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Optimize nuclear localization and intra-nucleus disassociation of the exogene for facilitating transfection efficacy of the chitosan

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ABSTRACT

Previously, we had reported improving transfection efficiency of the chitosan-plasmid DNA (CS/pDNA)complex *via* enhancing intracellular unpacking of the exogene by the utilization of phosphorylatable short peptide conjugated chitosan (pSP-CS). In this article, we addressed a novel strategy of nucleus localization signal linked nucleic kinase substrate short peptide (NNS) modification for further optimization of the transfection efficiency. NNS, consisting of "PKKRKVREEAIKFSEEQRFRR", contained a SV40 nucleus localization signal and a potentially phosphorylatable serine residue. The short peptide could be selectively phosphorylated in the nucleus in various mammalian cells. This phosphorylatable NNS (pNNS) was conjugated to chitosan and combined with Cy3 fluorescence labeled plasmid DNA to form a pNNS-CS/pDNA complex. *In vitro* phosphorylation and DNA releasing assays verified that pNNS could be effectively and selectively phosphorylated by nucleic lysate, hence promoting pDNA unpacking from the complex. Thereafter, C2C12 myoblast cells were transfected. Nuclear localization of the pDNA was represented by the fluorescence in the nucleus and transfection efficiency was determined by the expression of the *luciferase* reporter gene, which is carried by the plasmid DNA. The results revealed that, compared with lipofactamine2000 and the previously reported pSP-CS, pNNS-CS could transport more pDNA into the nucleus and intensively augment *luciferase* reporter gene expression. In conclusion, nucleus localization and unpacking from the delivery vector are both critical factors in influencing exogene expression, and pNNS modification is valuable in improving transfection efficacy of the chitosan.

1. Introduction

Up to now, enormous efforts have been made to develop safe and efficient gene delivery methods. Viral vectors are the most efficient gene delivery methods, taking advantage of the natural ability of viruses to penetrate host cells and transfer their genetic materials to the nucleus for transcription (Davidson et al., 2000; Auricchio et al., 2002). However, viral vectors have revealed a number of serious safety risks such as potential oncogenicity, toxicity and immunogenicity. Because of these drawbacks, non-viral strategies are becoming an alternative gene delivery method. Non-viral systems, especially polymers, have shown their advantages in multiple aspects as they can be specifically tailored for a proposed application with adequate safety profiles. The polymers can condense plasmid DNA (pDNA) into nano-sized particles, protect pDNA from degradation, shield pDNA particles against undesired interactions, and enhance cell binding and intracellular delivery. In recent years the potential of chitosan, a kind of cationic polysaccharide, 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). Currently, how-

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Table 1
Synthesized peptides potential as nuclear kinase substrate.

Name	Amino acid composition	Phospho-site	Protein name	Kinase
KU86	6His-REEAIKF**S**EEQRF	S	ATP-dependent DNA helicase II	Nuclear kinase NII
B025-B	6His-AKAKTRS**S**RAGLQ	2nd S	Histone H2A.1	S19
B014-C	6His-RKR**S**RKE**S**YSVYV	1st and 2nd S	Histone H2B	Histone kinase, S32
B014-B	6His-DGKKRKR**S**RKE**S**Y	Both S	Histone H2B	Histone kinase, S32
B059-B	6His-RGGVKRI**S**GLIYE	S	Histone H4	H4 PK1, S47

S: potential phosphorylatable serine residues.

ever, the transfection efficiency of CS/DNA microparticles is still not comparable with that of viral vectors or liposome reagent, hence obstructing its further application.

During the past decade, we have focused on improving chitosan to become an ideal non-viral gene delivery vector. In our opinion, there are three main steps involved in chitosan mediated exogene expression. These three steps are cell entrance, intracellular unpacking and nucleus localization. Previously, we had demonstrated that chitosan, especially low molecular weight chitosan, had the perfect ability of carrying plasmid DNA (pDNA) into mammalian cells; also, the expression of exogene could be significantly increased by enhancing pDNA unpacking in the cytoplasma (Liang et al., 2006; Zuo et al., 2008; Sun et al., 2010). The most promising result is that through phosphorylatable short peptide (pSP) conjugation, the transfection efficiency of chitosan was effectively improved to a large extent. The mechanism is in reference to the process of chromatin activation prior to the transcription, phosphorylated histone released DNA from the nucleic body and made it available for being transcribed. The phosphorylation of the short peptide could also significantly alternate electric charge of the chitosan-peptide compound. Introduced anionic phosphate group led to electric repulsion between pDNA and pSP conjugated chitosan and subsequently stimulated pDNA disassociation. Hence, the transfection efficiency of this pSP modified chitosan (pSP-CS) could be increased without exception.

Now, we focus on the procedure of nucleus localization. There were many reports indicating that nucleus entrance of the exogene could be significantly augmented *via* being complexed with different groups or modified polymers, such as poly amido ethylenimine (Jeong et al., 2010), Guanidinylated bioreducible polymer (Kim et al., 2010), PAMAM-triamcinolone acetonide (Ma et al., 2009), nuclear localization signal (Kanazawa et al., 2009; Moore et al., 2009) and some other groups. As reported, all these strategies can benefit the expression of the exogene to some extent as more DNA can be transported into the nucleus. Meanwhile, however, a controversy arose since most of the cationic polymer gene delivery vectors need to complex DNA into a condensed form in order to facilitate cell entrance as well as protect DNA from being digested. The vectors are always biodegradable or biosoluble in the cytoplasma, meaning DNA can release from the complex and freely enter the nucleus to be the transcribable form. Nevertheless, for the purpose of enhancing exogene nuclear localization, these nucleus localization signal (NLS) modified vectors must be kept in compact complex with DNA until the latter is being actively transported into nucleus. At once, while augmenting nucleus localization, DNA disassociation may be reduced and hence diminish the benefit of increased nucleus localization. An approach which could promote DNA unpacking after the nucleus localization may resolve this problem.

We had already demonstrated that phosphorylatable peptide conjugation is effective in enhancing DNA unpacking from the chitosan carrier, and there are various kinases enriched in the nucleus undertaking the job of histone phosphorylation. Therefore, it is reasonable to consider modifying chitosan with a short peptide carrying both motifs of nuclear localization signal and a substrate of nuclear kinase.

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%). Lipofactamine2000 was purchased from Invitrogen Corporation (Invitrogen Co., USA). Luciferase reporter gene plasmid pGL-3/control and chemical illumination kit were purchased from Promega Com (Promega, USA). Mouse anti phospho-serine and anti His-tag monoclonal antibodies were purchased from cell signaling (Cell signaling, USA). Cy3 labeled by the Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit was purchased from Mirus Bio LLC (Mirus, USA).

2.2. Selection of peptides as specific nuclear kinase substrate

As shown in Table 1, five His tagged peptides were synthesized. They all carry serine residues which could be potentially phosphorylated by kinases located in the nucleus. Cytoplasmic and nucleic lysates were prepared separately from *in vitro* cultured C2C12 mouse myoblast cells and subjected to *in vitro* kinase assay in triplicate. For the assay, anti His-tag antibody coated 96 well plate and anti phospho-serine antibody were used. Firstly, a 50 μL phosphorylation reaction system was created, which consists of 20 μM ATP, properly diluted cell lysate from cytoplasma or nucleus, 1.5 μM synthesized short peptide, 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 anti His antibody pre-coated and blocked wells, then incubated at room temperature for 60 min. After thoroughly washing with PBST, 1:1000 diluted mouse anti phospho-serine mAb was added to each well, incubated at room temperature for 60 min. Properly diluted HRP, labeled secondary antibody, was added after washing and incubated at room temperature for 60 min. After five washes, 100 μL TMB substrate solution was added to each well and then stopped by 100 μL 1 N H_2SO_4. The plate was read at 450 nm, and the phosphorylation level of each peptide was simply reflected by OD_{450}. Besides C2C12 cells, phosphorylation assay of KU86 and B059-B peptides to cytoplasmic and nucleic lysate treatment from other cell lines of 3T3, 2T3, MG63 and COS-7 were also tested.

Table 2
Features of NLS–NKS short peptides (NNS).

Name	Composition	IP	Label
pNNS	***PKKRKV***REEAIKFSEEQRF**RR**	11.0	None
bpNNS	***PKKRKV***REEAIKFSEEQRF**RR**[*b]	11.0	C terminal biotin labeled
npNNS	***PKKRKV***REEAIKFAEEQRF**RR**	11.0	None
bnpNNS	***PKKRKV***REEAIKFAEEQRF**RR**[*b]	11.0	C terminal biotin labeled

IP: isoelectric point, determined by the online free software of PROTEIN CALCULATOR v3.3. NLS is in italic and bold font, two arginine residues were added to the carboxy terminals of each peptide to keep the peptide being strongly basic.
*b: biotin labeled. ***PKKRKV***: nucleus localization signal. **RR**: two additional Arginine residues to modify the IP of the short peptides.

2.3. Phosphorylation assay of NLS–NKS fusion peptides

As shown in Table 2, a total of four kinds of NLS (nucleus localization signal)–NKS (nuclear kinase substrate) short peptides were synthesized and represented as NNS. SV40 peptide (PKKRKV), the most commonly used NLS, was fused to the amino terminal of KU86 and its derivants, phosphorylatable and nonphosphorylatable peptides, with or without biotin label. All the peptides were modified with two arginine residues at their carboxy terminal to keep the peptides strongly basic. For nonphosphorylatable peptides (np), the serine residue was replaced by alanine. For the convenience of following *in vitro* phosphorylation assay, parts of pNNS and npNNS were biotin labeled. These bpNNS and bnpNNS were subjected to the phosphorylation assay following the protocol in the above section, except the coating of the plate was changed from anti-His antibody to streptavidin.

2.4. In vitro DNA unpacking

Both pNNS and npNNS were conjugated to chitosan, following the previously described method exactly in order to form pNNS-CS and npNNS-CS complexes respectively (Sun et al., 2010). For *in vitro* DNA unpacking assay, pGL-3/control plasmid DNA was extracted and made into NNS-CS/DNA complexes as follows. We dissolved plasmid DNA into TE buffer to the final concentration of 0.2 mg/ml, while NNS-CS (pNNS-CS and npNNS-CS respectively) was dissolved in acetic buffer (pH 5.4) to the final concentration of 0.5 mg/ml. 1.0 μg plasmid DNA was mixed with an equal volume NNS-CS solution, then incubated at room temperature for 30 min to allow the complex to form. Thus, the weight ratio of NNS-CS:DNA in the complexes was controlled as 2.5:1, which was previously verified as the optimal weight ratio of short peptide conjugated chitosan to pDNA for facilitating complex formation and further transfection (Sun et al., 2010). Formation of the NNS-CS/DNA complexes were confirmed by the retardation in agarose gel electrophoresis and then subjected to DNA unpacking assay. Complexes of pNNS-CS/DNA and npNNS-CS/DNA were mixed with cell lysate (extracted from C2C12 cells, cytoplasmic and nucleic lysate separately), 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, instantly subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. Untreated pNNS-CS/DNA, npNNS-CS/DNA and naked plasmid were being electrophoresised at same time as controls.

2.5. pNNS-CS mediated pDNA transfection and nuclear localization

Plasmid pGL-3/control, which contained an intact expression cassette of luciferase reporter gene, was extracted and Cy3 labeled by the *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kit (Mirus 7020, USA). This Cy3-pDNA was applied to the transfection into C2C12 mouse myoblast cells by different approaches: lipofactamine2000, pNNS-CS, npNNS-CS and the previously described pSP-CS. pSP-CS is the only phosphorylatable short peptide that conjugated chitosan and the peptide could be phosphorylated in the cytoplasma, not in the nucleus, by corresponding kinase.

Cells were seeded at a density of 5×10^5/ml on 24-well microplates in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum), then cultured at 37 °C under a 5% CO_2 atmosphere. Once grown to half confluence, the culture medium was discarded and cells were rinsed three times with serum-free DMEM. Transfection procedure of different reagents was either following company protocol (lipofactamine2000) or what we described before (CS/DNA complexes) (Sun et al., 2010). Sextuplicate wells were established for each transfection – three wells were assigned to Cy3 fluorescence investigation and nucleus staining, and the remaining three were used for quantifying reporter gene expression. Intracellular Cy3 fluorescence was monitored under fluorescent microscope every 24 h. At 48 h after transfection, cell culture was terminated. Half welled of the cells were being lysised in RIPA buffer and luciferase activity in the cell lysate was detected by a chemical illumination kit (Promega, USA). Total protein concentration of each lysate sample was also determined by a BCA protein assay kit from PIERCE (PIERCE, USA). The luciferase activity was therefore represented as "RLUs/mg protein". The other three wells of cells of each transfection were washed three times with PBS and then stained with DAPI-methanol solution. Cy3 and DAPI fluorescence were investigated, while images of the same fields were taken and merged by photoshop program. Four fields of each well were analyzed, with an average number of Cy3 positive cells per field and the ratio of nucleus localized cy3 to total cy3 were calculated from all three wells of each transfection.

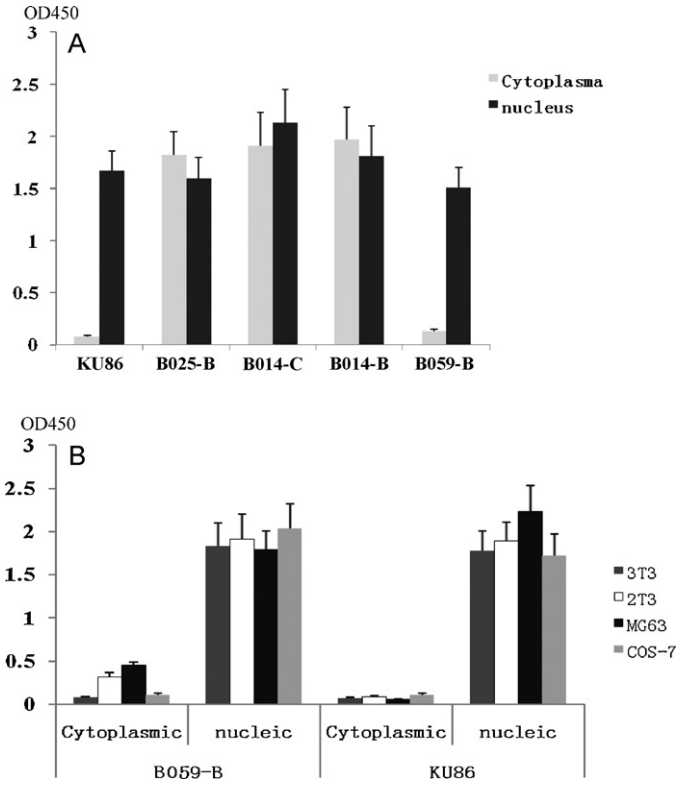

Fig. 1. *In vitro* phosphorylation of the short peptide. (A) Two kinds of short peptides of KU86 and B059-B could be selectively phosphorylated by nucleic but not cytoplasmic lysate. All the others were equally phosphorylated by either nucleic or cytoplasmic lysate. (B) Nucleic lysate from all the cell lines could phosphorylate B059-B and KU86 effectively, while some cytoplasmic lysate could phosphorylate B059-B to a small extent but could not on KU86.

3. Results and discussion

Cell permeability, nucleus localization and unpacking of DNA from the complex are regarded as the three critical factors that influence transfection efficiency of the gene carrier most. Some low molecular weight chemicals, including low molecular weight chitosan, can condense DNA intensively to form a tight carrier/DNA complex that was demonstrated to be of good cell permeability (Nimesh et al., 2010; Yang et al., 2009). For DNA unpacking by phosphorylatable peptide conjugation, as we described before, the disassociation of exogene from its chitosan carrier could be significantly augmented. In this article we focused on resolving the issue of nucleus localization and DNA disassociation. We hope

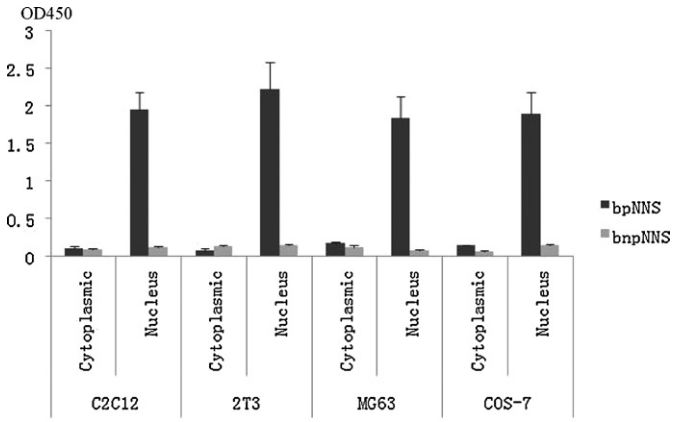

Fig. 2. *In vitro* phosphorylation of the NLS–NKS short peptides. Only biotin labeled phosphorylatable NLS–NKS short peptides(bpNNS), but not nonphosphorylatable NNS(bnpNNS), could be selectively phosphorylated by nucleus lysate extracted from multiple cellines.

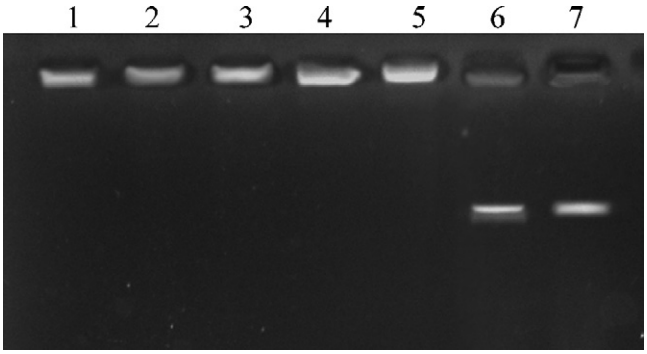

Fig. 3. NNS-CS/DNA complexes respond to cell lysate treatment. Lane 1: untreated npNNS-CS/DNA; lane 2: untreated pNNS-CS/DNA; lane 3: cytoplasmic lysate treated npNNS-CS/DNA; lane 4: nucleic lysate treated npNNS-CS/DNA; lane 5: cytoplasmic lysate treated pNNS-CS/DNA; lane 6: Nucleic lysate treated pNNS-CS/DNA; lane 7: naked plasmid control. In response to cell lysate treatment, DNA could be only released from pNNS-CS/DNA complex-received nucleic lysate and moved toward a node in the electric field.

to improve these two factors simultaneously by the strategy of NLS–NKS modification to the low molecular weight chitosan. To achieve this purpose, the phosphorylation of CS conjugated peptide must be strictly controlled so that it only occurs in the nucleus. Early phosphorylation in the cytoplasma would cause DNA unpacking from the chitosan carrier, hence NLS could not exert its ability of promoting DNA nucleus localization. Fig. 1A showed that two peptides out of five candidates could be selectively phosphorylated by C2C12 myoblast cell nucleic lysate. Fig. 1B further demonstrated that KU86 is more advantageous than B059-B for specific nucleic lysate phosphorylation across different cell lines.

To form phosphorylatable NLS–NKS short peptides (pNNS), KU86 was fusioned by a NLS at its amino terminal. pNNS is designed

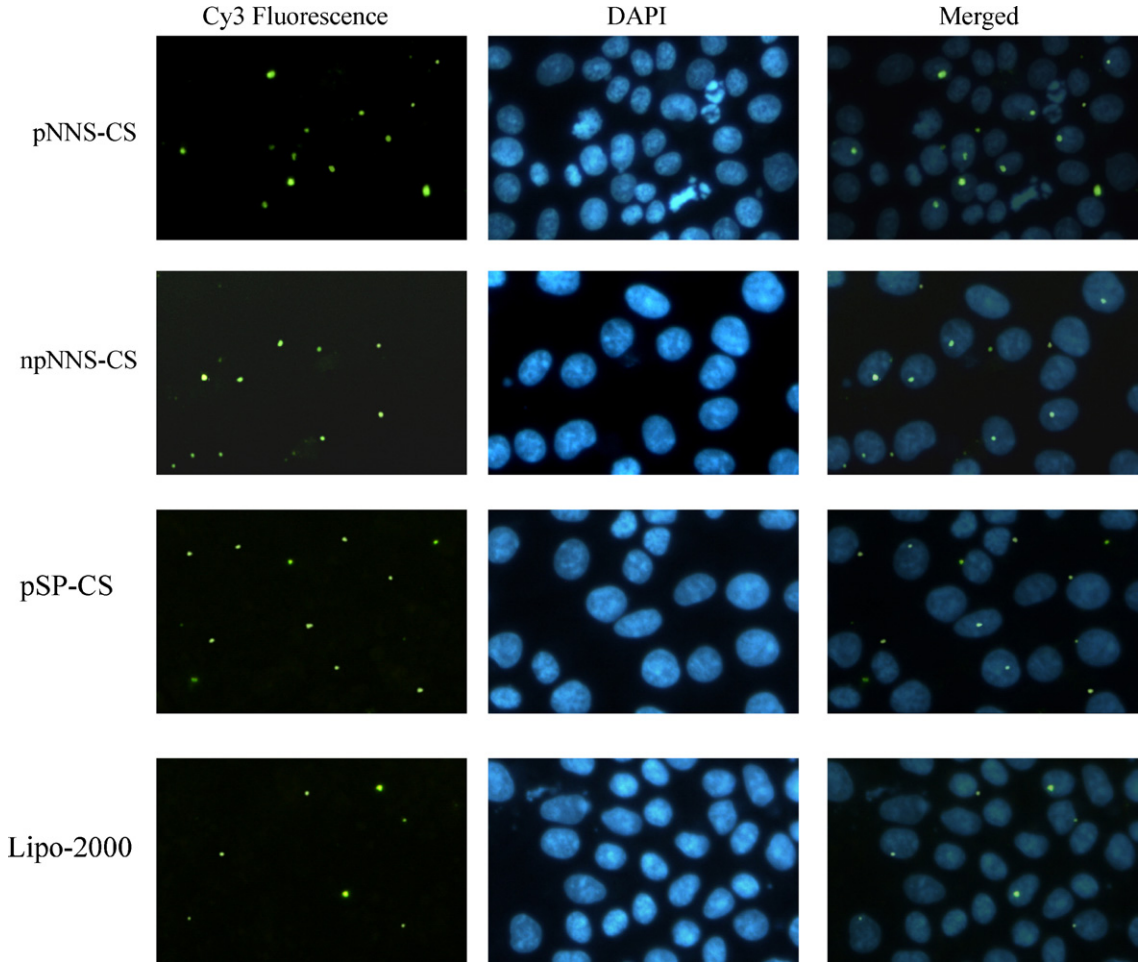

Fig. 4. Cy3 fluorescence and DAPI nucleus staining at 48 h after transfection (10 × 40). Lipofactamine2000 transfection yields less intracellular Cy3 fluorescence than the other three groups and shows no significant difference between pNNS-CS, npNNS-CS and pSP-CS transfections. Merged images show that pNNS-CS, npNNS-CS and lipofactamine had the superior ability of transforming combined DNA into the nucleus than pSP-CS transfection.

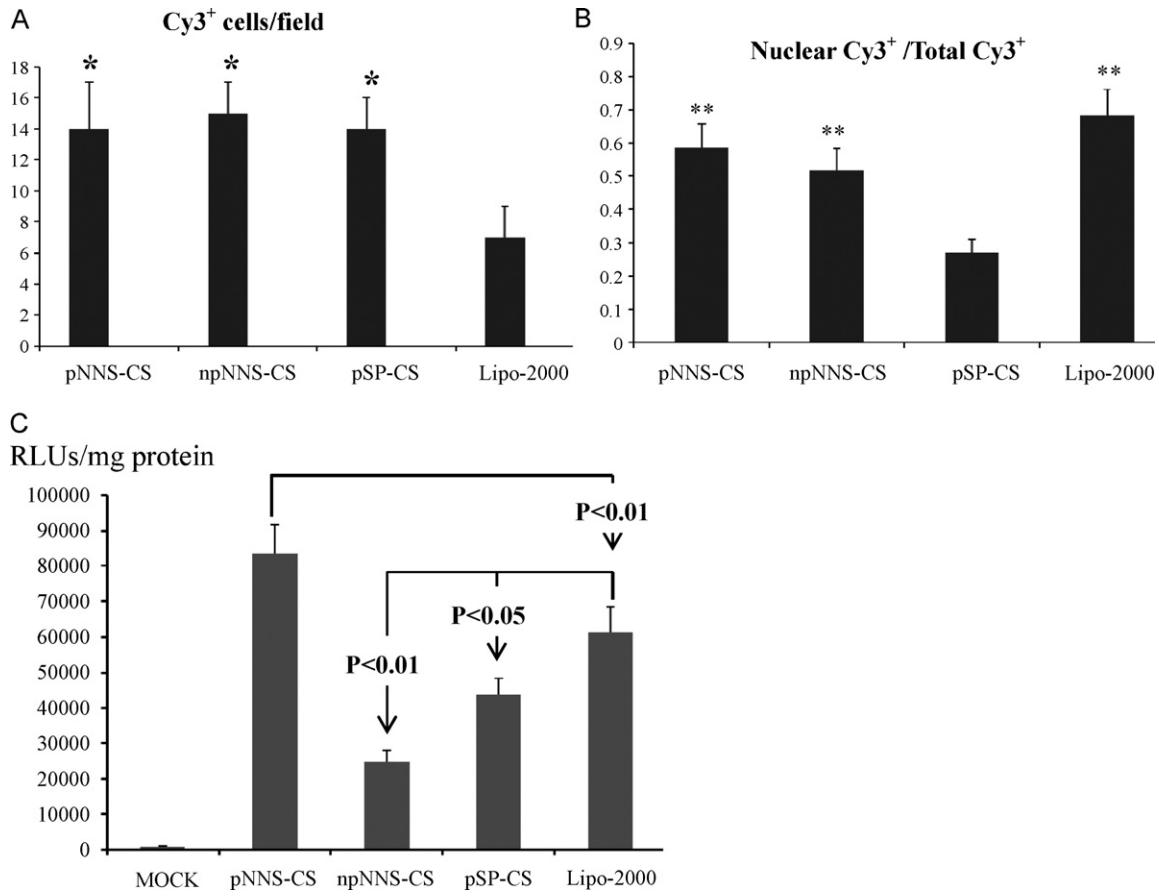

Fig. 5. Cy3 nucleus localization and luciferase activity in each transfection. (A) Compared with lipo-2000, peptide conjugated CS can yield more Cy3 positive cells regardless of the kind of conjugation (*$P < 0.05$). (B) Lipo-2000 has good ability of transforming DNA into nucleus, while, NLS modification can increase nucleus localization ability of CS/DNA complex near lipo-2000 (**$P < 0.01$). (C) Compared with npNNS-CS, pNNS-CS transfection significantly increased luciferase activity in the cell lysate, more so than lipo transfection.

to have the dual functions of enhancing nucleus localization mediated by NLS and intra-nucleus DNA unpacking mediated by NKS phosphorylation. The reason for selecting simian virus 40 (SV40) large T antigen (PKKKRKV) as the NLS is that the results of current studies reported PKKKRKV was far more efficient than other nuclear localization signals (Zhang and Mallapragada, 2010; Dixon et al., 2009). Two arginine residues were flanked to its carboxy terminal to make the short peptide basic. Thus, the peptide modified chitosan could complex with DNA in a highly condensed form.

Fig. 2 demonstrated that nucleic lysate of various cell lines could phosphorylate bpNNS with high efficiency; however, no phosphorylation of bnpNNS could be detected. This result confirmed that the phosphorylation of the peptide correctly occurred to the serine residue that we desired, as no phosphorylation could be detected when serine residue was substituted by alanine. *In vitro* DNA unpacking assay was shown in Fig. 3; in response to cell lysate treatment, plasmid DNA could only be released from nucleic lysate treated pNNS-CS/DNA complex. Released DNA could be visualized by ethidium bromide staining in the gel. Hence, there is no doubt that the phosphorylation of pNNS by nucleic lysate enhanced the DNA unpacking, and the method of pNNS conjugation is effective for facilitating DNA unpacking from the CS/DNA complex specifically in the nucleus.

Cy3 labeled pGL3/control plasmid DNA, which carried a luciferase reporter gene, was subjected to transfection assay to test the effect of NLS enhanced DNA nucleus localization and the improvement of transfection efficiency result from NKS facilitated DNA intra-nucleus unpacking. Figs. 4 and 5 show that all kinds of CS transfection, regardless of the type of peptide conjugation, could yield more $Cy3^+$ cells than lipofactamine2000. This result was in accordance with our previous findings that cell permeability of CS/DNA complex, especially made of low molecular weight chitosan, is much superior to that of liposome (Liang et al., 2006). Fig. 5B demonstrated the effect of NLS on enhancing nuclear localization of carried pDNA, showing that non NLS modified pSP-CS has the lowest "nucleic $Cy3^+$/total $Cy3^+$" ratio, which meant only a small part of exogenous DNA was being successfully imported into the nucleus after being transported into the cell. NLS modification significantly increased nucleus localization of CS/DNA complex to the level of near lipo-2000. Fig. 5C reflected the transfection efficiency of different approaches by the activity of luciferase reporter. pNNS-CS generated the highest luciferase activity, while npNNS-CS generated the least, which strongly supports the conclusion that either nucleus entrance or DNA unpacking plays critical roles in determining transfection efficiency. Due to insufficient unpacking, when transfection was performed by npNNS-CS, even compared with pSP-CS reporter gene could only be expressed in lower level, although more DNA was demonstrated being transported into the nucleus. NKS modification is effective in promoting intra-nucleus DNA unpacking. Thus, pNNS-CS improved transfection of CS/pDNA complex to the level of beyond lipofactamine2000 due to its NLS and NKS double modification.

In summary, pNNS conjugation gives us a novel method to enhance exogene expression by facilitating pDNA nucleus localization at the same time with intra-nucleus unpacking. More tests, such as using more effective nuclear kinase substrate to modify

chitosan or optimizing transfection conditions, need to be done for further improvement in increasing its efficiency.

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