to optimize the DNA condensation efficiency of gene delivery vectors to obtain better gene delivery efficiency, such as reducing the number of positive charges,[11] conjugating poly(ethylene glycol) (PEG) chains,[12] decreasing the polymer molecular mass,[13] etc. These synthetic methods are proved to be efficient; however, they incur a high cost and are time consuming.

Most synthetic methods optimize the interaction between DNA and gene delivery vectors by changing the structure of gene delivery vectors; but little effort has been made to optimize it by changing the structure of DNA. In fact, the structure of DNA has an extensive influence on the DNA condensation efficiency and gene transfection efficiency.[14] It has been well established that alkali treatment can alter the structure as well as some bioactivities of DNA.[15] For example, the alkali-treated phage DNA increased capacity to form new phage DNA.[16] We suppose that it is possible to improve the DNA condensation efficiency by alkali treatment.

In this study, we developed a method to improve the DNA condensation efficiency of plasmid DNA by alkali treatment. Electrophoresis assay and atomic force microscope (AFM) observation showed that the structure of plasmid DNA was greatly changed after alkali treatment. The alkali-treated DNA exhibited higher DNA condensation efficiency with spermidine and polyethylenimine (PEI) than the native DNA. Due to the improved interactions, the alkali-treated DNA could be delivered into mammalian cells in the presence of less PEI. The strategy for improving the DNA condensation efficiency by altering the structure of DNA is simple, inexpensive, and time saving, which is important in the field of gene therapy.



## MATERIALS AND METHODS

### Preparation of the Alkali Treated Plasmid DNA

Plasmid DNA pEGFP-C2 (4.7 kb) was purified from *E. coli* DH5$\alpha$ and was stored in TE (Tris-EDTA) buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH = 8.0) at $-20^{\circ}$C. The concentration of plasmid DNA was determined by spectrophotometric analysis using a DU-7 Spectrophotometer (Beckman, Fullerton, CA, USA). NaOH was dissolved in water to 0.2 mol/L. The NaOH solution and DNA solution were mixed at the ratio (v/v) of 1:1 and the samples were incubated at room temperature for 14 hours to obtain the alkali denatured DNA.

### Electrophoresis Assay

The alkali-treated plasmid DNA was prepared as above. The renatured DNA was prepared by adjusting the pH of the alkali-treated DNA to 7.2 and incubating the DNA sample at room temperature for 24 hours. The native DNA was used as a control. 0.8% agarose gel was used for electrophoresis assay, which contained 1 $\mu$g/mL ethidium bromide. Tris-borate-EDTA buffer (10 mmol/L Tris-HCl, 445 mmol/L boric acid, and 10 mmol/L EDTA) was used in all the electrophoresis experiments. Electrophoresis assay was performed at 90 V for 30 minutes. The result was visualized on a UV transilluminator (UVP, Inc., Upland, CA USA) equipped with UVP BioImaging Systems Lab Works 4.5TM software.

### AFM Observation

An amount of 100 $\mu$L of 2 mmol/L $MgCl_2$ solution was dripped onto a freshly cleaved mica slice. The slice was incubated at room temperature for 5 minutes, rinsed twice with 200 $\mu$L of distilled water, and dried with a stream of nitrogen gas. The AFM liquid cell was clamped over the $MgCl_2$-modified mica slice on a sample plate. An amount of 300 $\mu$L of the native DNA solution (2 ng/$\mu$L) or the alkali denatured DNA solution (2 ng/$\mu$L) was filled into the liquid cell. The solution was incubated at room temperature for 30 minutes before AFM observation. AFM observation was performed using a Pico AFM (Molecular Imaging, Corp., San Diego, CA, USA). Images were obtained with a magnetic AC drive (MacMode) using Type I MAClevers of nominal spring constant 0.6 N/m. The drive frequency was 30 kHz. The scan speed was 1.5 lines.



### Stability Measurement

We adjusted the pH of alkali-treated DNA to 7.2 and incubated the DNA samples at room temperature for 15, 30, 60, 90, and 120 minutes, respectively. Of each DNA sample, 5 $\mu$L (40 ng/$\mu$L) was loaded into the agarose gel for electrophoresis assay. The native plasmid DNA and the alkali-treated plasmid DNA were also loaded as controls.

### DNA Condensation with Spermidine

Spermidine was purchased from Sigma Corp. (St. Louis, MO, USA). In the experiment, the concentrations of spermidine solutions were 0, 0.1, 0.2, 1, 2, 10, 20, 100, 200, and 1000 mmol/L, respectively. The alkali-treated plasmid DNA and the renatured DNA were prepared as described above. The concentration of all the DNA samples was 40 ng/$\mu$L. A solution of 5 $\mu$L of DNA and 5 $\mu$L of spermidine was mixed thoroughly and the mixture

was incubated at room temperature for 90 minutes. Then the samples were investigated by electrophoresis assay as mentioned above.

### DNA Condensation with PEI

PEI was purchased from Sigma (MW = 25000). The concentrations of PEI solution were 0, 10, 20, 40, 60, 80, and 100 ng/$\mu$L, respectively. The concentration of all the DNA samples was 30 ng/$\mu$L. A solution of 5 $\mu$L of DNA and 5 $\mu$L of PEI were mixed thoroughly and the mixture was incubated at room temperature for 90 minutes. The samples then were investigated by electrophoresis assay as mentioned above.

### Cell Culture and Gene Transfection Experiment

293T cells (a human embryonic kidney cell line) were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Twenty-four hours before transfection, 293T cells were seeded in a 24-well plate. The complexes of PEI and plasmid DNA were prepared by mixing 2 $\mu$g DNA with 1.33 $\mu$g PEI in 100 $\mu$L of phosphate buffered saline (PBS) solution. The samples were incubated at room temperature for 30 minutes and then gently dripped onto the wells. The cells were cultured at 37°C for 48 hours. The gene transfection results were observed under a TE 2000-U fluorescent microscope equipped with Spot software (Nikon, Tokyo, Japan). The negative control was designed as 2 $\mu$g native DNA being directly added into 100 $\mu$L of PBS solution; the positive control was designed as 2 $\mu$g native DNA being mixed with 6 $\mu$g PEI in 100 $\mu$L of PBS solution.



## RESULTS AND DISCUSSION

### Observation of the Alkali Treated DNA

As a common method to investigate the topology of DNA, an electrophoresis assay was applied to investigate the alkali-treated plasmid DNA in this study. Plasmid DNA extracted from *E. coli* consists of two major components: component I is supercoiled DNA without single-strand break; component II is circular DNA with single-strand breaks. These two components were of different mobilities in electrophoresis assay (Figure 1a, lane 1). This difference in their mobilities is believed to be caused by their topology difference rather than any difference in their molecular weights. After alkali treatment, the bands of component I DNA and component II DNA disappeared and two new bands of DNA components with higher mobility appeared (Figure 1a, lane 2). We defined these two new components as component III DNA and component IV DNA, respectively.

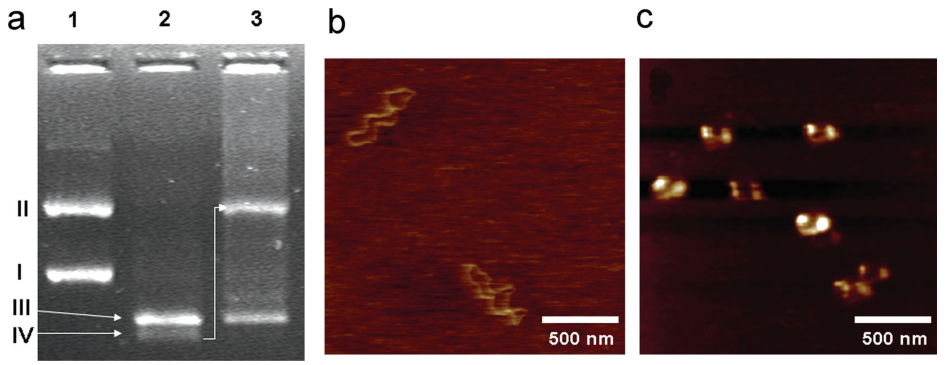


**FIGURE 1** The alkali treated plasmid DNA. a) Electrophoresis assay for the alkali treated DNA. Lane 1: the native DNA; lane 2: the alkali treated plasmid DNA; lane 3: renatured DNA. Component I DNA (I) is the circular supercoiled double-stranded DNA with no single-strand breaks; component II DNA (II) is the relaxed circular DNA arising from introducing single-strand breaks into component I DNA. Component III DNA (III) and component IV DNA (IV) are new DNA components resulting from alkali treatment. b) AFM image for the native DNA. c) AFM image for the alkali treated DNA.



This result is consistent with that of previous research by H.S. Jansz et al., which reports the appearance of the two new DNA components after alkali treatment.[16]

When the alkali-treated DNA was incubated at pH 7.2 for 24 hours, component IV DNA disappeared while component III DNA remained at its position in the agarose gel (Figure 1a, lane 3). This result indicated that component III DNA was more stable than component IV DNA. Previous research shows that under the optimal conditions for the renaturation of linear phage DNA, denatured double-stranded DNA arising from component I DNA cannot be renatured. However, introducing single-stranded breaks will allow the renaturation process to complete.[17] As is known, introducing single-strand breaks into component I DNA will result in component II DNA. That means that the DNA component arising from component I DNA should be more stable than the DNA component arising from component II DNA. In the electrophoresis assay, it was shown that component III DNA was more stable than component IV DNA. Consequently, we supposed that the component III DNA was converted from component I DNA and component IV DNA was converted from component II DNA.

The formation mechanism of component III DNA and component IV DNA is supposed to be as follows: During the alkali denaturation process, the two strands of plasmid DNA shift with respect to each other along the longitudinal axis of the helix with all of the hydrogen bonds broken. The two strands cross over each other hundreds of times to form entangled structures of component III DNA (without single-strand breaks) or component IV DNA (with single-strand breaks).[17]

The electrophoresis assay indicated that the structure of DNA would undergo some changes after alkali treatment. We used AFM to investigate

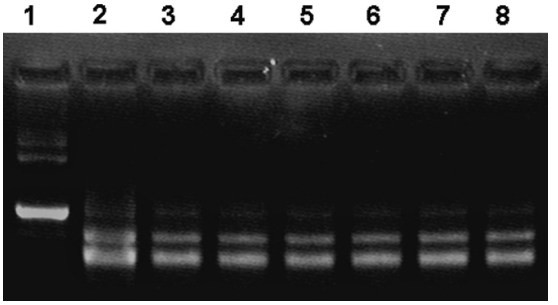


**FIGURE 2** Renaturation of the alkali denatured DNA at pH 7.2. DNA was treated with alkali and then incubated at pH 7.2 for a period of time. Lane 1 is the native DNA; lane 2 is the alkali treated DNA; lanes 3–8 are the renatured DNA. The renaturation time for lanes 3–8 was 0, 15, 30, 60, 90, and 120 minutes, respectively.



the detailed structure of the alkali-treated plasmid DNA (Figures 1b and 1c). The naked DNA, which was a circle about 1.6 $\mu$m in girth, twisted like the Arabic numeral 8. After alkali treatment, the DNA converted to compact particles, which had outer diameters of 185 nm on average (sample number = 7). These particles might receive less resistance in the electrophoresis assay and, thus, they had higher mobility. Theoretically, these particles should be a mixture of component III DNA and component IV DNA. Unfortunately, it was difficult to differentiate them by their shapes. We supposed that component III and component IV DNA had similar structures, since they were formed through a similar mechanism.[17]

## Stability of the Alkali Treated Plasmid DNA at Moderate pH

Most biological experiments associated with DNA are performed at moderate pH. As well, most biomaterials are often stored in moderate pH solutions. Consequently, the stability of the alkali-treated DNA at moderate pH is important for its applications in biological field. The double-stranded native DNA was treated with alkali to obtain component III DNA and component IV DNA. Then, we adjusted the pH of the alkali-treated DNA to 7.2 and incubated the samples for different periods of times. The renatured DNA samples were investigated by electrophoresis assay. As shown in Figure 2, both component III DNA and component IV DNA maintained their mobility in electrophoresis assay at pH 7.2 for at least 120 minutes. DNA's mobility is influenced by its structure. The result indicated that the alkali-treated plasmid could maintain its structure at pH 7.2 for at least 120 minutes. Although component IV DNA is reported to be able to convert back at moderate pH,[17] it can be still stable at pH 7.2 for a period of time. This stability of both alkali-denatured DNA components will endow the alkali-treated DNA with great applications in biological field.

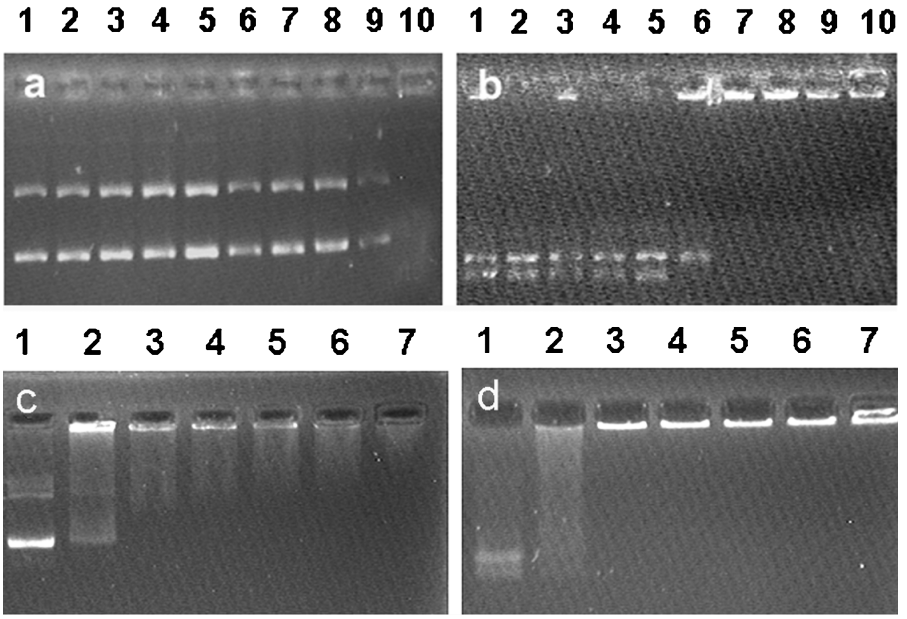


**FIGURE 3** Influence of alkali treatment on DNA condensation. a and b) DNA condensation induced by spermidine using the native DNA and the alkali treated DNA, respectively; here, the concentration of DNA solution is 40 ng/$\mu$L and the molar ratios of spermidine to DNA nucleotide from lane 1 to lane 10 are 0, 0.825, 1.65, 8.25, 16.5, 82.5, 165, 825, 1650, and 8250, respectively. c and d) DNA condensation induced by PEI using the native DNA and the alkali treated DNA, respectively; here, the concentration of DNA solution is 30 ng/$\mu$L and the ratios (w/w) between PEI and DNA from lane 1 to lane 7 are 0, 0.333, 0.667, 1.333, 2, 2.667, and 3.333, respectively.



## Influence of Alkali Treatment on DNA Condensation

As the requisite for gene delivery, DNA condensation has attracted many researchers ranging from DNA condensation study to designing gene delivery vector. DNA condensation has been proved to be possibly achieved with enzymes,[18] ions,[19] and polyamines.[20] It has been especially well documented that spermidine can efficiently induce DNA condensation.[21] Consequently, in this study we investigated the DNA condensation efficiency of the alkali-treated plasmid DNA by using spermidine as the DNA condensation reagent (Figures 3a and 3b). Spermidine exhibited obvious DNA condensation efficiency by decreasing the mobility of DNA bands. Especially, the native DNA was retained in the gel well at the ratio (spermidine/DNA) of 8250. However, after alkali treatment it could be retained in the gel well at the ratio of 165. This result indicated that after alkali treatment, the DNA condensation efficiency with spermidine was improved.

The decrease of DNA's mobility in the gel well was supposed to be caused by the interactions between spermidine and DNA. The naked DNA molecules have negative charges due to the anionic nature of the phosphate groups. These negative-charged DNA get a force toward the anode and

will move to the anode in the electrophoresis assay. The spermidine has positive charges due to the protonation of the amino groups. In the DNA condensation process, these positive charges will neutralize the negative charges of DNA and make DNA collapse into large complexes. As a result of the interaction, DNA will get greater resistance and less force in the electrophoresis assay. Consequently, the DNA's mobility will decrease.

We observed a similar result in a DNA condensation experiment with PEI. As shown in Figures 3c and 3d, PEI could decrease the mobility of both the native DNA and the alkali-treated DNA. When the ratio (w/w) of PEI/DNA reached 0.667, the alkali-treated DNA was entirely retained in the gel wells. However, at this ratio, part of the native DNA could still move out of the gel well. This result indicated that the alkali-treated DNA had better DNA condensation efficiency than the native DNA.

Increased DNA condensation efficiency of the alkali-treated DNA was supposed to be related to its structure. As shown in AFM image (Figure 1c), the structure of DNA underwent great changes after alkali treatment. This structure endowed the alkali-treated DNA with strong interactions with DNA condensation reagents and, therefore, increased its DNA condensation efficiency. However, the detailed mechanism about how the structure of alkali DNA influenced their DNA condensation efficiency was still unclear.



When double-stranded DNA is treated with alkali, the hydrogen bonds between double stands are broken to produce single-stranded DNA. Previous research shows that some other treatments, such as increasing the temperature, can also convert the double-stranded DNA into a single-stranded coil state.[22,23] But the research with single-, double-, and triple-stranded DNA shows that the ability of spermine to provoke DNA precipitation is in the following order: triplex DNA > duplex DNA > single-stranded DNA.[24] The result shows that single-stranded DNA has lower DNA condensation efficiency with spermidine than that of double-stranded DNA. However, from our results, the alkali-treated DNA had better DNA condensation efficiency than the native double-stranded DNA. So, we supposed that the single-strand transition could not be the main reason for the enhanced interactions between the alkali-treated DNA and DNA condensation reagents. After alkali treatment, the double strands of DNA do not simply separate but cross over each other to form a complex structure. This structure might contribute to their enhanced interaction with spermidine and PEI.

## Gene Transfection Experiment

Improved DNA condensation efficiency can be applied in many fields related to DNA condensation study and gene therapy. In this study, gene transfection experiment was used as an example to show the advantage of the improved DNA condensation efficiency. Plasmid DNA pEGFP-C2 can express green fluorescent proteins in mammalian cells in the presence

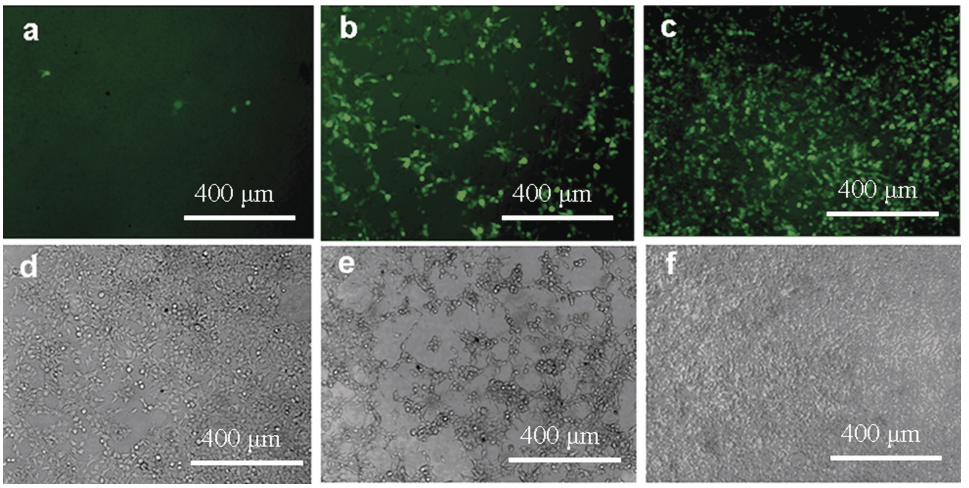


**FIGURE 4** Fluorescent image of gene transfection result using: a) 2 $\mu$g of the native plasmid DNA and 1.33 $\mu$g of PEI; b) 2 $\mu$g of the alkali treated plasmid DNA and 1.33 $\mu$g of PEI; c) 2 $\mu$g of the native plasmid DNA and 6 $\mu$g of PEI. d, e, and f) The corresponding phase contrast images of a, b, and c.



of PEI.[25] The native DNA exhibited good transfection efficiency with the proper amount of PEI (6 $\mu$g per cell well), which was indicated by a great number of green cells seen with the fluorescent microscope (Figure 4c). When the amount of PEI was decreased to 1.33 $\mu$g per cell well, the gene transfection efficiency of the native DNA decreased dramatically (Figure 4a). It was supposed that the interactions between DNA and PEI were weakened in the presence of insufficient PEI. In this case, DNA could not be well packaged and delivered into cells. Consequently, the gene transfection efficiency was decreased. However, in the presence of 1.33 $\mu$g PEI per cell well, the alkali-treated DNA showed admirably high gene transfection efficiency (Figure 4b). After alkali treatment, the interactions between DNA and PEI were improved. As a result, the alkali-treated DNA could be still well packaged and have an admirable gene transfection efficiency in the presence of less PEI.

It is noteworhty that alkali treatment only allows a gene transfection experiment being performed in the presence of less PEI. However, whether alkali treatment can increase the optimum gene transfection efficiency of PEI is still uncertain. Long-time alkali treatment will destroy the structure of DNA more or less, and consequently decrease the gene transfection efficiency. Consequently, the gene transfection efficiency of the alkali-treated plasmid DNA was greatly influenced by the experiment conditions such as the alkali treating time, the optimum ratio of DNA/PEI, the mixing process of DNA and alkali, etc. However, from the experimental data, the transfection efficiency of the alkali-treated DNA was still high after alkali treatment, meaning our method should be an effective strategy for gene

transfection without destroying DNA significantly. In addition, in this study we just used PEI as the gene delivery vector. Much work is still needed to explore whether alkali treatment can be used as a universal method to improve the gene delivery efficiency with other gene delivery vectors.

## CONCLUSION

We developed a method to improve the DNA condensation efficiency of plasmid DNA by alkali treatment. The alkali-treated DNA exhibited higher DNA condensation efficiency with spermidine and PEI. Due to the enhanced interactions, the alkali-treated DNA could be used for a gene transfection experiment in the presence of less PEI. This research found an efficient way to increase the DNA condensation efficiency by alkali treatment, which is not only useful in DNA condensation study but also of considerable potential in the gene therapy field.



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