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Pre-deliver chitosanase to cells: A novel strategy to improve gene expression by endocellular degradation-induced vector unpacking

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Abstract

A radio-labeled plasmid pTracer/Bsd/LacZ containing LacZ reporter gene was complexed with different molecular weights of chitosans (CS). Mouse myoblast cell line C2C12 was transfected by these chitosan–plasmid DNA complexes, and lipofectamine 2000 was used as control. Forty-eight hours after transfection, the activity of β-galactosidase and radioactive count of cell lysis were determined. It was found that chitosan, especially low molecular weight species, had a surprising ability to deliver DNA into cells, since the radioactive count of cells transfected by chitosan–DNA complexes was even two times that of cells transfected by lipofectamine 2000. But the β-galactosidase activity of chitosan/DNA complexes was much lower compared to that of lipofectamine 2000. Chitosanase which could degrade chitosan in specific mode was transported into C2C12 cells by osmotic lysis prior to gene delivery. Then these chitosanase-modified cells were transfected by CS–DNA complexes. The results indicated that β-galactosidase activity in these cells increased markedly to 425.4 ± 45.1 U/mg protein, nearly two-fold as that of cells transfected by liposome. This transfection protocol was also applied to 3T3 mouse fibroblast, 2T3 mouse osteoblast and MG63 human osteosarcoma cell lines, and an increased gene expression level was observed without exception. It is thought that the incorporated chitosanase could aid in chitosan degradation, which would promote gene unpacking, consequently increasing gene expression.

Keywords: Chitosan; Non-viral vector; Gene transfection; Unpacking

1. Introduction

Chitosan is a natural biodegradable mucoadhesive polysaccharide derived from crustacean shells, and a biocompatible polymer that has been widely used in controlled drug delivery (Ko et al., 2002; Lee et al., 2002; Shimono et al., 2002; Vila et al., 2002). Chitosan can effectively bind to negative surfaces because of its positive charge, so it may also provide a less immunogenic and non-toxic carrier for successful DNA delivery. At acidic pH, the amine groups of chitosan become positively charged, electrostatically attractive to DNA and condense it to nano/microparticles (Lee et al., 1998; Ishii et al., 2001). The size and shape of particles can be changed depending on the conditions of the formulation process (Mao et al., 2001). Chitosan microparticles containing reporter gene are being used for the transfection of mammalian cells both in vitro and in vivo conditions (Corsi et al., 2003; Iqbal et al., 2003; Dastan and Turan, 2004). However, the transfection efficiency of chitosan–DNA microparticles reported to date is quite low. And a variety of strategies are being employed, such as coupling deoxycholic acid, galactose, poly(vinyl pyrrolidone), urocanic acid and alkyl chainsto chitosan (Kim et al., 2001, 2004, 2003; Park et al., 2003; Liu et al., 2003). Although there were several reports supporting that the modification of chitosan could really increase the transfection efficiency of chitosan–DNA microparticles and there were even in vivo studies on using chitosan as gene delivery vector for gene therapy (Chen et al., 2004; Mansouri et al., 2004), the transfection efficiency of chitosan–DNA complexes was relatively lower compared with that of liposome (Aral and Akbuga, 2003; Sato et al., 2001; Romoren et al., 2003).

There are two key steps that could influence the transfection efficiency of chitosan–DNA complexes, one is cell membrane transport of the complexes, and the other is the unpacking of DNA from its carriers. To our best knowledge, up to now,

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much work has been focused on improving transmembrane ability of chitosan via conjugation of ligands. However, few of researchers have paid attention to the unpacking issue associated with chitosan-mediated gene transfection. In this work, we adopted for the first time a novel strategy to increase the level of chitosan-mediated gene expression by pre-delivering chitosanase to cells prior to transfection. It is expected that the incorporated chitosanase could accelerate chitosan degradation, which would facilitate gene unpacking, consequently improving gene expression.

2. Materials and methods

2.1. Materials

Chitosan (CS) was purchased from Haihui Bioengineering Co. (Qingdao, China) in MW = 2000, 5000 and 50 kDa (degrees of deacetylation approximate to 80%). Liposome transfection reagent Iipofectamine 2000 and Dulbecco's Eagle Modified Medium (DMEM) cell culture medium powder were purchased from Invitrogen Co. (USA). Plasmid pTracer-CMV/Bsd has two different promoters, one promoter controls the expression of Green Fluorescence Protein (GFP) reporter gene, the other locates at the upstream of multi-clone site (MCS) and regulates the expression of inserted gene. Plasmid pTracer-CMV/Bsd/LacZ is the control plasmid of pTracer-CMV/Bsd, which has a LacZ gene inserted into the MCS of plasmid pTracer-CMV/Bsd. So this plasmid has two reporter genes controlled by two different promoters, and the expression of LacZ reporter gene could be estimated quantitatively by detecting the activity of β-galactosidase. These two plasmids were also purchased from Invitrogen Co. Chitosanase powder, aminoglucose (NAG) and 4-dimethylaminoazobenzene-4′-sulphonyl chloride (DABS-C1) were purchased from Sigma (USA). β-Galactosidase activity assay kit and plasmid purification kit were purchased from Promega Co. (USA). ^{3}HTdR (^{3}H thymine deoxyriboside) was purchased from Institute of Atomic Energy, China.

2.2. Radioactive label of plasmid DNA

E. coli JM109 containing plasmid pTracer-CMV/Bsd/LacZ was cultured in 3 mL LB medium. Sixteen hours later, the medium was transferred into another 100 mL LB medium and further cultured until the absorbance at 600 nm, A_{600}, is equal to 0.6. Then ^{3}HTdR of 600 μCi was added into the medium. The culturing was stopped after 3 h, and the plasmid DNA was extracted, of which 2 μg of DNA was added into 3 mL scintillation liquid and liquid scintillation counting was performed. The radioactivity counting of this 2 μg labeled plasmid pTracer/Bsd/LacZ was 16,436.00 cpm, confirming the incorporation of ^{3}HTdR into the plasmid.

2.3. Formation of CS/DNA polyelectrolyte complex

Chitosans of different molecular weight were separately dissolved in 0.1 M sodium acetate buffer to form a solution of 1 mg/mL, and a plasmid DNA solution of 0.1 mg/mL dissolved in TE was also formed. Chitosan/DNA complexes at various molar ratios were prepared by mixing chitosan solution with DNA solution, vortexing for 15 s and incubated at room temperature for 30 min. The complex formation was confirmed by electrophoresis on a 1.0% agarose gel with Tris–acetate (TAE) running buffer at 100 V for 30 min. DNA was visualized with ethidium bromide.

2.4. Particle size measurement of CS/DNA complexes

Particle size of chitosan/DNA complexes was measured by dynamic light scatting (DLS). DLS measurement was carried out with an argon ion laser system tuned at 514 nm. The complex 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.

2.5. DNA-transforming ability and the expression of LacZ reporter gene of CS/DNA complexes

C2C12 mouse myoblast cells were seeded at a density of 5×10^{5} mL^{-1} on 24-well microplates in DMEM containing 10% fetal bovine serum (FBS). Cells were cultured at 37 °C under 5% CO_2 atmosphere. When the cells were grown to half confluence, the culture media were extracted and the cells were rinsed three times with serum-free DMEM. The complexes between DNA and chitosans of different molecular weights were diluted with 1 mL serum-free DMEM and added into corresponding wells (2 μg of radioactively labeled plasmid DNA/well). After 4 h incubation, the complexes were removed and the culture media were replaced by fresh FBS-containing DMEM media and incubated for another 48 h. Each kind of CS/DNA complexes transfected eight wells of cells, of which four wells were used for the detection of β-galactosidase activity and the others for radioactivity counting. β-Galactosidase activity was detected by the kit (Promega, E-2000). As regards radioactivity counting, cells were lysed in 1 mL PBS solution containing 1% NP40 and 1 mmol/L PMSF by subjecting to three cycles of deep freezing and thawing. Cell lysate was added into 3 mL scintillation liquid for liquid scintillation counting. At same time, the scintillation counting of 2 μg ^{3}H labeled plasmid was detected directly to confirm the radio-labeling of plasmid; by comparing the scintillation counting of cell lysate in the above cases with that of ^{3}H labeled plasmid, the percentage of DNA internalized into the cells could be calculated. The total protein concentration of cell lysate was also detected by BCA method. The naked DNA and lipofectamine 2000 were used as control.

2.6. Degradation activity of chitosanase (Csn)

Chitosanase was dissolved in PBS to form a solution of 20.0 μmol/L. Fifty microliters chitosanase solution was added into 1 mL 0.5% chitosan solution (acetic buffer, pH 5.4), incubated for 15 min at 37 °C and then boiled for 5 min to stop the reaction. The amount of reduced sugar was detected by modified Schales method using aminoglucose of different concentrations as standard curve. Firstly, a Schales solution of 0.1% potassium ferricyanide ($K_3Fe(CN)_6$) in 0.5 mol/L Na_2CO_3 was prepared. The reaction solution and NAG of different concentrations were transferred into 1 mL Schales solution, boiled for 10 min, and the absorbance at 420 nm (A_{420}) was detected after the solution being cooled. Chitosan degradation ratio was calculated according to the equation below; lysozyme of equal molar quantities with the chitosanase was used as a control.

$$\text{CS degradation ratio (\%)} = \frac{\text{concentration of reducing sugar (mg/ml)} \times 161.16}{\text{primary concentration of chitosan (mg/ml)} \times 179.17} \times 100$$

2.7. In vitro determination of chitosan degradation-induced DNA release from CS–DNA complex

Different amounts of chitosanase (0.1, 0.5, 2.0, 10.0, 50.0 and 200.0 μg) or different amounts of lysozyme (1.0, 4.0, 16.0, 64.0, 256.0 ng and 1.0 μg) were separately introduced to 1.0 μg of CS/DNA complex made from plasmid and chitosan of molecular weight 5000. A total volume of 20.0 μL solution was incubated for 30 min at 37 °C. As a control, the same CS/DNA complex was also subjected to different volumes of cell lysate (0.1, 0.2, 0.5, 2.0, 5.0 and 10.0 μL). Herein, 5×10^5 cultured C2C12 cells were lysed in 100 μL lysis buffer (supplied in β-galactosidase enzyme assay system, purchased from Promega) to generate the cell lysate which had a protein concentration of 50 mg/mL determined by BCA (with BSA of different concentrations as standard). After chitosan degradation, the release of DNA from CS/DNA complex was determined by electrophoresis on a 1.0% agarose gel.

2.8. Chitosan-mediated gene transfection with pre-delivery of chitosanase into C2C12 cells

Osmotic lysis pinosomes, as developed by Okada and modified by Raja (Okada and Rechsteiner, 1982; Raja et al., 1989), was applied to transport chitosanase into C2C12 cells. Briefly, C2C12 cells were suspended and washed one time with DMED/5% FBS. After centrifugation, the cells were incubated at 37 °C in a hypertonic medium (50 μL/10^7 cells) containing 0.9 M sucrose, 0.5 mg/mL chitosanase and 10% PEG in DMEM (pH 6.4) buffered with 10 mM HEPES. After 6 min, the incubation medium was diluted 50-fold with buffered DMEM (pH 6.4) containing 10% FBS, and incubated for 2 min at 37 °C. The medium was removed by centrifugation, and the cells were washed three times with DMED/10% FBS and seeded on 24-well plate in this medium. Then these chitosanase-pre-delivered cells were transfected with naked plasmid DNA, lipofectamine 2000 and CS–DNA complexes from plasmid pTracer/Bsd/LacZ and chitosans of molecular weights 2000 and 5000. The normal C2C12 cells without chitosanase modification were also transfected in same manner as controls. With the identical transfection protocol, chitosan of molecular weight 5000 was applied to 3T3 mouse fibroblast cell line, 2T3 mouse osteoblast cell line and MG63 human osteosarcoma cell line.

2.9. Effect of the pre-delivered chitosanase on chitosan degradation inside C2C12 cells

Although the above experiments demonstrated that pre-delivered chitosanase was able to increase the expression of *LacZ* reporter gene, which was supposedly due to the increased chitosan degradation caused by chitosanase, no direct evidence confirmed that pre-delivered chitosanase increased chitosan degradation inside the cells. So HPLC was applied to detect the concentration of aminoglucose, a monosaccharide as the product of chitosan degradation (Livia et al., 1993). Briefly, glucose, aminoglucose and chitosan of molecular weight 5000 were separately dissolved in cell lysis buffer (TE buffer contains 1% NP40, pH 8.0) to a final concentration of 1 mg/mL each. Forty micrograms of 4-dimethylaminoazobenzene-4′-sulphonyl chloride in 40 μL of acetonitrile was added into 20 μL of each sample. Vials were sealed and incubated in a drying oven at 70 °C for 15 min. Ethanol–water (440 μL, 1:1, v/v) was added to the samples and 6–12% of the resulting solution was injected into the HPLC column (C_{18} Ultrasphere-DABS column, 250 mm × 4.6 mm). Ultraviolet–vis detector module was operated at 436 nm, and the solvent consists of 45% acetonitrile, 0.32% *N*,*N*-dimethylformamide and 3.2 mM sodium citrate with flow-rate of 1.4 mL/min. By osmotic lysis pinosomes, chitosanase was pre-delivered into C2C12 cells and transfected with CS–DNA complexes made of chitosan of molecular weight 5000. After washed three times with PBS, cells were lysed in 100 μL cell lysis buffer by three cycles of deep freezing and thawing. Then aminoglucose in the cell lysis was determined by HPLC in the same manner.

3. Results and discussion

If chitosan is complexed with DNA, the neutralization and/or increase in molecular size of the complex will result in the complete retardation of DNA migration toward the anode in the electric field. Ethidium bromide contained in the gel is bound to DNA while it moves toward the cathode in the electric field. Since the complex was detained in agarose wells, the fluorescence in the wells could be seen under ultraviolet wavelength. Fig. 1 shows the agarose gel electrophoresis results of CS–DNA complexes. One can see that a complete retardation of DNA migration occurs at molar ratio of 1/2 for CS of $M_v = 2000$ and 1/4 for CS of $M_v = 5000$ and 50,000, indicating the formation of complexes. In the following study, in the formation of CS/DNA

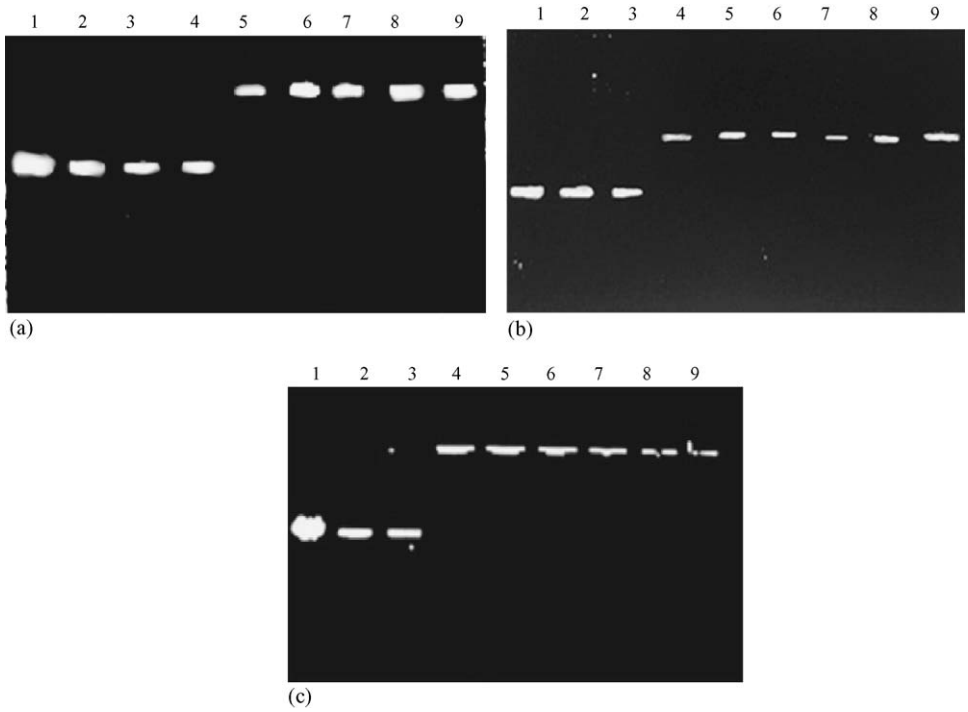

Fig. 1. Electrophoresis of CS/DNA complexes on an agarose gel. M_v of CS: (a) 2000; (b) 5000; (c) 50,000. Lane 1: plasmid DNA; lane 2: CS/DNA = 1/20; lane 3: 1/10; lane 4: 1/4; lane 5: 1/2; lane 6: 1/1; lane 7: 2/1; lane 8: 4/1; lane 9: 6/1.

Table 1
Mean diameter of CS/DNA complex particles (nm)

	Molecular weight of chitosan (M_v)		
	2000	5000	50,000
Mean diameter (nm)	110 ± 18	120 ± 22	105 ± 12

The values are the results of three measurements and denoted as mean ± S.D.

complexes, the molar ratios of CS/DNA were selected as 1/2 and 1/4 for chitosans of M_v = 2000, 5000 and 50,000, respectively. From Table 1, it is seen that the mean particle sizes of complexes from different molecular weights of chitosans range from 105 to 120 nm. Thus, it is reasonable to consider that this slight difference will not influence the transfection level to be discussed below.

The results of radioactivity counting and β-galactosidase activity exhibit a distinct difference in the ability of different chitosans to deliver DNA into C2C12 cell, and chitosan with relatively low molecular weight shows a strong ability to deliver DNA into C2C12 cell, even surprisingly higher than lipofectamine 2000, as shown in Fig. 2. Table 2 shows the percentage of DNA internalized into C2C12 cells. CS-5000 transported 12.7% DNA, much higher than lipofectamine 2000 with only 5.8% DNA internalization. It is evident that CS-5000 has the highest capability of transporting DNA into C2C12 cell.

Table 2
The percentage of DNA internalized into C2C12 cells

	^{3}H labeled plasmid	Cell plasmid lysate of different transfection methods			
		Naked plasmid	CS2k	CS5k	Lipofectamine
CPM	8580.28	184.48	756.84	1091.93	494.08
Internalization ratio (%)		2.2	8.8	12.7	5.8

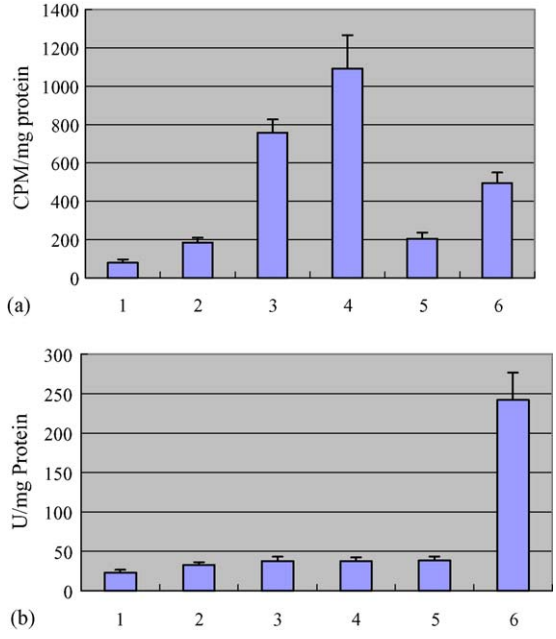

Fig. 2. Liquid scintillation counting (a) and β-galactosidase activity (b) of cell lysate. Each experiment was performed in triplicate. Lane 1: cell without transfection; lane 2: cell transfected with naked plasmid DNA; lane 3: CS-2000; lane 4: CS-5000; lane 5: CS-50,000; lane 6: lipofectamine 2000.

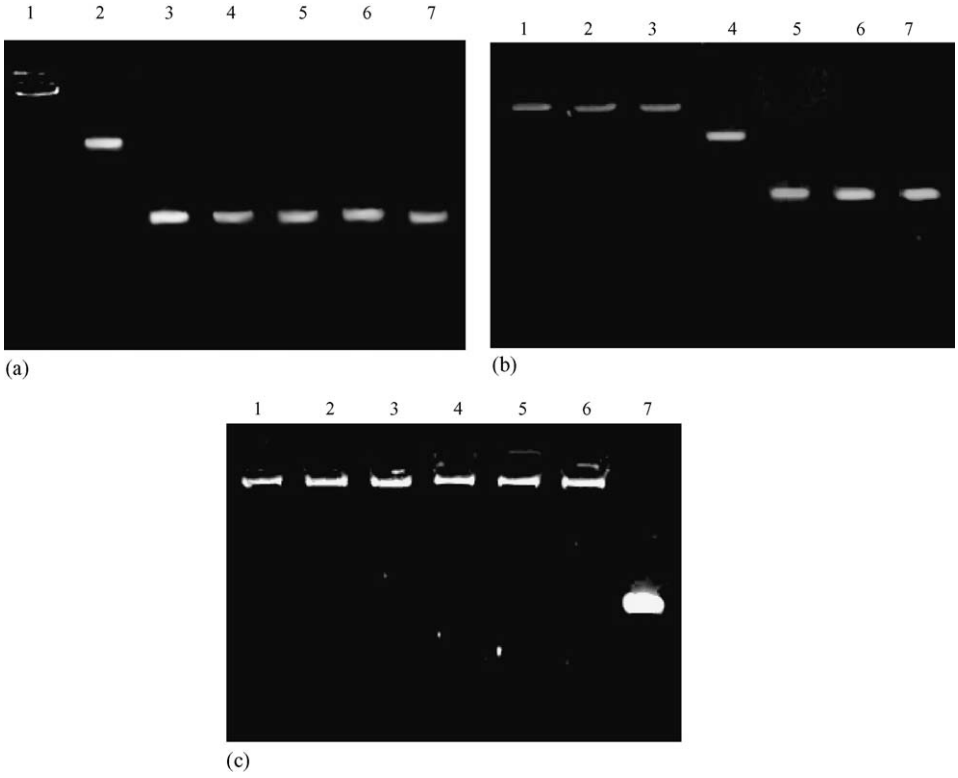

Fig. 3. Electrophoresis of CS/DNA complexes after chitosan degradation. (a) Treated with chitosanase; lane 1: 0.1 ng chitosanase; lane 2: 0.5 ng; lane 3: 2.0 ng; lane 4: 10.0 ng; lane 5: 50.0 ng; lane 6: 200.0 ng; lane 7: plasmid without treatment. (b) Treated with lysozyme; lane 1: 1.0 ng lysozyme; lane 2: 4.0 ng; lane 3: 16.0 ng; lane 4: 64.0 ng; lane 5: 256.0 ng; lane 6: 1.0 μg; lane 7: plasmid without treatment. (c) Treated with cell lysate; lane 1: 0.1 μL cell lysate; lane 2: 0.2 μL; lane 3: 0.5 μL; lane 4: 2.0 μL; lane 5: 5.0 μL; lane 6: 10.0 μL; lane 7: plasmid without treatment.

Table 3
β-Galactosidase activity in C2C12 cell line without and with pre-delivery of chitosanase (U/mg protein)

	Normal ($n=3$)	Chitosanase-modified C2C12 cells ($n=3$)
Cell itself	27.7 ± 4.1	25.3 ± 5.2
Naked DNA	32.4 ± 5.5	34.2 ± 4.7
CS2k	37.9 ± 6.0	349.6 ± 48.0
CS5k	36.5 ± 5.1	425.4 ± 45.1
Lipofectamine	224.6 ± 35.1	280.4 ± 45.8

But as to the expression of LacZ reporter gene, no significant difference between the chitosans used was detected, and β-galactosidase activity of cell transfected with chitosans is much lower than that with lipofectamine. It is surmised that a strong electrostatic interaction between chitosan and DNA prevents the unpacking of gene from its carrier, unfavorably lowering gene expression.

Herein, it was experimentally determined that chitosan degradation ratios of chitosanase and lysozyme were 25.9 and 0.4%, respectively, implying that chitosanase is more powerful in degrading chitosan. In mammalian cell, there are also some enzymes such as lipase and lysozyme which only degrade chitosan unspecifically. We had also detected the chitosan-degradation activity of C2C12 cell lysate in the absence of chitosanase. The results demonstrate that merely limited amount of chitosan was degraded. Therefore, this degradation may be not enough for the complete release of DNA from CS–DNA complexes, still impeding the expression of reporter gene.

As shown in Fig. 3, when the quantity of chitosanase is up to 0.5 ng, free DNA is dissociated from the complexes and migrates out of starting slots; in comparison, only at 16.0 ng can lysozyme

Table 4
β-Galactosidase activity in other cell lines (U/mg protein) ($n=4$)

	2T3		3T3		MG63	
	Csn-modified	Normal	Csn-modified	Normal	Csn-modified	Normal
Cell itself	21.2 ± 3.3	22.4 ± 3.8	26.4 ± 3.1	24.3 ± 3.9	13.2 ± 3.2	12.1 ± 2.3
Naked DNA	30.4 ± 4.5	31.6 ± 3.7	31.4 ± 2.8	33.5 ± 4.0	20.3 ± 1.9	21.3 ± 2.8
CS5k	362.5 ± 58.1	26.4 ± 4.1	223.7 ± 38.9	37.8 ± 4.6	213.7 ± 42.8	15.3 ± 2.7
Lipofectamine	254.3 ± 35.4	280.2 ± 40.3	210.4 ± 33.8	260.7 ± 42.1	165. ± 24.7	143.5 ± 12.5

cause the dissociation of DNA, further verifying its inferior ability to degrade chitosan. Whereas for cell lysate, all complexes remain immobile in the starting slots, that is, no free DNA is liberated from its vector, so the release of DNA by cell lysate can be reasonably excluded.

Table 3 lists the β-galactosidase activity in C2C12 cell line without and with pre-delivery of chitosanase. It is obvious that after chitosanase was pre-delivered into the cells, the β-galactosidase activity in these cells was increased tens of fold compared with the control, and even significantly higher than lipofectamine 2000 transfected C2C12 cell. In order to prove whether this protocol is a special case for C2C12 or applicable to other cell lines, we performed the transfection of 3T3, 2T3 and MG63 cells using the above strategy. From Table 4, it is seen that although transfection efficiency of CS–DNA complex is different for these cell lines, the pre-delivered chitosanase increases

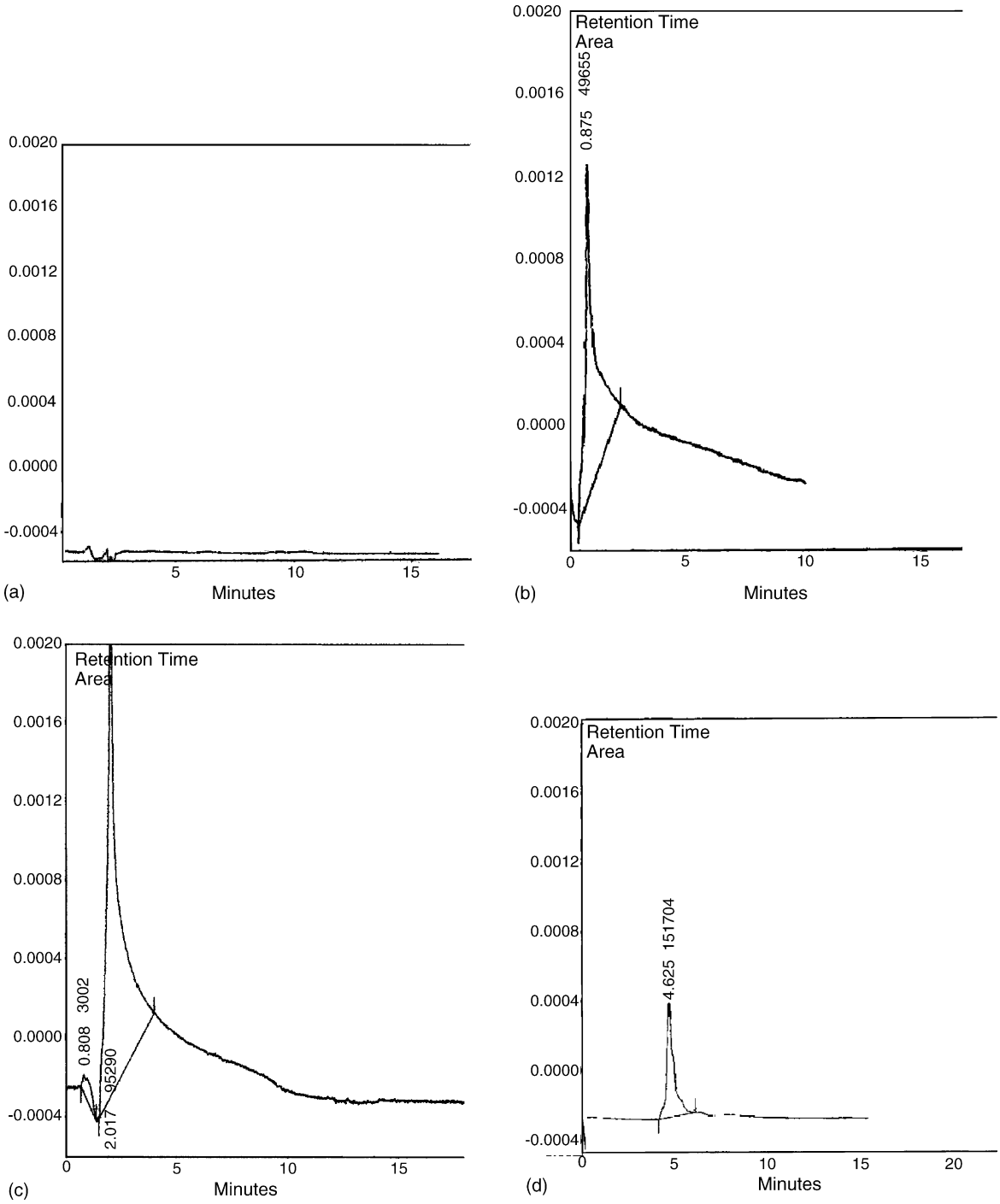

Fig. 4. HPLC detection of aminoglucose: (a) blank cell lysis buffer; (b) cell lysis buffer with 1 mg/mL glucose; (c) cell lysis buffer with 1 mg/mL aminoglucose; (d) cell lysis buffer with 1 mg/mL chitosan of molecular weight 5000; (e) cell lysis of C2C12 cell; (f) cell lysis of chitosanase modified C2C12 cell transfected by CS–DNA complex; (g) cell lysis of C2C12 cell transfected by CS–DNA complex.

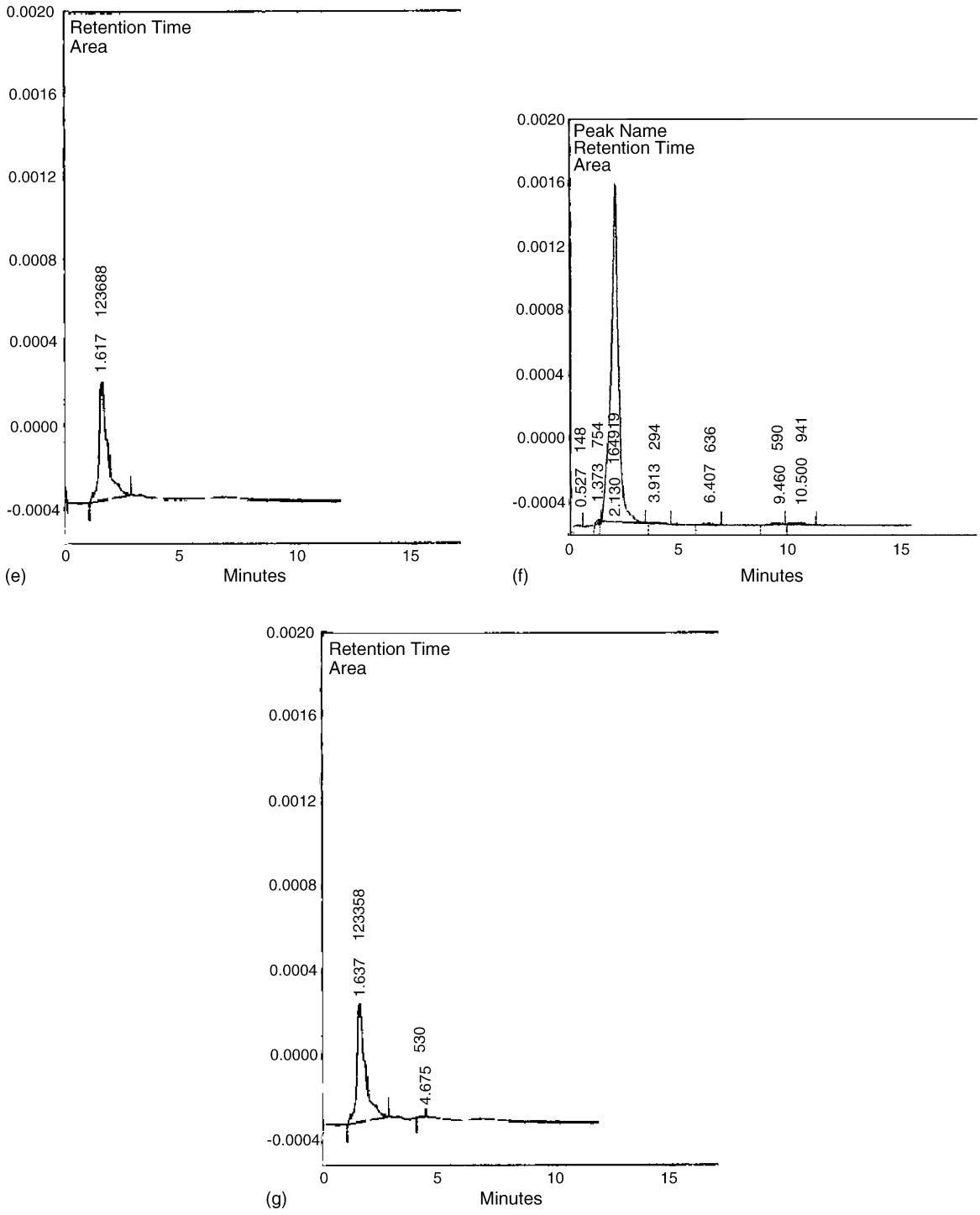

Fig. 4. (*Continued*).

the expression of LacZ reporter gene markedly without exception. Therefore, it is rational to consider that with chitosanase incorporation, this built-in enzyme facilitates the degradation of chitosan carrier after it transports gene into cells; hence, the exogenes are readily dissociated from the vectors, accordingly increasing the expression of reporter gene.

The determination of aminoglucose is shown in Fig. 4. In order to ensure that glucose, aminoglucose and chitosan of molecular weight 5000 could well be distinguished from each other in this HPLC manner, glucose, aminoglucose and chitosan were first separately dissolved in cell lysis buffer to a final concentration of 1 mg/mL each. It was verified by HPLC that the retention times of these three samples are significantly different. In same way, aminoglucose in cell lysis were detected by HPLC. Compared with the untransfected C2C12 cell and CS–DNA complex transfected C2C12 cell, the cell lysis of

chitosanase modified C2C12 cell demonstrated a high concentration of aminoglucose, as shown by a high peak with retention time of 2.13 min. This result further confirmed that the pre-delivered chitosanase really promoted chitosan degradation. Of course, the degradation rate of chitosan must be strictly controlled to meet the requirements of gene ferry and ultimate expression. The detailed work involving confocal microscopical inspection is underway in our laboratory.

4. Conclusion

The quantity of DNA ferried into the cell was an important factor influencing the expression of the exogenous gene. But the release of DNA from its vector may play a decisive role in ultimate expression level. In this paper it was revealed that chitosan with relatively low molecular weight had a strong ability to transport exogene into cells, but its transfection efficiency was relatively lower, which was supposedly due to the strong electrostatic interaction between chitosan and DNA, and that restricted gene expression. It was previously reported that chitosan was degraded by lysosome in host cell, and then exogene was liberated and expressed (Erbacher et al., 1998). In this paper it was found that cell plasma could hardly perform this process (Fig. 3). Thus, the deficient chitosan degradation in the absence of chitosanase only caused tiny amount of exogene dissociation from the complex and resulted in low expression level. In contrast, the incorporated chitosanase was able to lead to a noticeable degradation of chitosan. In turn, the degraded chitosan promoted gene unpacking from its vector, so the level of gene expression was considerably raised.

Note that transfection efficiency should be defined as how many DNA are delivered into the cells or how many cells are transfected. Since transfection efficiency is normally determined by the expression of some reporter genes, such as GFP, CAT, LacZ and luciferase, in this paper we use β-galactosidase activity to represent transfection efficiency. In fact, the pre-delivered chitosanase had no effect on the amount of DNA internalized into the cells, but chitosanase is able to promote DNA unpacking, thereby increasing the expression of LacZ reporter gene and β-galactosidase activity. Although we could not exploit these findings routinely to improve gene transfection, it indeed provides a novel approach in theory to improve the transfection efficiency of chitosan-based vectors via endocellular unpacking.

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