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# Phosphorylatable short peptide conjugation for facilitating transfection efficacy of CS/DNA complex

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ABSTRACT

Previously, we had demonstrated that enhancing intracellular unpacking of exogene from its chitosan carrier by promoting chitosan degradation could markedly improve transfection efficiency of the CS/DNA complex. In this article we addressed a novel strategy of phosphorylatable short peptide modification for further facilitating intracellular DNA unpacking and optimizing transfection efficiency of the CS/DNA complex. A short peptide (SP) with the amino acid composition of "LLLRRRDNEY*FY*VRRLL" containing two potentially phosphorylatable tyrosine residues was synthesized. The short peptide could be phosphorylated by constitutively expressed cytoplasmic protein kinase Jak2. The SP was conjugated to chitosan and combined with *GFP/luciferase* reporter gene plasmid DNA to form SP-CS/DNA complex. *In vitro* phosphorylation and DNA releasing assays verified that mammalian cell lysate could effectively phosphorylate SP and hence promote plasmid DNA unpacking from the SP-CS carrier. Thereafter, C2C12 myoblast cells were transfected by SP-CS/DNA and the transfection efficiency was presented by the expression of GFP and luciferase reporters. Further more, multiple cell lines were transfected by SP-CS/DNA complexes loading luciferase reporter gene. Results revealed that, compared with CS, SP-CS could intensively augment the transfection efficiency to the level of near lipofectamine 2000.



## 1. Introduction

In recent years, the potential of chitosan as a non-viral gene carrier has been extensively considered by several research groups. Chitosan/DNA (CS/DNA) microparticles containing therapeutic genes or siRNA were being used for the transfection of mammalian cells both *in vitro* and *in vivo* (Mao et al., 2010; Techaarpornkul et al., 2010; Klausner et al., 2010). However, up to date the transfection efficiency of CS/DNA microparticles reported is lower and just this relatively low transfection efficiency obstructs its further use as a perfect gene therapy vector (Prabaharan and Mano, 2005). A variety of strategies had been employed, such as coupling galactose (Kim et al., 2004), poly(vinyl pyrrolidone) (Park et al., 2004), urocanic acid (Kim et al., 2003), glycol (Yoo et al., 2005), PEG (Yun et al., 2005) and alkyl chains (Liu et al., 2003) to chitosan or using condensed chitosan and nuclear localization signal peptide modification (Morris et al., 2009; Opanasopit et al., 2009). Although, several reports suggest that the modification of chitosan could really increase the transfection efficiency of CS/DNA microparticles to some extent (Chen et al., 2004; Zhang et al., 2006; Mansouri et al., 2004, 2006). But still is not comparable with liposome reagent or virus gene carrier (Aral and Akbuga, 2003; Romoren et al., 2003; Ozgel and Akbuga, 2006; Wong et al., 2006).

To the best of our knowledge, much work has been focused on improving the cell membrane permeability of CS/DNA complex *via* condensation of the chitosan or conjugation of various ligands. Increased transmembrane ability could let chitosan deliver more DNA into host cell and hence augment its tranfection efficiency. No doubt, cell membrane permeation is the precondition of transfection, but after being transported into the cell the unpacking of exogene from its chitosan carriers may also play an equal or even more important role in determining the transfection efficiency (Liang et al., 2006). The strong electrostatic interaction between chitosan and DNA might prevent exogene unpacking from its carrier and unfavorably hinder its expression. Recent results also demonstrated that, compared with depolymerized chitosan, highly polymerized chitosan cause intense interaction between chitosan and DNA leading to decreased transfection. By means

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of using uncharged oligosaccharides for the substitution of chitosan can augment exogene expression (Supaprutsakul et al., 2010). However, excessive reduction of the CS–DNA interaction will on the opposite abolish the transfection (Strand et al., 2010). So the balance between cell membrane permeability and intracellular unpacking seems to be really critical for the transfection.

We previously reported that extremely low molecular mass chitosan (5000–800 Da) was a perfect vehicle for transporting DNA across cell membrane. Inducing DNA unpacking from the chitosan carried by co-transfected chitosanase gene (*csn*) caused chitosan degradation could 3-fold times increase transfection. Although co-transfected *csn* could effectively benefit the transfection (Aijun et al., 2008), it might be potentially unsafe of introducing a enzyme of chitosanase into the host cell. So in the present work, we tried a new method of phosphorylatable short peptide modified chitosan (SP-CS). Like chromatin activation prior to the transcription, phosphorylated histone released DNA from the nucleic body and made it available for being transcribed. We hope the intracellular phosphorylation of the short peptide could release exogene from the chitosan and hence stimulating its expression.

## 2. Materials and methods

### 2.1. Materials

Chitosan (CS) was purchased from Sigma (Sigma–Aldrich Co., USA) in molecular mass = 5000 Da (degrees of deacetylation approximate to 80%). Lipofectamine 2000 and DMEM (Dubelco's Eagle Modified Medium) cell culture medium powder were purchased from Invitrogen Corporation (Invitrogen Co., USA). EGFP reporter gene plasmid pEGFP-C1 was purchased from Clontech (Clontech, USA). Luciferase reporter gene plasmid pGL-3 basic and streptavidin coated 96 well plate were purchased from Promega Com (Promega, USA). Mouse antiphospho-tyrosine monoclonal antibody was purchased from Cell Signaling (Cell Signaling, USA).

### 2.2. Synthesis of the short peptides

Not only phosphorylatable short peptide($^{p}$SP) but also a non-phosphorylatable short peptide($^{np}$SP) were being designed and synthesized at the same time. For $^{p}$SP, the amino acid composition was "LLLRRRDNEY*FY*VRRLL". The core peptide of NEY*FY*V containing two potentially phosphorylatable tyrosine residues is the substrate of Jak2 kinase. For $^{np}$SP, those two tyrosine residues were substituted by glutamic acid and glycine, thus the composition was changed into "LLLRRRDNEEFGVRRLL". For following *in vitro* phosphorylation and DNA releasing assay, parts of $^{p}$SP and $^{np}$SP were biotin labeled and represented as $^{bp}$SP and $^{bnp}$SP.

### 2.3. In vitro phosphorylation of the short peptides by mammalian cell lysate

Mammalian cell lysate was prepared from *in vitro* cultured C2C12 mouse myoblast cells and diluted serially with PBS (1:1, 1:4, 1:20, 1:100,1:400 and 1:1000), triplicate samples were subjected to the assay. For phoshorylation assay a streptavidin coated 96 well plate and antiphospho-tyrosine antibody were used. Briefly, a 50 μL phoshorylation reaction system was created, which is consist of 20 μM ATP, differently diluted cell lysate sample, 1.5 μM $^{bp}$SP or $^{bnp}$SP, appropriate phosphorylation assay buffer and distilled water. After incubation at room temperature for 30 min, 50 μL 50 mM EDTA was added to stop the reaction. 25 μL each reaction and 75 μL $H_2O$ were transferred to biotin coated wells, incubated at temperature for 60 min. After thoroughly washing with PBST, 1:1000 diluted mouse antiphospho-tyrosine mAb was added to each well, incubated at temperature for 60 min. After thoroughly washing, properly diluted HRP labeled secondary antibody was added, incubated at room temperature for another 60 min. After five times wash, 100 μL TMB substrate solution was added to each well and then stopped by 100 μL 1N $H_2SO_4$.The plate was read at 450 nm, SP phosphorylation level was simply reflected by $OD_{450}$.

**Table 1**
Diameter of the SP-CS/DNA complex($n = 3$).

| | PS-CS:DNA ration | | |
|---|---|---|---|
| | 2.5:1 | 5:1 | 10:1 |
| $^{bp}$SP-CS/DNA | 124 ± 17 nm | 117 ± 21 nm | 108 ± 16 nm |
| $^{bnp}$SP-CS/DNA | 121 ± 19 nm | 131 ± 18 nm | 124 ± 20 nm |

$p > 0.05$, no significant difference between any paired groups.

### 2.4. Chitosan conjugation of the short peptides

All those four kinds of short peptides were conjugated to chitosan to form $^{p}$SP-CS, $^{np}$SP-CS, $^{bp}$SP-CS and $^{bnp}$SP-CS respectively. Briefly, dissolve 0.5 g chitosan (molecular mass = 5000 Da) in 50 ml TEMED/HCl solution (pH 5.0). Short peptide was dissolved in 0.9% NaCl solution to the final concentration of 6.5% (w/v). SP and CS solution of equal volume was mixed together to form the conjugation solution, the molar ratio of "SP/chitosan carried amino group" is roughly equal to 1/2. Proper amount of EDC solution (10 mg/ml, in distilled water) was added to the conjugation solution and kept stirring at room temperature for 5 h. Then, the solution was dialyzed against PBS for 48 h, in a dialysis tube with the exclusion molecular weight of 20 kDa, to remove EDC, free peptide and low efficiently conjugated chitosan. The solution was freeze-dried to obtain the SP-CS powder.

### 2.5. In vitro unpacking of DNA from SP-CS carrier respond to cell lysate treatment.

Luciferase reporter gene plasmid pGL-3/basic was extracted and SP-CS/DNA complex was prepared as follows. Dissolved plasmid DNA into TE buffer to the final concentration of 0.2 mg/ml, above prepared SP-CS ($^{bp}$SP-CS and $^{bnp}$SP-CS respectively) was dissolved in acetic buffer (pH 5.4) to the different final concentration of 2.0, 1.0, 0.5, 0.2 and 0.1 mg/ml. 1.0 μg plasmid DNA was mixed with equal volumed SP-CS solution of different concentrations, vertexed instantly, incubated at room temperature for 30 min to let the complex formation. Thus, the weight ratio of SP-CS:DNA in the SP-CS/DNA complexes was controlled as 10:1, 5:1, 2.5:1, 1:1 and 1:2 respectively. SP-CS/DNA complexes were subjected to agarose gel electrophoresis for verifying the complex by gel retardation. Particle size of SP-CS/DNA complexes made at SP-CS:DNA weight ratio of 2.5:1, 5:1 and 10:1 were measured as previously described (Liang et al., 2006). The mean diameter was evaluated by the Stokes–Einstein relationship, shown in Table 1.

For DNA unpacking assay, $^{p}$SP/DNA and $^{np}$SP/DNA complexes were prepared at SP-CS:DNA ratio of 2.5:1 separately. Thereafter the complexes were mixed with 5.0 μl cell lysate, appropriate phosphorylation buffer and $H_2O$ to form a 50 μl reaction system. After incubation at room temperature for 30 min, the reaction was stopped by 50 μl 50 mM EDTA solution and instantly subjected to agarose gel electrophoresis and then visualized by ethidium bromide staining. Untreated $^{p}$SP-CS/DNA and $^{np}$SP-CS/DNA complexes were being electrophoresised at same time, established as control.

### 2.6. SP-CS mediated reporter genes transfection

EGFP and luciferase reporter gene plasmids were extracted and SP-CS/DNA complexes ($^{p}$SP-CS/DNA and $^{np}$SP-CS/DNA) were prepared following above-mentioned method with SP-CS:DNA weight

**Table 2**
Luciferase activity(LUCs/mg protein) in multiple cell lines 72 h after transfection, $n = 3$.

| | Mock | CS/DNA | $^{np}$SP-CS/DNA | $^{p}$SP-CS/DNA | lipofectamine 2000 |
|---|---|---|---|---|---|
| 2T3 | 87 ± 11 | 21373 ± 3824 | 22167 ± 2531 | 41876 ± 5023# | 55388 ± 5721* |
| 3T3 | 101 ± 17 | 15357 ± 1719 | 17643 ± 2531 | 53472 ± 7029# | 65359 ± 7251* |
| MG63 | 142 ± 24 | 23730 ± 3137 | 21753 ± 2429 | 48398 ± 7764# | 57823 ± 6347* |
| COS-7 | 113 ± 17 | 22539 ± 3019 | 23782 ± 3108 | 49740 ± 4372# | 60325 ± 6827* |

Within each cell line, there are significant difference between # and * marked results (# *vs* *, $p < 0.05$); # and * marked results have significant difference with other unmarked ones ($p < 0.05$).

ratio of 2.5:1. C2C12 mouse myoblast cells were seeded at a density of $5 \times 10^5$/ml on 24-well microplates in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum). Cells were cultured at 37 °C under 5% $CO_2$ atmosphere. When the cells were grown to half confluence, culture medium was discarded and the cells were rinsed three times with serum-free DMEM. SP-CS/DNA complexes were diluted with 1 ml serum-free DMEM (pH 7.2) and added into corresponding wells. After 4 h incubation, the complexes were removed and the medium were replaced by fresh FBS-containing DMEM medium and incubated for another 48 h, 72 h or 96 h. Meanwhile, cells were also transfected by lipofectamine 2000 and unmodified CS as control. Triplicate wells were assigned for experimental group and 1 μg plasmid DNA was used for each well. At different time points (48 h, 72 h and 96 h) after transfection, plasmid pGL-3/basic transfected cells were being lysised in RIPA buffer and luciferase activity in the cell lysates was detected by a chemical illumination kit (Promega, USA). Total protein concentration of each cell lysate sample was also determined by a BCA protein assay kit from PIERCE (PIERCE, USA). The luciferase activity was then represented as "RLUs/mg protein". For plasmid pEGFP-C1 transfection, GFP was investigated under fluorescent microscope every 24 h. For each group, 30 (10 fields/well) fields ($10 \times 10$) were totally investigated and the average numbers of GFP positive cells per field were calculated. Subsequently, multiple cell lines of 3T3, 2T3, MG63 and COS-7 were transfected by SP-CS/DNA complexes loading luciferase reporter genes with same strategy. 72 h after transfection, luciferase activity in the cell lysate was detected and shown in Table 2.

## 3. Results and discussion

The idea of using phosphorylatable short peptide ($^{p}$SP) conjugated CS for facilitating intracellular DNA unpacking was proposed by referencing to the process of chromatin activation prior to transcription. Phosphorylation of the serine and threonine residues could efficiently release DNA from histone octamer by the variation of electric interaction between DNA and protein. By coupling a phosphorylatable short peptide to the chitosan, we hope that after $^{p}$SP was being phosphorylated DNA could be released from the $^{p}$SP-CS/DNA complex due to the electric repulsion. Chitosan, as a polymerized glucosamine, is abundant of amino groups which make it easily to be conjugated by the peptide. The short peptide used in this article was NEY*FY*V which is the substrate of Jak2 protein kinase. The reason for chose this peptide is that Jak2, as a cytoplasmic kinase, was approved of being constitutively expressed in multiple tissues (Wallace et al., 2004). So the peptide could be efficiently phosphorylated in the host cell regardless of cell type being transfected. Some basic amino acids residues, arginine and lysine, were flanked to the core substrate to make the short peptide to be a basic peptide. The isoelectric point of the $^{p}$SP and $^{np}$SP were 10.88 and 11.50 respectively. Thus these basic peptides modified chitosan could complex with DNA in a highly condensed form. Fig. 1 demonstrated that highly diluted C2C12 cell lysate could still phosphorylate $^{p}$SP efficiently and no phosphorylation of the $^{np}$SP could be detected.

As far as the formation of SP-CS/DNA complex was concerned, when SP-CS:DNA reach or beyond 2.5:1 fully retardation of the SP-CS/DNA complexes in the electrophoresis were observed due to DNA carried anion was completely neutralized by SP-CS, shown in Fig. 2a and b. Particle size evaluation, shown in Table 1, revealed that when SP-CS:DNA ratio is equal to or larger than 2.5:1 SP-CS and plasmid DNA formed similar-sized nanoparticles, the diameter of the complexes are all around 120 nm. In the following study, the SP-CS/DNA complex was all prepared at the SP-CS:DNA ratio of 2.5:1.

For *in vitro* DNA unpacking assay, shown in Fig. 3, responded to cell lysate treatment only $^{p}$SP-CS/DNA could release DNA from the complex. The released DNA could be visualized by ethidium bromide staining in the gel. Hence, a conclusion could be made that it is the phosphorylation of phosphorylatable short peptide by cell lysate enhanced DNA unpacking and the way of $^{p}$SP conjugation might effective for facilitating DNA unpacking from the SP-CS/DNA complex in the cytoplasm.

Reporter genes applied us convenient way for evaluating transfection efficiency and in this article two kinds of reporter genes were used. By detecting luciferase activity in cell lysate the transfection efficiency could be evaluated quantitatively, while *GFP* reporter gene in the construct gave us a convenient way to visualize the expression of exogene. Figs. 4 and 5 showed the expression of these two reporter genes respectively. The most clear fluorescence was observed at 48 h after transfection, shown in Fig. 4. To clearly show the variation of GFP expression across differently transfected cells, we counted GFP positive cells under fluorescence microscope. Five fields were counted for each well and the relative GFP expression was represented as the average numbers of GFP positive cells per field ($10 \times 10$). As a result, there was no significant difference between CS/DNA and $^{np}$SP-CS/DNA transfected cells ($17.6 \pm 3.4$ *vs* $18.5 \pm 4.2$). While, $^{p}$SP conjugation significantly increase the numbers of GFP positive cells to $33.4 \pm 5.4$ ($p < 0.05$), however, which was still significantly less than $42.4 \pm 6.2$ of lipo-

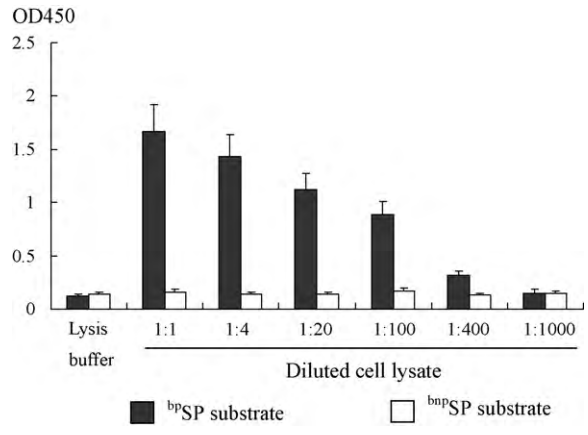


**Fig. 1.** *In vitro* phosphorylation of the short peptide. Compared with $^{bnp}$SP, which cannot be phosphorylated by either lysis buffer or mammalian cell lysate, $^{bp}$SP could be effectively phosphorylated by the cell lysate and even 1:100 dilution can phosphorylate $^{bp}$SP significantly.

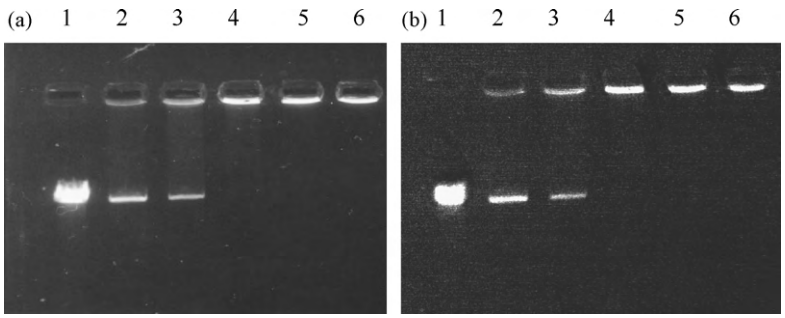


**Fig. 2.** Agarose gel electrophoresis of the SP-CS/DNA complexes. (a) $^{bp}$SP-CS/DNA complexes. (b) $^{bnp}$SP-CS/DNA complexes. Lane 1: free plasmid DNA; lane 2: SP-CS:DNA (w/w) = 1:2; lane 3: 1:1; lane 4: 2.5:1; lane 5: 5:1; lane 6: 10:1. When SP-CS:DNA reached 2.5:1, both $^{bp}$SP-CS/DNA and $^{bnp}$SP-CS/DNA complexes lost their mobility in the gel.

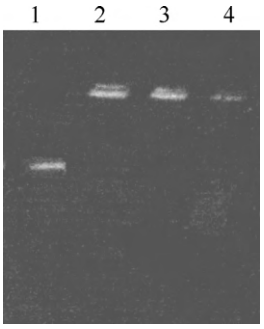


**Fig. 3.** SP-CS/DNA complexes respond to cell lysate treatment. Lane 1: cell lysate treated $^{p}$SP-CS/DNA; lane 2: untreated $^{p}$SP-CS/DNA; lane 3: cell lysate treated $^{np}$SP-CS/DNA; lane 4: untreated $^{np}$SP-CS/DNA. Respond to cell lysate treatment, DNA was released from $^{p}$SP-CS/DNA complex and moved toward anode in the electric field.

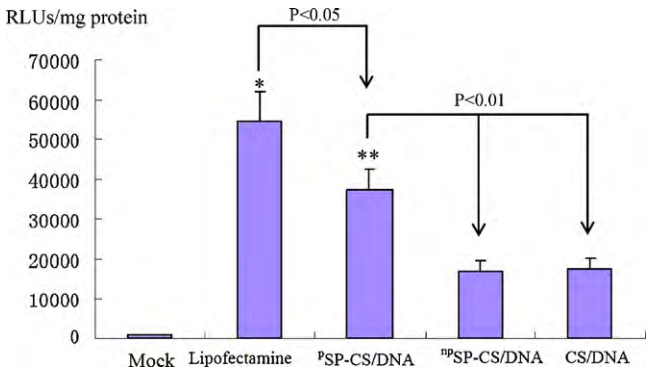


**Fig. 5.** Luciferase activity in the cell lysate 72 h after transfection. Compared with $^{np}$SP-CS/DNA, $^{p}$SP-CS/DNA can significantly increase the luciferase activity in the cell lysate. However, no significant difference was observed between $^{np}$SP-CS/DNA and CS/DNA transfection.

fectamine 2000 transfection ($p < 0.05$). As far as luciferase activity was concerned, its peak was appeared at 72 h after transfection for all plasmid pGL-3/basic transfected cells in regardless of transfection method, shown in Fig. 5. In comparison with unconjugated CS, $^{p}$SP conjugation could significantly increase transfection efficiency of the CS/DNA, as much more green fluorescence could be seen and markedly increased luciferase activity could be detected. While, $^{np}$SP brought no change to the expression of these two reporter genes. Therefore, it is rational to consider that after $^{p}$SP was being phosphorylated by Jak2 kinase the introduce anion repelled DNA from the complex, accordingly increasing the expression of reporter genes. From Table 2, it was seen that although transfection efficiencies were slightly varied among those cell lines, the $^{p}$SP conjugation increases the expression of luciferase reporter gene markedly without exception.

Comparing with other studies we found out that, at first, medium molecular weight chitosan(HMWCs, 40–100 kDa) were commonly used as gene carrier. HMWCs could always result in higher transfection efficiency than low molecular weight chitosan(LMWCs, <10 kDa), although LMWCs showed stronger DNA binding affinity, higher physiological pH solubility and better biodegradability than HMWCs. Recently more studies focused on improving transfection efficiency of the LMWCs through various strategies. By optimizing pH and serum concentration (Nimesh et al., 2010) and hydrophobic modification (Zhang et al., 2008) to promote cellular uptake or by hyaluronic acid conjugation to ultra low molecular weight chitosan (5 kDa) (Duceppe and Tabrizian, 2009) to prevent DNA and chitosan forming tight compact they

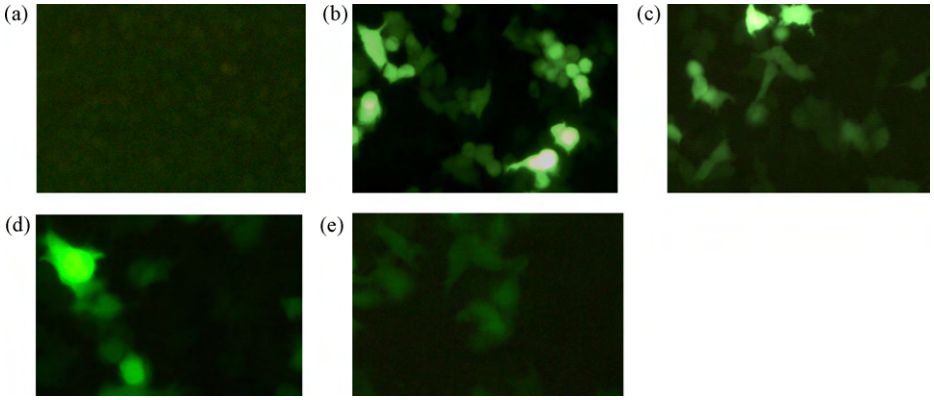


**Fig. 4.** GFP investigation at 48 h after transfection (10×10). (a) Untransfected cell control; (b) lipofectamine 2000 transfection control; (c) $^{p}$SP-CS/DNA tranfection; (d) $^{np}$SP-CS/DNA transfection; (e) CS/DNA transfection. Compared with CS/DNA transfection, $^{np}$SP-CS/DNA did not result in any enhancement of the GFP expression, however, $^{p}$SP-CS/DNA can significantly increase GFP content in the cells.

all improved transfection efficiency of LMWCs to some extend. In this paper we proposed a new strategy for facilitating transfection efficiency of the chitosan *via* enhancing intracellular unpacking.

In sum, $^{p}$SP conjugation apply us a method to enhance exogene expression by facilitating intracellular DNA unpacking. Although $^{p}$SP-CS improved transfection efficiency of the chitosan to a large extend, it is still not comparable with lipofectamine 2000. More works, such as using more effective kinase substrate to modify chitosan or optimizing transfection conditions, need to be done for further increasing its efficiency.

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