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Phosphorylatable short peptide conjugated low molecular weight chitosan for efficient siRNA delivery and target gene silencing

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ABSTRACT

Small interfering RNA (siRNA) has been widely investigated as a potential therapeutic approach for diseases with genetic defects. However, its application was greatly hampered by the rapid degradation and poor cellular uptake. Recently, chitosan (CS) and its derivant have been considered as a promising siRNA transporter with the advantages of low toxicity, good biodegradability and biocompatibility. Chitosan of different molecular weight (Mw) and degrees of deacetylation (DD) showed significantly varied target gene silencing efficacy, and it is still not well clarified how these characteristics influence CS mediated siRNA transfection. To compare the aspects of cell permeability and intracellular unpacking of CS/siRNA complex on the effect of CS/siRNA transfection. A radiolabeled siRNA, targeting firefly luciferase gene, was loaded by chitosan of different molecular weight (varying from 2000 to 800,000 Da) and subjected to the transfection against MDA-MB-231/Luc human breast cancer cell line which stably expressed knocked in firefly Luciferase reporter gene. Following transfection intracellular radioactivity was measured to represent cell entrance ability of the CS/siRNA, while, luciferase activity in the cell lysate was also determined to reflect target gene silencing effect. The results revealed that although low molecular weight chitosan (LMWC) condensed siRNA has the highest cell permeability of almost two folds of medium molecular weight chitosan and lipofactamine, its target gene silencing effect is really low of almost eight times less than lipofactamine. This conspicuous contradiction gave us the hypothesis that LMWC generated more condensed CS/siRNA complex to facilitate cell entrance but the tight electrostatic interaction probably limited intracellular siRNA unpacking as well and unfavorably hindered target gene silencing as the final consequence. To approve this hypothesis a phosphorylatable short peptide conjugated LMWC was adopt to promote intracellular siRNA unpacking. Which was demonstrated of perfect target gene knock down ability to the extent of being even superior to lipofactamine 2000. In a conclusion, low molecular weight chitosan has the great potential to be an ideal siRNA vehicle if the issue of siRNA unpacking could be properly resolved.

1. Introduction

RNA interference (RNAi) represents a powerful tool for specific gene silencing. It is mediated through a 21–23mer duplex siRNA with 2 nucleotides (nt) overhang at the 3′ends. siRNA sequence specifically triggers the cleavage and degradation of their target mRNA by the Dicer enzyme (Sharp, 2001). siRNA has gained a high potential of new drug development to silence various pathologic genes. For siRNA therapeutics, the first step is the selection of an ideal siRNA delivery vector. Up to now, a number of gene delivery systems have been developed, including viral-based vector, non-viral-based vector, cationic lipids and cationic polymers. Although viral vectors are the more powerful vehicles for gene transfer than non-viral vectors, their applications are limited due to safety issues. Several non-viral vectors, including lipofectamine 2000, L-alpha-dioleoyl phosphatidylethanolamine (DOPE), poly-L-lysine (PLL), polyethyleneimine (PEI), atelocollagen, and chitosan (CS) have been successfully investigated for gene delivery *in vitro* and *in vivo* (Lee et al., 2001; Ishii et al., 2001; Weecharangsan et al., 2006; Zhao et al., 2006; MacLaughlin et al., 1998). Among them,

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Table 1
Dabcyl labeled peptides and FAM labeled siRNA.

Abbreviation	Full name	Composition	Label
dlpSP	Dabcyl labeled phosphorylatable SP	^{Da}LLLRRRDNEY*FY*VRRLL	N terminal Dabcyl
dlnpSP	Dabcyl labeled non-phosphorylatable SP	^{Da}LLLRRRDNEEFGVRRLL	N terminal Dabcyl
FsiRNA1	FAM labeled LusiRNA1	5′-^{F}GCTTGAAGTCT TTAATTAAtt-3′ 3′-ggCGAACUUCAGAAAUUAAUU^{F}-5′	5′ FAM

Y*: phosphorylatable tyrosine residues.

chitosan-derived vectors have gained increasing interest as a safer and more cost-effective vehicle for gene materials, such as plasmid DNA (pDNA), oligonucleotide (ODN) as well as proteins and peptides. Chitosan and its derivatives showed beneficial qualities of low toxicity, low immunogenicity, excellent biodegradability, biocompatibility as well as highly positively charged that makes chitsosan can easily form polyelectrolyte complexes with negatively charged nucleotides by electrostatic interaction.

Although chitosan has been studied for more than a decade as a gene delivery vector for DNA, so far, there have been only a few studies carried out to investigate the use of chitosan to deliver siRNA. To improve chitosan and its derivatives to be the optimal siRNA vehicle, various measures, including optimizing molecular weight and degree of deacetylation (Ji et al., 2009; Techaarpornkul et al., 2010), RGD peptide conjugation (Han et al., 2010), poly-L-arginine modification (Noh et al., 2010) and polyguluronate coacervation (Lee et al., 2009), have been adopted to enhance the target gene silencing effect of chitosan mediated siRNA delivery.

The concept was widely accepted that the synergism of good cell permeability and sufficient intracellular unpacking were critical factors on determining the efficiency of chitosan mediated DNA transfection. And we had also reported that low molecular weight chitosan (5000–8000 Da) was perfect at transporting pDNA across the cell membranes (Liang et al., 2006) and the exogene expression could be extensively augmented when the intracellular unpacking of pDNA from the chitsosan carrier was successfully enhanced by the means of facilitating chitosan degradation or alternating the electrostatic interaction between chitosan and DNA (Zuo et al., 2008; Sun et al., 2010). For siRNA transfection, the intracellular unpacking might also be a key criteria on determining target mRNA degradation. To our knowledge, it is still not well clarified whether different molecular weight chitosan has significantly varied siRNA transporting abilities, and what is the contribution of intracellular unpacking to the consequence of target gene silencing. Here, we demonstrated that low molecular weight chitosan (LMWC) could pack siRNA into more condensed form and delivered more siRNA into the host cell. However, the strong electrostatic interaction between LMWC and siRNA extremely limited siRNA unpacking and unfavorably hindered target gene knockdown. Enhancing siRNA intracellular unpacking by the utilization of phosphorylatable short peptide conjugation could significantly promote target gene silencing.

2. Materials and methods

2.1. Materials

Commercially available chitosan of different molecular weight (Mw 2 kDa, 5 kDa, 20 kDa, 40 kDa and 80 kDa respectively) were purchased from Sigma (Sigma–Aldrich Co., USA), the degrees of deacetylation (DDA) of them are all around 85%. Lipofectamine2000 was purchased from Invitrogen Corporation (Invitrogen Co., USA). The luciferase activity chemical illumination assay kit was purchased from Promega Com (Promega, USA). MDA-MB-231/Luc cell line was purchased from Cell Biolabs (Cell Biolabs Inc., USA). Radioactive labeled/unlabeled siRNA and short peptides were self designed and synthesized by ShineGene Corporation (Shinegene Co., China).

2.2. Target gene silencing effect comparison of the siRNAs

To test the efficacy of those two kinds of siRNAs, LusiRNA1 and LusiRNA2, MDA-MB-231/Luc human breast cancer cells with knocked in firefly luciferase gene were transfected by Lipo/siRNA complexes following the reagent protocol. Briefly, when the cells were grown to half confluence, the culture medium was extracted and the cells were rinsed three times with serum-free DMEM.

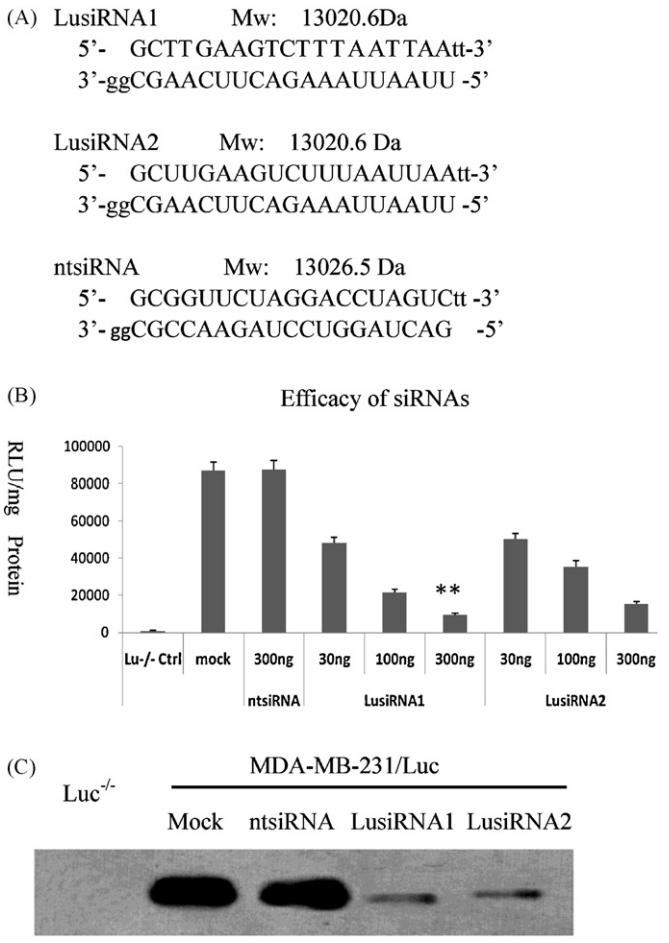


Fig. 1. Composition and silencing effect of siRNA targeting *Luciferase* gene. (A) Composition of the siRNAs targeting firefly *Luciferase* gene and one non-targeting control. (B) Compared with *Luciferase* gene null control cells (Luc$^{-/-}$ Ctrl), high luciferase activity could be detected in MDA-MB-231/Luc human breast cancer cells which stably express knocked in firefly *Luciferase* reporter gene. Non-targeting siRNA did not show any *luciferase* gene silencing effect, while, both LusiRNA1 and LusiRNA2 could effectively decrease luciferase activity in a dose dependent manner and LusiRNA1 is more effective than LusiRNA2. (C) Western blot demonstrated that both LusiRNA1 and LusiRNA2 significantly decreased the amount of Luciferase molecule in the cell lysate, all the siRNAs were used at 300 ng. **Statistically difference with unmarked groups, $p < 0.01$.

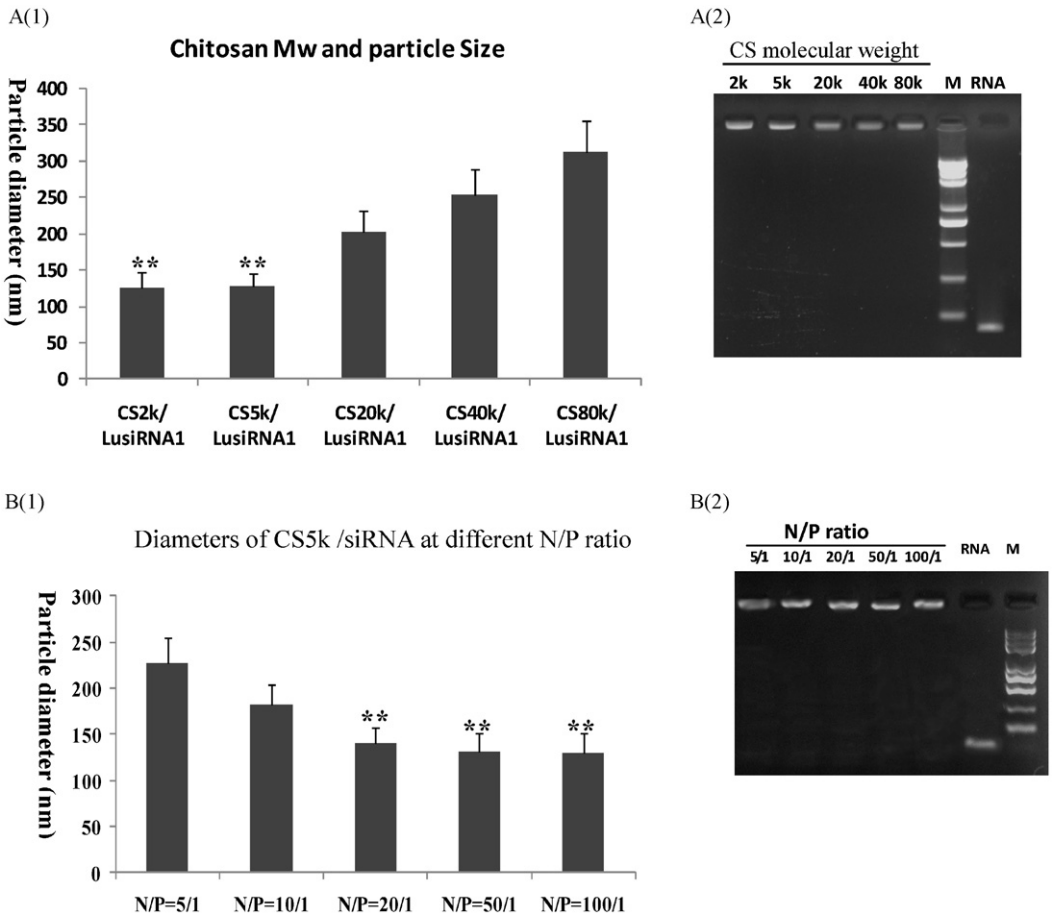


Fig. 2. Influence of the chitsosan molecular weight and N/P ratio on the particle diameter of CS/siRNA complexes. (A) CS/siRNA complexes formed at charge (N/P) ratio of 50/1 from different Mw chitosan: (1) Low Mw chitosan (Mw = 2k or 5k) formed CS/siRNA complexes had significantly smaller particle size. (2) Electrophoresis retardation confirms the formation of the complexes. (B) Effect of N/P ratio on the particle size of CS_{Mw5k}/siRNA: (1): CS/siRNA complexes were formed at different charge (N/P) ratio of 5/1, 10/1, 20/1, 50/1 and 100/1 respectively, from Mw 5000 chitosan. Although higher N/P ratio tend to form CS/siRNA complexes with more condensed particle size, when N/P beyond 20/1 there is no significant difference any more. (2) Complete retardation occurs at all charge ratios, indicating the formation of CS/siRNA complex. **Statistically significant difference with unmarked groups, $p < 0.01$.

Diluted different amounts of siRNA (from 30 to 300 ng) and appropriate amounts of Lipofectamine 2000 in 50 μl DMEM separately, combine the solutions, mix gently and incubate for 20 min at room temperature. Add 100 μl of Lipo/siRNA complexes to each well and incubate at 37 °C under 5% CO_2 atmosphere, replace the medium with DMEM containing 10% FBS after 4 h of incubation. Meanwhile, Luciferase gene null ($Luc^{-/-}$) cell, untransfected 231/Luc cell and non-targeting siRNA transfected 231/Luc cells were established as controls. Cells were lysised in RIPA buffer at 48 h after transfection, luciferase activity in the cell lysates was detected by a chemical illumination kit (Promega, USA). Total protein concentration of each cell lysate was also determined by a BCA protein assay kit from PIERCE (PIERCE, USA). Luciferase activity was normalized to the protein concentration and represented as "RLUs/mg protein". Cell lysate was also subjected to western blot assay to determine the quantity of luciferase molecule.

2.3. Formation of chitosan/siRNA nanoparticles

Chitosan of different molecular weight (Mw 2k, 5, 20, 40 and 80 kDa) was dissolved in sodium acetate buffer (0.2 M NaAc, pH 4.5) to obtain a 1.0 mg/ml working solution. Twenty microliters of LusiRNA1 (140 μM) was added to 1 ml of filtered chitosan working solution as above while stirring, incubate at room temperature for 1 h. The complex formation was confirmed by electrophoresis on a 2.2% agarose gel with Tris–acetate (TAE) running buffer at 100 V for 45 min. Double strands siRNA was visualized with ethidium bromide. Chitosan/siRNA (CS/siRNA) nanoparticles were used directly for subsequent cell transfection assay and physicochemical characterization.

To calculate charge (N/P) ratio (defined as the molar ratio of chitosan amino groups/RNA phosphate groups), a mass per phosphate (P) of 325 Da for RNA and mass per amino group (N) of 167.88 Da for chitosan (85% deacetylation) was used. Following the above formulation, the N/P ratio of the CS/siRNA complex was 50.7/1, and LusiRNA1 concentration in the solution was 36.5 μg/ml. Particle diameters of the CS/siRNA complexes were measured by dynamic light scatting (DLS). DLS measurement was carried out with an argon ion laser system tuned at 514 nm. The CS/siRNA solutions were filtered through a 0.5 μm filter (Millipore) directly into a freshly cleaned 10 mm-diameter cylindrical cell. The intensity of autocorrelation was measured at a scattering angle (θ) of 90° with a Brookhaven BI-9000AT digital autocorrelator at room temperature. When the difference between the measured and the calculated baselines was less than 0.1%, the correlation function was accepted. The mean diameter was evaluated by the Stokes–Einstein relationship. To further study the influence of N/P ratio on the diameter of CS/siRNA complexes, particle sizes of a series of CS5k/LusiRNA1 complexes formed at different N/P ratios were measured with the same method.

2.4. Cellular uptake and target gene silencing of the CS/siRNA complexes made of different Mw chitosan

Firstly, LusiRNA1 was ^{3}H labeled by thymidine incorporation during the process of synthesis, named as LusiRNA1^{3H}. At the N/P ratio of 50/1, LusiRNA1^{3H} was compounded with chitosan of different molecular weights to form a set of complexes: CS2k/LusiRNA1^{3H}, CS5k/LusiRNA1^{3H}, CS20k/LusiRNA1^{3H}, CS40k/LusiRNA1^{3H} and CS80k/LusiRNA1^{3H} respectively. Then, MDA-MB-231/Luc cells were seeded at a density of 5×10^5 ml^{-1} on 24-well microplates, in DMEM containing 10% FBS. When the cells were grown to half confluence, the culture medium was extracted and the cells were rinsed three times with serum-free DMEM. Proper amounts of various CS/siRNA complexes all containing 300 ng LusiRNA1^{3H} were diluted with 1 ml serum-free DMEM and added into corresponding wells. Lipo/LusiRNA1^{3H}, naked LusiRNA1^{3H} transfection were established as controls. Sextuplicate wells were set for each kind of transfection, three wells were assigned to Luciferase activity evaluation, the others were used for intracellular radioactivity counting. After 4 h of incubation, cells were thoroughly rinsed and the culture medium was replaced by fresh FBS-containing DMEM. As regards of radioactivity counting, at 4 h after transfection three wells of cells in each transfection were lysised in 1 ml PBS solution (containing 1% NP40 and 1 mmol/L PMSF) by being subjected to three cycles of deep freezing and thawing. Cell lysate was added into 3 ml scintillation solution for liquid scintillation counting. For luciferase activity assay, at 48 h after transfection the cells were lysised in RIPA buffer and luciferase activity in the lysate was detected as previously described. Either lucifersase activity or radioactivity counting results were normalized to the total protein concentration of each sample, and represented as "RLU/mg protein" and "CPM/mg protein" individually.

2.5. pSP conjugation of the chitsosan and the features of pSP–CS/siRNA complex

In order to enhance intracellular siRNA unpacking from the chitsosan, a phosphorylatable short peptide conjugated chitsosan (pSP–CS) was tested. For conveniently investigating the disassociation of siRNA and pSP–CS a modified fluorescence resonance energy transfer (FRET) technique was adopted with FAM and Dabcyl as a pair of molecular beacon. As shown in Table 1, two kinds of Dabcyl labeled peptides were synthesized. The unique difference between them is whether containing the phosphorylatable tyrosine residues. All of these peptides were conjugated to chitosan to form dlpSP–CS (dabcyl labeled phosphorylatable short peptide conjugated chitosan) and dlnpSP–CS (dabcyl labeled non-phosphorylatable short peptide conjugated chitosan) respectively. Briefly, dissolve 0.5 g chitosan (Mw 5000) in 50 ml TEMED/HCl solution (pH 5.0). Dabcyl labeled short peptides were dissolved in 0.9% NaCl solution to the final concentration of 6.5% (W/V). Peptide and chitosan solutions of equal volume were mixed together to form the conjugation solution. 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 whole solution was dialyzed against PBS for 48 h in a dialysis tube with the exclusion molecular weight of 20 kDa to remove EDC reagent, free peptide, free chitosan and low efficiently conjugated chitosan. The sizes of the dlpSP–CS and dlnpSP–CS particles were further normalized by being centrifuged in an ultrafiltration tube (Millipore Corp, USA) with cut-off molecular mass of 50 kDa. The spin down solution was freeze-dried to obtain the SP–CS powder.

To evaluate whether dlpSP or dlnpSP modification bring significant characteristic alteration to the CS, radio-labeled LusiRNA1^{3H} was compounded with dlpSP–CS, dlnpSP–CS and regular CS to form dlpSP–CS/LusiRNA1^{3H}, dlnpSP–CS/LusiRNA1^{3H} and CS5k/LusiRNA1^{3H} respectively. All these complexes were made from Mw 5000 chitosan and at the N/P ratio of 50/1. Particle diameters of the complexes were measured, electrophoresis retardation assay was performed on a 2.2% agarose gel to confirm the formation of the complexes. Radioactivity and luciferase activity in the cell lysate were also detected following the transfection against MDA-MB-231/Luc cells in sextuplicate wells, 300 ng siRNA was used in each transfection with lipofactamine 2000 as control.

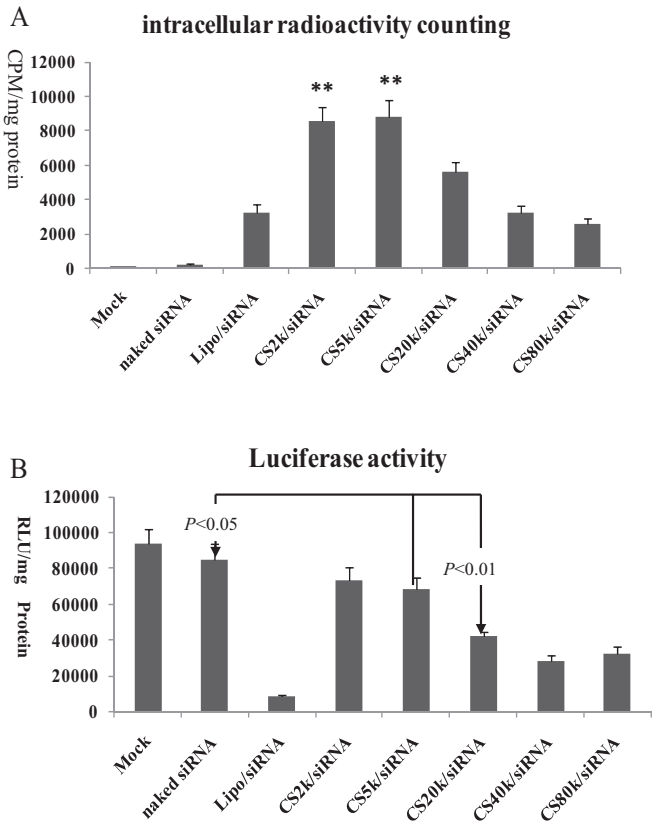


Fig. 3. cellular uptake and target gene silencing of the CS/LusiRNA1^{3H}. CS/siRNA complexes made of different molecular weight chitosan were all at charge (N/P) ratio 50/1. (A) CS/siRNA made of Mw 2k and Mw 5k chitosan showed the highest ability of delivering radioactive siRNA into the host cell. (B) Lipo/siRNA could greatly decrease luciferase activity in the cell lysate. CS5k/siRNA and CS2k/siRNA could only slightly decrease luciferase activity and there is no significant difference between each other. ** Statistically significant difference with unmarked groups, $p < 0.01$. siRNA stand for LusiRNA1^{3H} in this figure.

2.6. In vitro unpacking assay of dlpSP-CS and siRNA

FAM labeled LusiRNA1 (FsiRNA1) was commercially synthesized and compounded with dlpSP–CS or dlnpSP–CS at N/P ratio of 50/1. For *in vitro* disassociation assay, dlpSP-CS/FsiRNA1 and dlnpSP-CS/FsiRNA1 complexes were mixed with an aliquot of MDA-MB-231 cell lysate, proper amounts of phosphorylation assay buffer and ATP, incubated in a fluorescent real-time PCR machine with periodic fluorescence detection. The incubation temperature was set at 37 °C, the fluorescence in each reaction tubes was monitored every 30 s with excitation/emission wavelength of 494/522 nm. Triplet tubes were established for each kind of reaction and the fluorescence baseline was set according to the mean fluorescence in the no cell lysate control tubes, incubated at room temperature for 3min. Fluorescence from the free FsiRNA1, as the same amount as in the complex, was also detected as the positive

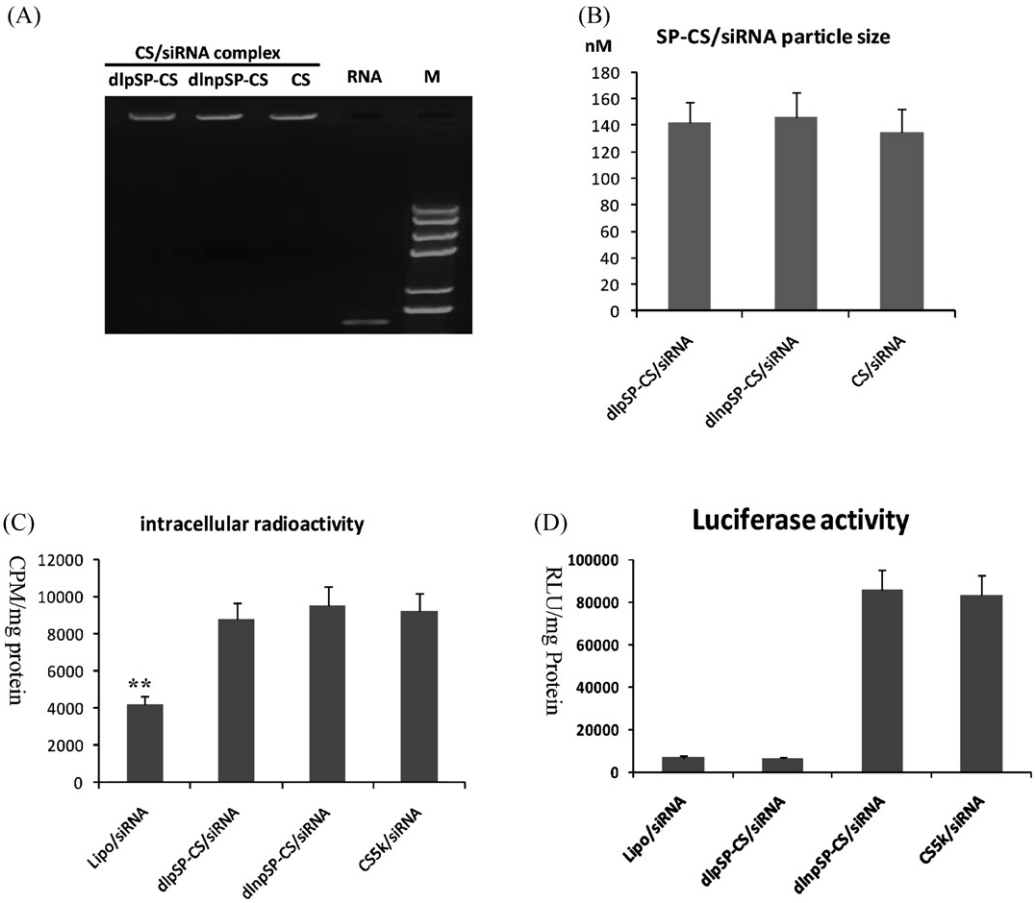


Fig. 4. Comparison of pSP-CS/siRNA with CS/siRNA. All kinds of CS/siRNA complexes were made of molecular weight 5000 chitosan and radio-labeled LusiRNA1^{3H} at the charge (N/P) ratio of 50/1. (A) Complete electrophoresis retardation indicated the formation of CS/siRNA complexes. (B) All the CS/siRNA complexes had same particle diameters. (C) All kinds of CS/siRNA complexes yield same intracellular radioactivity, which were significantly higher than Lipo/siRNA. (D) Only dlpSP-CS/siRNA, but not other CS/siRNA forms, could markedly decrease luciferase activity to the level of around Lipo/siRNA.

control. Disassociation of the FsiRNA1 in response to multi-typed cell lysate treatment were also carried out.

2.7. Intracellular disassociation and target gene silencing of the dlpSP-CS/FsiRNA1

In vitro cultured MDA-MB-231/Luc cells were transfected by dlpSP-CS/FsiRNA1, dlnpSP-CS/FsiRNA1 and Lipo/FsiRNA1 respectively, sextuplicate wells were established for each kind of transfection. Intracellular disassociation of the complexes was represented by intracellular fluorescence, which was monitored every 4 h after transfection. 16 h after transfection, three wells of cells of each group were rinsed three times with PBS and then stained with DAPI-methanol solution. FAM and DAPI fluorescence were investigated successively under fluorescence microscope. Images of the same fields were taken and merged by photoshop program. After fluorescence investigation the cells were harvested and resuspended in PBS (pH7.4), then FAM fluorescence positive cells were quantified by flow cytometry. At 48 h after transfection, the rest of the cells were lysised and the firefly luciferase gene silencing effect was represented by decreased luciferase activity in the cell lysate.

3. Results and discussion

3.1. Selection of siRNA for effective target gene silencing

Small interfering RNA (siRNA) has been recognized as a new therapeutic tool for gene expression-implicated disease (Elbashir et al., 2001). Generally, siRNA is a class of 20–25 nucleotide-long double-stranded RNA molecules that are complimentary to given mRNAs and sequences specifically destroy that particular mRNA, thereby diminishing or abolishing target gene expression. However, recent studies discovered that the combination of a DNA analog with a rigid RNA analog in siRNA duplexes or incorporation of DNA-like nucleotides can knockdown several genes with much higher potency than native RNA duplexes. Based on previous reports (Glen et al., 2010), two kinds of siRNA, all targeting firefly luciferase gene, were primarily evaluated in this paper. As shown in Fig. 1B, both LusiRNA1 and LusiRNA2 could specifically suppress luciferase activity in the MDA-MB-231/Luc cell lysate in a dose dependent manner. The DNA–RNA duplexes form of LusiRNA1 was more effective than the RNA–RNA form of LusiRNA2. Thus, LusiRNA1 was selected in the following study. To confirm that the significantly decreased luciferase activity is due to LusiRNA induced mRNA degradation and subsequent protein synthesis obstruction, but not direct interference to the activity of luciferase molecule. The western blot result in Fig. 1C indicated that either LusiRNA1 or LusiRNA2 decreased luciferase protein content in the cell lysate.

3.2. Effect of chitosan molecular weight and N/P ratio on the particle size of CS/siRNA complex

What was shown in Fig. 2A indicated that at the constant N/P ratio of 50/1, chitosan of different molecular weight compound siRNA into the nanoparticles of different diameters, varying from 130 nm to 330 nm, and low molecular weight chitosan (LMWC) of

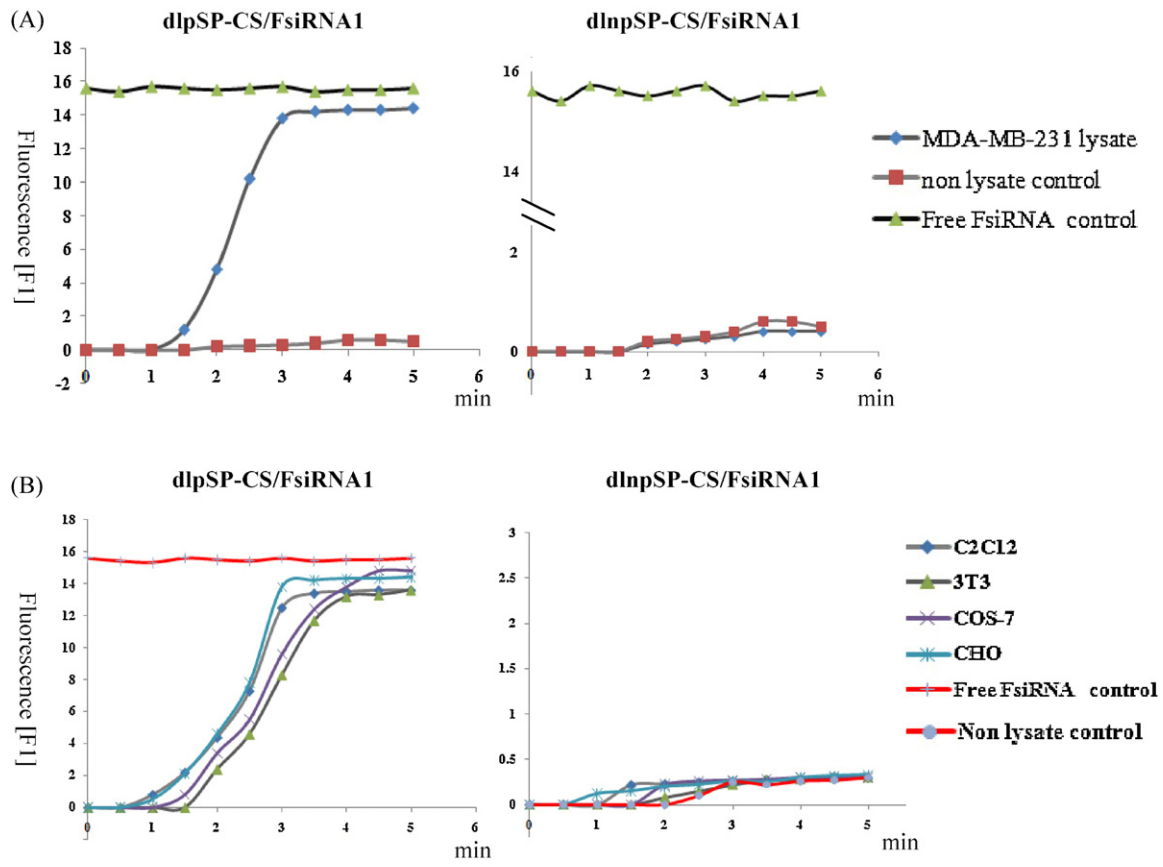


Fig. 5. *In vitro* disassociation of the SP-CS/FsiRNA1 complexes. The complexes were made of Dabcyl labeled short peptide conjugated chitsosan and FAM labeled siRNA at the charge (N/P) ratio of 50/1. (A) MDA-MB-231 cell lysate induced SP–CS/FsiRNA1 disassociation: In response to MDA-MB-231 cell lysate treatment, FAM labeled siRNA could be effectively disassociated from dlpSP-CS/FsiRNA1 complex, but not dlnpSP-CS/FsiRNA1, and hence generate detectable fluorescence. (B) Effect of cell lysate from different cell types: all we tested cell lyasate could lead to efficient disassociation of siRNA from the dlpSP–CS, but not dlnpSP–CS.

Mw 2000 and Mw 5000 tend to form CS/siRNA complexes with the smaller particle size. The reason for primarily selecting an N/P ratio of 50/1 to form the CS/siRNA complexes is based on previously reported studies verified that an N/P ratio of chitosan:siRNA around 50/1 could always yield optimal particle size and better target gene suppression effects (Kenneth et al., 2008; Liu et al., 2008; Wu et al., 2010). Our result in Fig. 2B also confirmed that although higher N/P ratios tend to form CS/siRNA complexes with smaller particle sizes, when N/P is beyond 20/1, there is no significant difference of the particle diameters.

3.3. Cell uptaking and target gene silencing of the CS/siRNA complexes made of different molecular weight chitosan

As shown in Fig. 3A and B, the results of radioactivity counting and luciferase activity exhibited an apparent contradiction. As far as the siRNA transporting ability which was represented by the radioactivity in the cell lysate was concerned, low molecular weight chitosan (Mw 2000 or Mw 5000) showed their superior ability, even surprisingly much higher than lipofectamine 2000. But as to the capacity of diminishing luciferase activity, lipofectamine 2000 loaded siRNA is the most effective one. Compared with low molecular weight chitosan (LMWC), medium molecular weight chitosan (from Mw 20,000 to Mw 80,000) loaded siRNA showed lower cell permeability but higher target gene silencing effect. It is apparent that although LMWC has perfect siRNA transporting ability, the introduced siRNA is poorly functional to induce target mRNA degradation. Considering that only free siRNA is the functional form and LMWC always compound siRNA compactly, it is reasonable to surmise that the strong electrostatic interaction between LMWC and siRNA truly facilitates cell uptaking of the LMWC/siRNA complex, but on the opposite, also intensively hindered the intracellular unpacking of siRNA from its LMWC carrier to become the freely functional form. And unfavorably obstructed target gene silencing as a final result. Hence, stimulating siRNA disassociation from the LMWC carried after being efficiently delivered into the host cell might be beneficial for enhancing target gene knockdown. To conduct this study Mw 5000 chitosan was used as an example in the further study. As for LMWC/siRNA complexes made of CS_{Mw2000} and CS_{Mw5000}, they shared indistinctive characteristics of particle size, siRNA transporting ability and target gene silencing effect. The reason of selecting CS_{Mw5000} as the example is due to our hypothesis that lower molecular weight chitsosan tend to compound siRNA more tightly and hence leading to insufficient intracellular unpacking and poor target gene knockdown. To be compared with CS_{Mw2000}, the relatively larger molecular weight of CS_{Mw5000} may has the looser interaction with the siRNA and the complex might be more easily to be disassociated. And although there is no significant difference CS5k/siRNA led to lower luciferase activity than CS2k/siRNA in Fig. 3B.

3.4. Features of pSP-CS formed siRNA complex

For enhancing siRNA unpacking from the LMWC, a pSP–CS (phosphorylatable short peptide modified chitosan) was investigated. In our previous work on optimizing chitosan mediated pDNA transfection (Sun et al., 2010), by the means of pSP conjugation the transfection efficiency of chitosan was improved successfully.

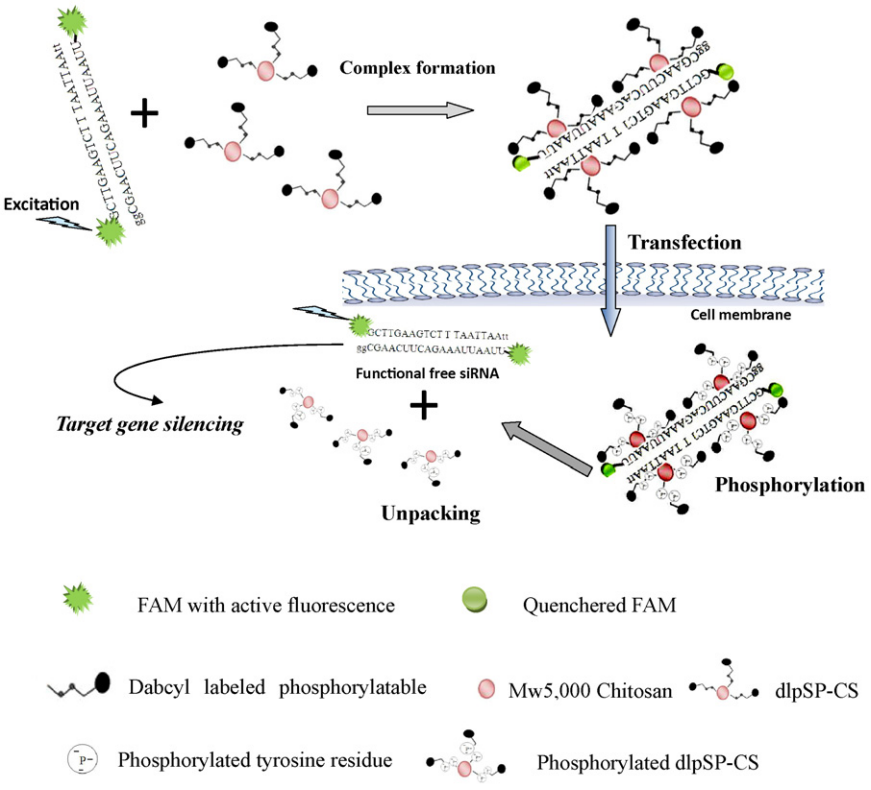


Figure6, Schematic presentation of intracellular dlpSP-CS phosphorylation determined siRNA unpacking and fluorescence generation

Fig. 6. Schematic presentation of intracellular dlpSP-CS phosphorylation determined siRNA unpacking and fluorescence generation. In the *in vitro* formed dlSP-CS/siRNA complexes at the charge (N/P) ratio of 50/1, Dabcyl was tightly connected to FAM and quenched FAM's fluorescence. Only after being delivered into the cell the phosphorylatable short peptide contained in the SP-CS could be phosphorylated by cytoplasmic Jak2 kinase, and hence repelled siRNA from the chitosan complex to be the functional free form.

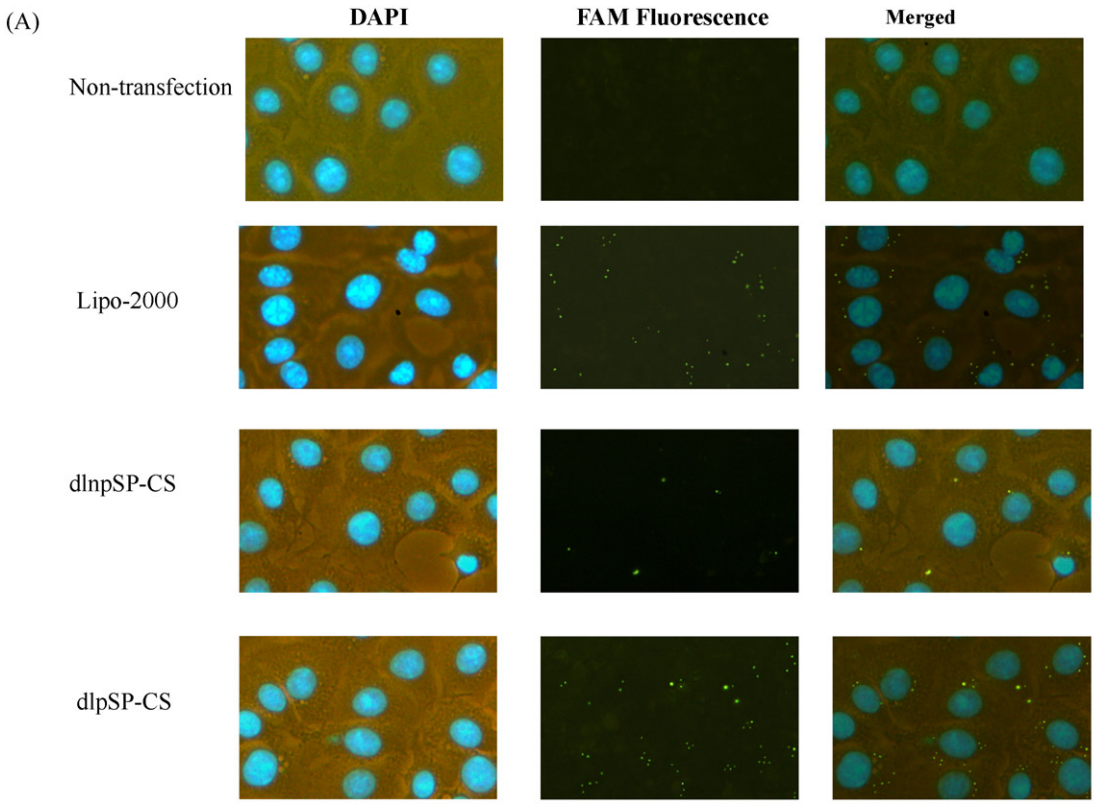
Intracellular phosphorylation of the short peptide, being coupled to chitsosan, intensively alternated electrostatic interaction between pSP–CS and pDNA. Being introduced negatively charged phosphate group caused electric repulsion between pDNA and pSP–CS and resulted in pDNA disassociation. This mechanism refers to the process of chromatin activation prior to the transcription of a given gene. Phosphorylated histone released DNA from the nucleic body and made it available for being transcribed. Since chitosan compounds siRNA also *via* the electrostatic interaction as the same way as pDNA, it might be also an effective way to facilitate siRNA unpacking from the LMWC by the means of pSP conjugation.

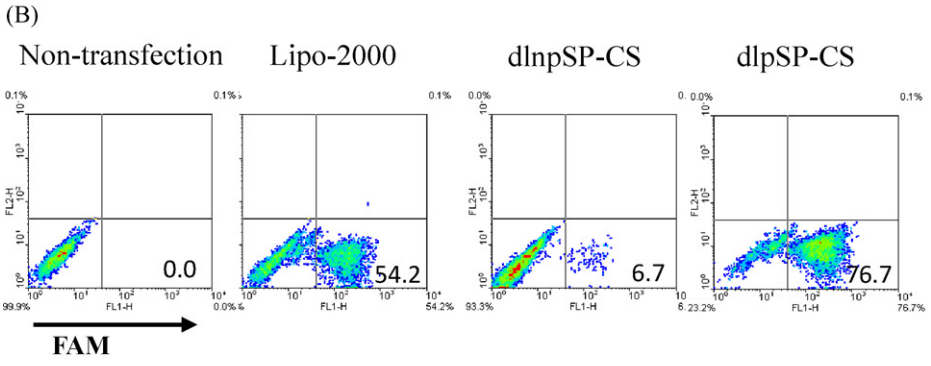
To conveniently estimate whether pSP–CS conjugation could really enhance siRNA unpacking, a carefully designed experiment was carried out referring to the technique of fluorescence resonance energy transfer (FRET). FAM, as the fluorescence reporter, was labeled to LusiRNA1 to form FsiRNA1. Dabcyl, as the fluorescence quencher, was conjugated to the amino terminal of the short peptides. As shown in Table 1, two kinds of Dabcyl labeled short peptides were commercially synthesized: Dabcyl labeled phosphorylateble SP (dlpSP) and Dabcyl labeled non-phosphorylateble SP (dlnpSP). The core motif in the pSP, with the amino acid composition of NEY*FY*V, is the substrate of Jak2 protein kinase. Jack2, as a cytoplasmic kinase, was approved of being constitutively expressed in multiple mammalian tissues (Wallace et al., 2004; Sandberg et al., 2004). Thus the peptide could be efficiently phosphorylated in most mammalian cells. Some basic amino acid residues of arginine and lysine were flanked to this core substrate to make the short peptide to be basic as well as to maintain the N/P ratio of SP-CS:siRNA to be around 50/1. The mass per amino group (N) is 167.88 Da for the chitosan and 196 Da for the short peptide which will not cause significant N/P ration altertion when SP–CS/siRNA was prepared following the same formulation of CS/siRNA preparation.

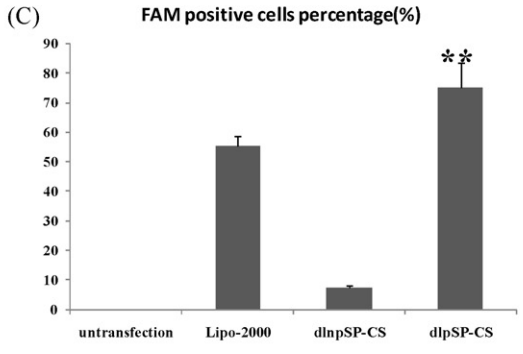
Fig. 4A–D showed convincingly data that compared with regular Mw 5000 chitosan, dlpSP or dlnpSP modification did not bring any significant effect to the particle size and siRNA transporting ability of the CS/siRNA complexes. Only dlpSP–CS, but not dlnpSP–CS, loaded siRNA lead to effective target silencing to the level of equal to lipofactamine 2000. Thus it has great possibility that the effective target gene silencing was resulted from the enhanced intracellular siRNA unpacking caused by the phosphorylation of the short peptide. The mechanism was discussed in detail in the following sections.

3.5. *In vitro* disassociation of siRNA from thecomplex

FRET relies on the sensitive distance-dependent transfer of energy from a donor molecule to an acceptor molecule. In the SP-CS/siRNA complex, whether Dabcyl could completely abolish the fluorescence emission from the FAM is sensitive to their inter-distance. When Dabcyl is tightly connected with FAM in the complex, *via* the strong electrostatic interaction between dlpSP and FsiRNA, no fluorescence could be generated. Once FsiRNA1







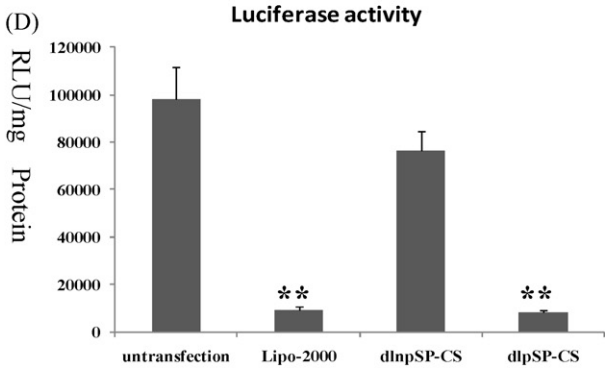


Fig. 7. Intracellular disassociation and target gene silencing of the SP-CS/FsiRNA1 complexes. All kinds of CS/siRNA complexes were made at the charge (N/P) ratio of 50/1. (A) In dlSP-CS/FsiRNA transfected cells intracellular fluorescence reflected the amount of disassociated siRNA, the fluorescence intensive was markedly augmented in dlpSP–CS transfection than dlnpSP–CS transfection. (B and C) Determined by flow cytometry, the highest rate of FAM fluorescence positive cells was observed in dlpSP-CS transfection. (D) Compared with dlnpSP–CS, dlpSP–CS/siRNA transfection effectively decreased luciferase activity.

unpacking from the complex and being separated from the Dabcyl, FAM group could generate green fluorescence under proper excitation. Fig. 5A and B demonstrated that in response to various cell lysate treatment significantly elevated fluoresce could only be detected in dlpSP–CS/FsiRNA1 complex but not the dlnpSP–CS/FsiRNA1. The results support the conclusion that it is the phosphorylation of the dlpSP by cytoplasmic kinase repelled siRNA from the dlpSP–CS carrier.

3.6. *In vivo disassociation of the siRNA and target gene silencing*

The mechanism of siRNA unpacking determined fluorescence generation was schematically illustrated in Fig. 6. After being delivered into the cell, the pSP containing in pSP-CS was phosphorylated by cytoplasmic Jak2 kinase and thereafter repelled siRNA from the complex to be the freely functional form. As shown in Fig. 7A, the intracellular fluorescence represented the amount of free FsiRNA1 being unpacked from the SP–CS carrier. Compared with dlnpSP–CS, dlpSP–CS extremely increased intracellular fluorescence to the level of being even superior to lipofactamine 2000. Flow cytometry results in Fig. 7B and C applied us quantitative numerus to evaluate the intracellular fluorescence. They all confirmed that dlpSP–CS loaded FsiRNA could generate much more fluorescence positive cells than dlpSP-CS, and even superior to lipofactamine 2000. Fig. 6D indicated that dlpSP–CS/FsiRNA, but not dlnpSP–CS/FsiRNA, transfection significantly decreased luciferase activity in MDA-MB-231/Luc human breast cancer cells. All theses datas supported the conclusion that enhance intracellular siRNA unpacking from the chitsosan carrier by the means of phosphorylatable short peptide conjugation could virtually benefit target gene silencing.

4. Conclusion

In this paper we reported that low molecular weight chitosan (LMWC) loaded siRNA had superior cell entrance ability but poor target gene silencing effect which may due to the strong electrostatic interaction between positively charged LMWC and anionic siRNA intensively hindered siRNA unpacking from the CS/siRNA complex to be the free functional form. By the utilization of phosphorylatable short peptide conjugated chitsosan (pSP-CS) to promote intracellular siRNA disassociation significantly augmented target gene silencing effect of the CS/siRNA. It indicated that low molecular weight chitosan has the great potency to be an ideal siRNA vehicle if the issue of siRNA unpacking could be properly resolved.

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