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Chitosan N-betainates/DNA self-assembly nanoparticles for gene delivery: In vitro uptake and transfection efficiency

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ABSTRACT

The aim of this work is to investigate the effect of betaine substitution degree of chitosan N-betainates (CsB) on cellular uptake, cytotoxicity and transfection efficiency of CsB/DNA complex nanoparticles (CsBNs) against COS-7 and MDA-MB-468 cells. The polymers with three substitution degrees (CsB12, CsB47 and CsB85) complexed with pDNA formed CsBN12s, CsBN47s and CsBN85s. The CsBNs showed less pH dependency with smaller particle size and higher zeta potential than that of chitosan/pDNA complex nanoparticles (CsNs) at neutral pH. CsBN85s showed stronger cellular uptake than that of CsBN47s or CsBN12s. CsBNs showed higher cytotoxicity than CsNs, and a trend increasing toxicity with substitution degree increasing. In COS-7 cells, the transfection efficiency increased with the substitution degree increasing, while the opposite result was observed in MDA-MB-468 cells. Chitosan modified with betaine could increase its ability to facilitate DNA uptake and its cytotoxicity, both of which showed the influence on transfection efficiency. It was able to increase cellular uptake and transfection efficiency of complex nanoparticles in COS-7 cells to increase betaine substitution of CsB, however, the higher sensitivity of MDA-MB-468 cells to CsBs led to decreased transfection efficiency due to the increased cytotoxicity with betaine substitution increasing. The predominant role of cellular uptake or toxicity in affecting transfection efficiency was different in two cell lines. These results provided an important guidepost for further development of chitosan derivatives/pDNA complexes as non-viral gene vectors.

1. Introduction

Chitosan (poly-1,4-β-D-glucosamine) is a biocompatible and biodegradable polysaccharide that attracts high interest in the pharmaceutical research area as a versatile excipient and a promising polymeric carrier of peptides and vaccines due to its safety and low cost (Sakkinen et al., 2003; Kim et al., 2008; Jiang et al., 2007). Besides these applications, chitosan has been investigated as non-viral vector for gene delivery because of its ability to condense gene into nanoparticles that are appropriate to be endocytosed by cells, and subsequently be released from endosomes and enter nucleus (Zhao et al., 2006; Lavertu et al., 2006). However, chitosan shows the pK_a of 5.6 and is only soluble in acidic solution with pH value lower than 6.0, and the transfection efficiency is still required improvement for its practical use. These major drawbacks of chitosan limit its use for gene delivery.

Quaternary ammonium derivatives of chitosan have been tried to overcome above disadvantages because these derivatives show the higher solubility over a broader pH range and more permanent positive charge on the polysaccharide backbone than those of unmodified chitosan. So far, quaternary chitosan has been used as antimicrobials or permeation enhancers in transepithelial delivery of both low molecular weight compounds and proteins (Holappa et al., 2006; Zambito et al., 2008; Di Colo et al., 2004; Boonyo et al., 2007). As gene delivery vector, it has been reported that the transfection efficiency of quaternary chitosan was affected by the degree of quaternization (Thanou et al., 2002; Kean et al., 2005; Germershaus et al., 2008), and quaternary chitosan was mainly prepared with methyl iodide, which reacted with the amino group of chitosan backbone under alkaline condition. This strategy cannot obtain structurally uniform polymer because the degree of N-alkylation in the first step by reductive alkylation is not complete, and the free amino group can also be mono-, di- or tri-methylated after the quaternization (Sieval et al., 1998). In addition, it is necessary to extend the time of derivatization reaction to obtain polymer with high degree of quaternization, which could cause the polymer to break up and form smaller fragments (Kean et al., 2005), and could also inevitably lead to total alkylation of the hydroxyl groups in chitosan (Holappa et al., 2006). Thus, this procedure will actually produce the diverse heteropolymers with a wide variety of different monomers and molecular weights, which could produce unreliable structure–activity relationship.

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Another synthetic route for quaternary chitosan derivatives is to attach a substituent with simple quaternary betaine moiety because betaine is a natural non-toxic product, and the derivatives could have more uniform structure than *N*-trimethylchitosan (Holappa et al., 2004). It has been reported that subtle chemical and structural change of polymer can bring about a profound impact on the biological property (Liu et al., 2004; Zelikin et al., 2002). So we supposed that chitosan N-betainates could show different physicochemical, biological and pharmaceutical properties. Also, it could be more accuracy to employ this synthetic approach to calculate the substitution degrees because it is very important to investigate their impacts on cellular uptake, cytotoxicity and transfection efficiency of chitosan derivatives when they are used for gene delivery.

We are interested in investigating the relationship of structure–activity of Cs derivatives for gene delivery. In this work, we selected chitosan N-betainates (CsB)/DNA self-assembly nanoparticles (CsBNs) for gene delivery, investigated the characterization of CsBNs and tried to find the correlation of the substitution degree with cellular uptake, cytotoxicity and the transfection efficiency of CsBNs on COS-7 and MDA-MB-468 cells.

2. Materials and methods

2.1. Materials

Chitosan (Cs, MW = 60 K) was purchased from Boao Biotechnologies Co., Ltd. (Shanghai, China). The degree of deacetylation (90%) was confirmed by ^{1}H NMR spectroscopy (AM-400 Bruker-Spectrospin, USA). Phthalic anhydride and hydrazine monohydrate were got from Sinopharm Group Chemical Reagent Co., Ltd. (China). Triphenylchloromethane was purchased from GL Biochem Ltd. (Shanghai, China). Betaine hydrochloride was bought from Sunwin Chemicals Co., Ltd. (China). N-Chlorobetainyl chloride was fleshly prepared before use with Vassel's method (Vassel and Skelly, 1963). Trypsin-EDTA, phosphate buffered solution (PBS) and agarose were obtained from Gibco-BRL (Burlington, ON, Canada). The Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium, antibiotics, YOYO-1 and fetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue and branched polyethylenimine (PEI, 25 kDa) were purchased from Sigma (St. Louis, MO, USA). Other chemicals if not mentioned were of analytical grade.

Chitosan was modified by a quaternary betaine moiety using an efficient five-step synthetic route according to Holappa's method (Holappa et al., 2004). The products were characterized by ^{1}H NMR spectroscopy and the substitution degree of betaine moiety in Cs was calculated. Plasmid EGFP-N1 (4.7 kb) encoding enhanced green fluorescent protein driven by immediate early promoter of CMV was purchased from Clontech (Palo Alto, CA, USA). The plasmid DNA (pDNA) grown in DH5α strain of *E. coli* was isolated with the EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). The purity was confirmed by spectrophotometry (A260/A280), and DNA concentration was measured by UV absorption at 260 nm.

2.2. Cell culture

The cell lines COS-7 (African green monkey kidney cell) and MDA-MB-468 (human breast cancer cell) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). COS-7 was grown in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. MDA-MB-468 was cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin). Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.3. Formation and characteristics of CsBNs

CsBNs were prepared by following procedure, briefly, pDNA was added to the CsB solution diluted in 5 mM sodium acetate/acetic acid buffer (pH 5.5) with equal volume to obtain the desired polymer nitrogen to DNA phosphate (N/P) ratio. Subsequently, solution was immediately vortexed for 30 s (XW-80A Votex mixer, Shanghai, China). The resulting complexes were allowed to sit at room temperature for 30 min. As control, Cs/DNA self-assembly nanoparticles (CsNs) were prepared by the same process as described for CsBNs above. As positive control, PEI/DNA formed nanoparticles (PEINs) with different mass ratios (polymer/DNA) were prepared as described above except that PEI was diluted in pure water.

The formation of CsBNs was evaluated by agarose gel electrophoresis. CsBNs containing 0.5 μg DNA at various N/P ratios were applied to a 1.0% agarose gel in Tris–Acetate–EDTA (TAE) buffer containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out at 80 V for 45 min in TAE buffer. The resulting DNA migration pattern was revealed under UV irradiation.

The particle size and zeta potential of CsBNs were determined by the laser light scattering measurement using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA). The CsBNs were diluted five times with sodium acetate/acetic acid buffer (pH 5.5) or PBS (pH 7.4) at a final DNA concentration of 50 μg/ml. Analyses were performed after 10 min.

2.4. Cellular uptake study

In order to know the cellular uptake of the CsBNs, DNA was fluorescently labeled with an intercalating nucleic acid stain YOYO-1. The DNA/YOYO-1 complex was prepared with 1 nM dye molecule/μg DNA and an incubation of 1 h at room temperature in the dark. Nanoparticles were prepared at N/P ratio 10 for Cs or CsB and mass ratio 4 for PEI with DNA/YOYO-1 concentration of 50 μg/ml. COS-7 and MDA-MB-468 cells were seeded in a 24-well plate with 0.5 ml growth medium (DMEM containing 10% FBS, supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin for COS-7, RPMI 1640 containing 10% FBS, supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin for MDA-MB-468, respectively) and allowed to attach for 24 h. Then, cells were incubated with nanoparticles (DNA concentration 2.5 μg/well) for 1.5 h at 37 °C followed by washing three times with PBS (pH 7.4). To visualize the internalization of the nanoparticles, each cell monolayer was observed under a fluorescence microscope. To measure the fluorescence intensity of the cells, cells after treated with complexes were carefully washed three times with PBS, then trypsinized and resuspended in the medium. The residual fluorescence out of the cell membrane was quenched with 0.4% trypan blue for 2 min (Hashimoto et al., 2006). Then cells were centrifuged and washed three times with PBS. The cell resuspension was finally subjected to flow cytometry and analyzed with CellQuest software through fluorescence channel 1 (FL1).

2.5. Cytotoxicity assays

COS-7 and MDA-MB-468 cells were seeded at a density of 1×10^4 cells per well in 150 μl growth medium in 96-well plates and grown overnight. Immediately after growth medium was removed, CsNs and CsBNs (N/P ratio 10) were applied onto the cells in fresh culture media with 10% FBS at polymer concentration range from 2 to 100 μg/ml. Cells treated with fresh culture media only were used as control. Incubation was performed for 8 h before removal of media containing complexes and replacement with fresh culture media. After 24 h, 20 μl MTT solution (5 mg/ml in PBS) was added, and the cells were incubated for additional 4 h at 37 °C for MTT formazan formation. Then, the medium was removed and DMSO was

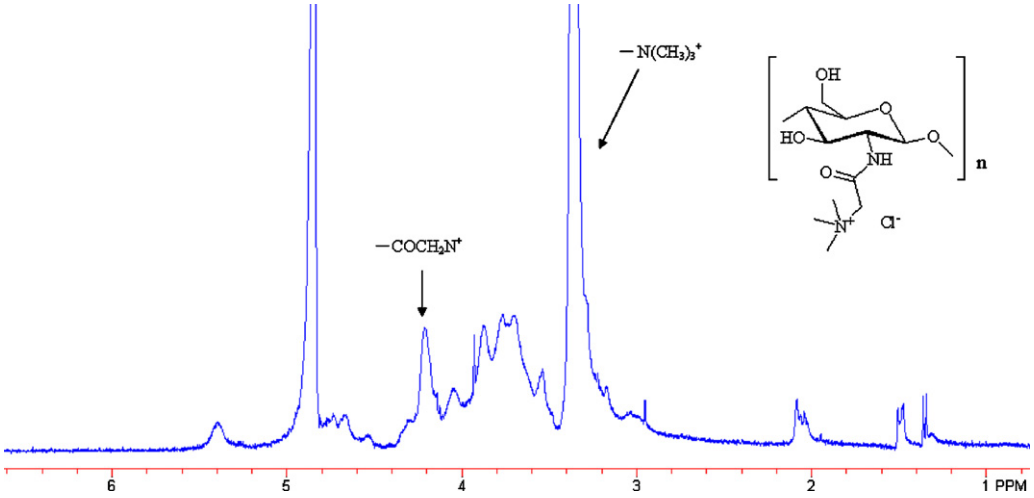

Fig. 1. ^{1}H NMR spectra of CsB85 in D_2O.

added to dissolve the crystal. The plates were mildly shaken for 10 min to ensure the dissolution of formazan. The absorbency values were measured using microplate reader (Bio-RAD, model 550) at wavelength 570 nm, blanked with DMSO solution. Three replicates were counted for each sample. The mean value of the three times was used as the final result.

2.6. In vitro transfection experiment

Cells (COS-7 and MDA-MB-468) were seeded in 24-well plates at a density of 1×10^5 cells per well in 500 μl of complete medium and incubated for 24 h prior to transfection. Then, the media was replaced with fresh growth medium containing CsBNs with DNA concentration 2.5 μg/well at various N/P ratios. Media were replaced by fresh culture medium (without polymer) after 8 h and cells were incubated for an additional 48 h. The expressed green protein could be observed under a fluorescence microscope. Finally, cells were collected and resuspended in PBS (pH 7.4). The transfection results were measured using a FACSCalibur (Becton Dickinson, USA) through fluorescence channel 1 (FL1). As control, CsNs and naked DNA were used on cell cultures and examined as described above. As positive control, PEINs with mass ratio 4 were prepared and transfected with media without FBS for 2 h before the medium was replaced with fresh complete growth medium. All transfection experiments were performed in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for $p < 0.05$ and $p < 0.01$ indicative of a very significant difference.

3. Results and discussion

3.1. Physicochemical characterization of CsBNs

In this study, chitosan has been successfully modified with betaine moiety by a five step synthetic route. The CsBs with three substitution degrees of betaine were obtained, which was 12.3% (CsB12), 46.8% (CsB47) and 84.7% (CsB85) estimated by NMR measurements (Fig. 1), respectively.

CsBNs were successfully prepared by a self-assembly procedure. The sizes of the CsNs and CsBNs prepared at various N/P ratios were shown in Fig. 2. When complex nanoparticles were diluted in acetate buffer (pH 5.5), chitosan or CsB12 showed the most efficient condensation of DNA with the smallest particle sizes (<200 nm) while N/P ratio ≥5. When complex nanoparticles were diluted in PBS (pH 7.4), the CsB85 showed the strongest DNA binding ability compared with Cs, CsB12 or CsB47. The particle sizes decreased with betaine substitution increasing in PBS (pH 7.4), which indicated that the DNA condensation ability of Cs increased with betaine substitution increasing. To CsBN47 and CsBN85, increase of the N/P ratio caused a slight increase of particle size, which might attribute to that more betaine moieties within the nanoparticle caused the higher intra-charge repulsion, and CsB47 and CsB85 demonstrated less dependency on pH than Cs or CsB12 when complexed with DNA. At N/P ratio 10, CsNs showed particles size of 167.4 nm in pH 5.5 or 498.6 nm in pH 7.4, while CsBN85 showed the particles size 241.5 nm or 174.2 nm at pH 5.5 or pH 7.4, respectively.

The pH dependency of nanoparticles could also be observed by the change of ζ potential. Under acidic conditions (pH 5.5), CsNs at N/P ratio 10 showed ζ potential of 8.6 ± 2.1 mV, while the potential decreased to 0.8 ± 1.4 mV at pH 7.4. As for CsBN12s, the pH dependency of ζ potential was similar with CsNs due to its low substitution of betaine in chitosan backbone, which was 10.8 ± 1.4 mV and 1.9 ± 1.6 mV for pH 5.5 and pH 7.4, respectively. In contrast, CsBN85s showed the highest ζ potential with 14.7 ± 2.1 mV at pH 5.5 and 9.4 ± 1.3 mV at pH 7.4.

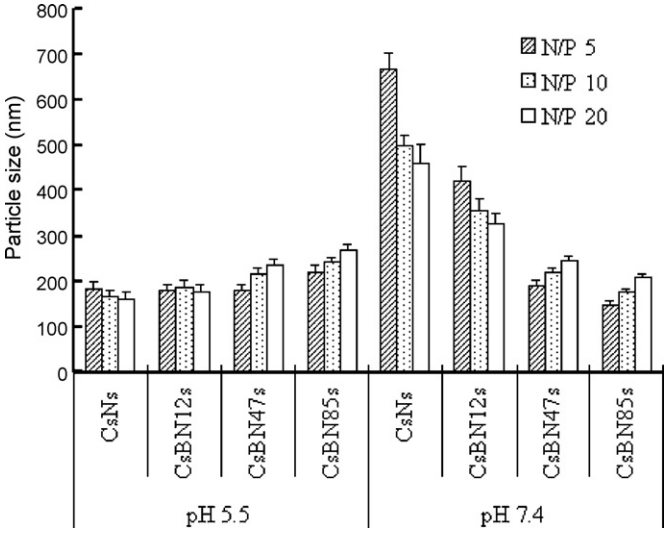

Fig. 2. Effect of pH on the size of complexes nanoparticles with different N/P ratio.

It was reported that the formation of small and stable nanoparticles in physiological solution was an important prerequisite for efficient gene delivery using cationic polymers (Mansouri et al., 2004). Due to low pK_a, the amino groups in chitosan backbone are only partially protonized at pH 7.4, as a result, chitosan is unable to efficiently condense DNA into enough small particles in physiologic solution. When pH increased to 7.4, the surface charge of CsNs decreased, which resulted in the charge repulsion between particles decreasing. It has been reported that the electric repulsion between particles is a very important factor to evaluate the stability of colloidal dispersion because particles with relatively high absolute value of zeta potential can remain stable (Müller et al., 2001). In contrast, $-N(CH_3)_3^+$ group had a very high pK_a (pK_a > 11). Modifying chitosan with betaines could increase chitosan charge density and solubility because the $-N(CH_3)_3^+$ group presented good solubility and positive charge in physiological solution. So CsBNs could condense DNA into smaller particles (Fig. 2) with higher charge potential and stability than CsNs under physiological solution.

In order to investigate the ability of CsB with different substitution degrees of betaine to condense DNA, CsBNs prepared at various N/P ratios from 1 to 20 were electrophoresed on an agarose gel. CsNs showed the retarded DNA migration at N/P ratio ≥2 (Fig. 3A), while CsBs showed stronger condensation ability with retarded DNA migration at N/P ratio ≥1 (Fig. 3B–D).

3.2. Cellular uptake of CsBNs

The cellular uptake was investigated with pDNA labeled with YOYO-1 on COS-7 and MDA-MB-468 cells. YOYO-1 as an intercalating dye with high affinity to DNA has been widely used in cellular uptake study (Hashimoto et al., 2006; Zaric et al., 2004; Mehier-Humbert et al., 2005). The internalization of CsBNs in MDA-MB-468 cells was first observed under a fluorescence microscope (Fig. 4A). After 1.5 h incubation, CsBN12s and CsBN47s were distributed into inside of the cells as spot-like fluorescence, in particular, CsBN85s showed some fluorescence detected in the nucleus, which revealed that CsBN85s had stronger ability to deliver DNA into the cells compared with CsBN12s or CsBN47s, while CsNs were detected only as some large aggregates on the cell surface. These results demonstrated that chitosan modified with betaine could improve its cellular uptake, and the uptake efficiency increased with betaine substitution degree increasing. In addition, the significant differences between chitosan derivatives and PEI were observed. PEINs showed DNA profused throughout whole cytoplasms and accumulated in the nucleus. This different subcellular distribution also was detected in COS-7 cells (Fig. 4B).

To estimate the amount of DNA internalized into cells and measure the fluorescent intensity, the fluorescence of the complexes adsorbed on the cell surface was first quenched by trypan blue. Then cells were subjected to FACSCalibur. Fig. 5 showed significant difference between CsBNs and PEINs in mean fluorescence intensity (MFI) in two cell lines. For CsBNs, the MFI increased with substitution degree increasing, which was accordant with the observed results under fluorescence microscope (Fig. 4).

It is reported that there are at least two different roles in cellular uptake of Cs or Cs derivatives, one is the electrostatic interaction between positive charged polymer and the negative charged cell membrane (Mao et al., 2007). Particles with stronger positive

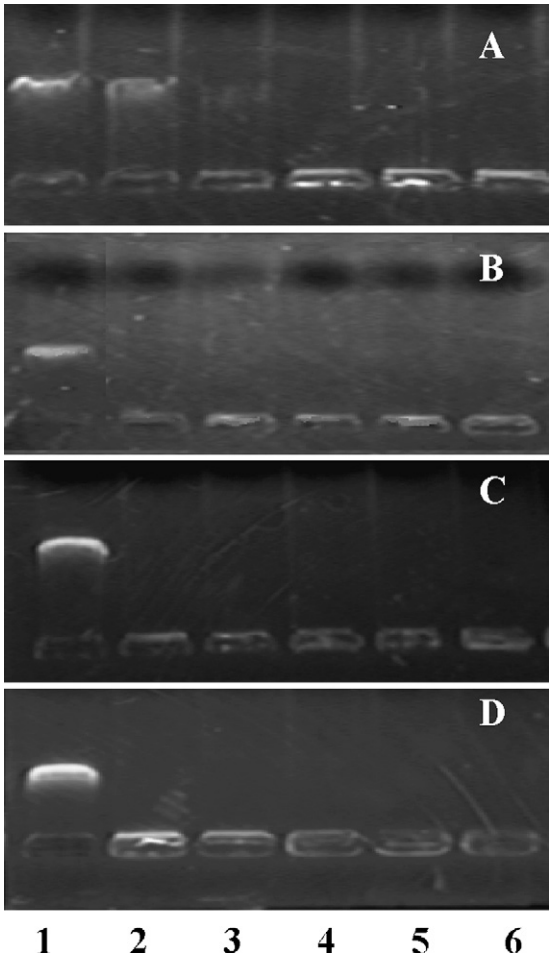

Fig. 3. Agarose gel electrophoresis of CsNs (A), CsBN12s (B), CsBN47s (C) and CsBN85s (D). Lane 1: naked DNA control; Lanes 2–6: complexes nanoparticles prepared at N/P ratios of 1, 2, 5, 10, and 20 in pH 5.5 acetate buffer, respectively.

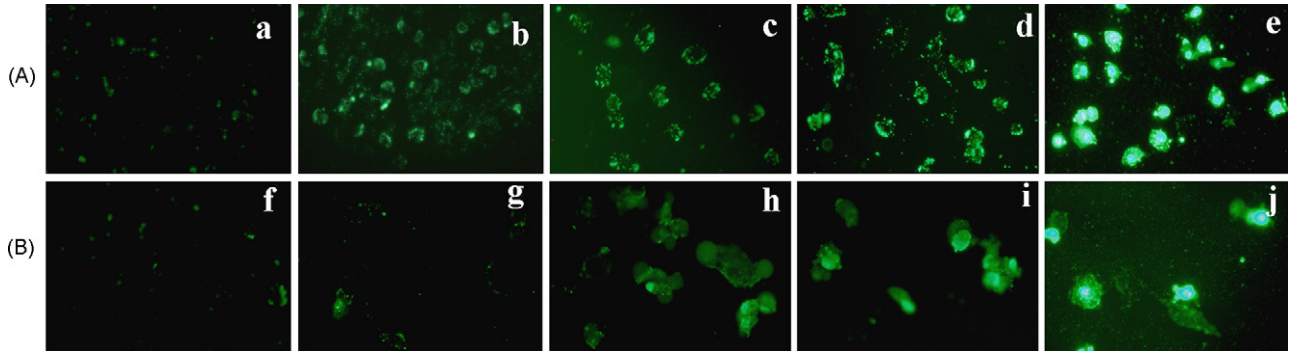

Fig. 4. Fluorescent images of MDA-MB-468 (A) and COS-7 (B). Cells were incubated with CsNs, CsBN12s, CsBN47s or CsBN85s at N/P ratio 10 and PEINs at mass ratio 4 for 1.5 h at 37 °C. Complexes nanoparticles were formed with polymer and YOYO-1 labeled DNA. CsNs (a and f), CsBN12s (b and g), CsBN47s (c and h), CsBN85s (d and i) and PEINs (e and j).

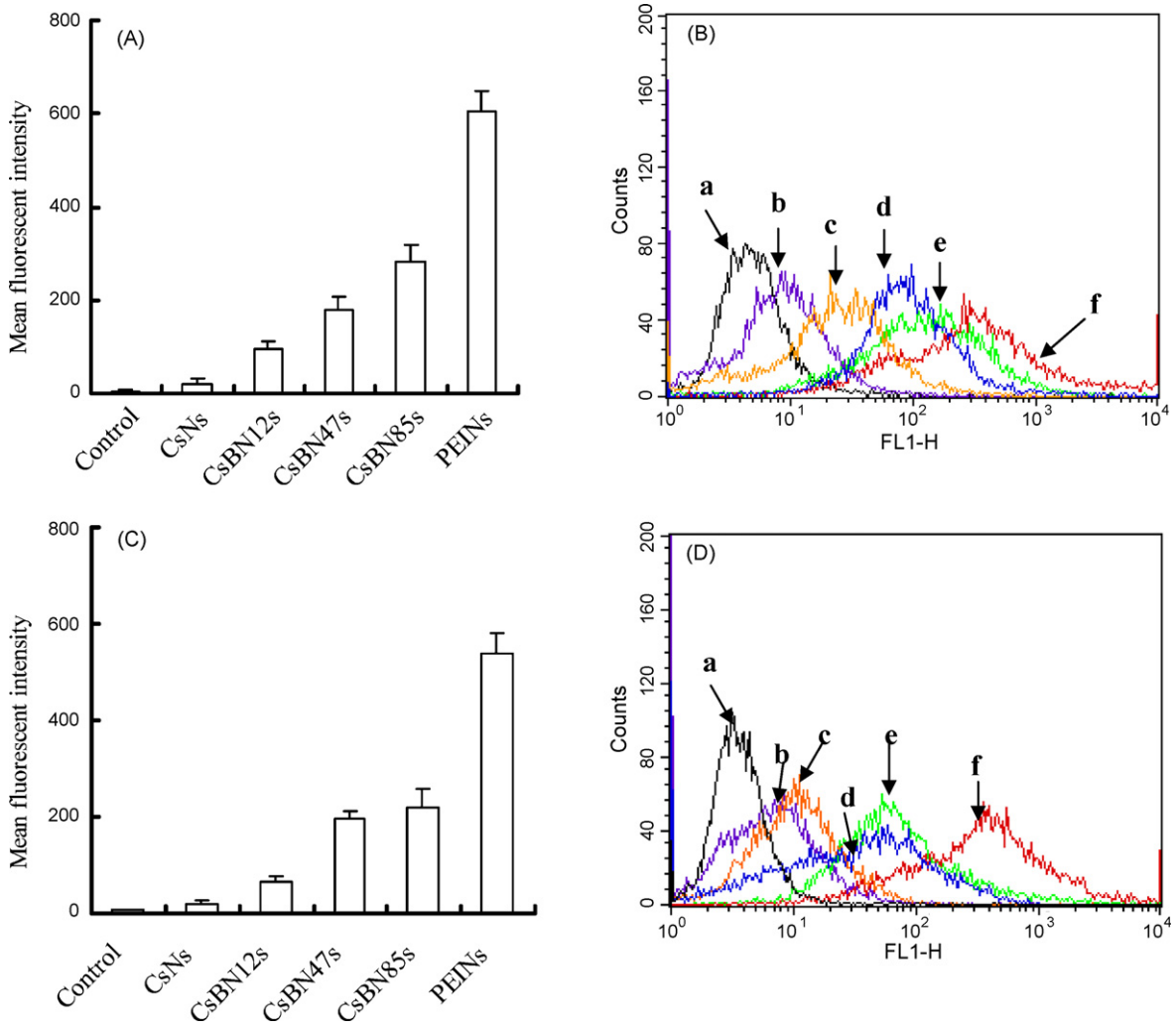

Fig. 5. FACSCalibur flow cytometry analyzed the mean fluorescent intensity of COS-7 cells (A) and MDA-MB-468 cells (C) incubated with CsNs, CsBN12, CsBN47 and CsBN85 at N/P ratio 10 or PEINs at mass ratio 4 for 1.5 h at 37 °C. Untreated cells were used as negative control. The FCM pictures of control cells (a), cells treated with CsNs (b), CsBN12s (c), CsBN47s (d), CsBN85s (e) or PEINs (f) of collected COS-7 cells (B) and MDA-MB-468 cells (D).

charge are preferably adsorbed with a higher rate. The other is the non-ionic interaction between chitosan backbone and the cell surface proteins (Venkatesh and Smith, 1998). It was reported that this interaction played a more important role in cellular uptake than the electrostatic interaction in primary chondrocytes, and the CsNs got the highest transfection efficiency when particles were neutral (Zhao et al., 2006). From our experimental results, the electrostatic interaction was the dominant in cellular uptake on CsBNs against both cell lines. The increase of the substitution degree of betaine could increase the positive charge on the complex nanoparticles thus increasing cellular uptake. Moreover, it was reported that quaternary chitosan was capable of opening tight junctions of cells, which led to an increased paracellular permeability (Thanou et al., 2000; Mao et al., 2005). So the betaine moieties in chitosan backbone might also have this ability that led to increased cellular uptake.

3.3. Cytotoxicity of CsBNs

Polycations are considered to be cytotoxic because cationic macromolecules could interact with cell membranes, extracellular matrix proteins and blood components leading to side effect (Kircheis et al., 2001). The cytotoxicity of polycation was greatly influenced by polymer structures (Fischer et al., 2003). In order to investigate the cytotoxicity of CsBNs on COS-7 and MDA-MB-468 cell lines, the MTT assay was used. The complex nanoparticles at N/P ratio 10 with various polymer concentrations from 2 to 100 μg/ml were applied to cells for 8 h, which was indicative of the toxicity experiment over time of *in vitro* transfection. As shown in Fig. 6, CsNs and CsBN12s showed very low toxicity at tested concentrations. However, the cytotoxicity of CsB increased with degree of betaine substitution increasing, and MDA-MB-468 cells showed higher sensitive to CsBN47s and CsBN85s. At 100 μg/ml, the cell viability of CsBN85s was only 47.6% and 15.4% for COS-7 and MDA-MB-468 cells (Fig. 6), respectively. As for PEI, compared with MDA-MB-468 cell, COS-7 cells showed a little less sensitive, in particular, due to its severe toxicity, almost no cells were viable when treated with PEINs (20 μg/ml) after 8 h incubation (data not shown).

3.4. Transfection efficiency of CsBNs

So far, it has been reported that quaternized chitosan/DNA complexes could transfect many cell lines such as mouse embryo cells (NIH/3T3), epithelial breast cancer cells (MCF-7), COS-1 and Caco-2 cells, etc. (Thanou et al., 2002; Kean et al., 2005; Germershaus et al., 2008), but the transfection efficiency depends on the quaternization degree. In this study, uniform structural chitosan N-betainates were successfully prepared to investigate the relationship of structure–activity. Transfection efficiency of CsBNs was evaluated in COS-7 and MDA-MB-468 cells using plasmid encoding

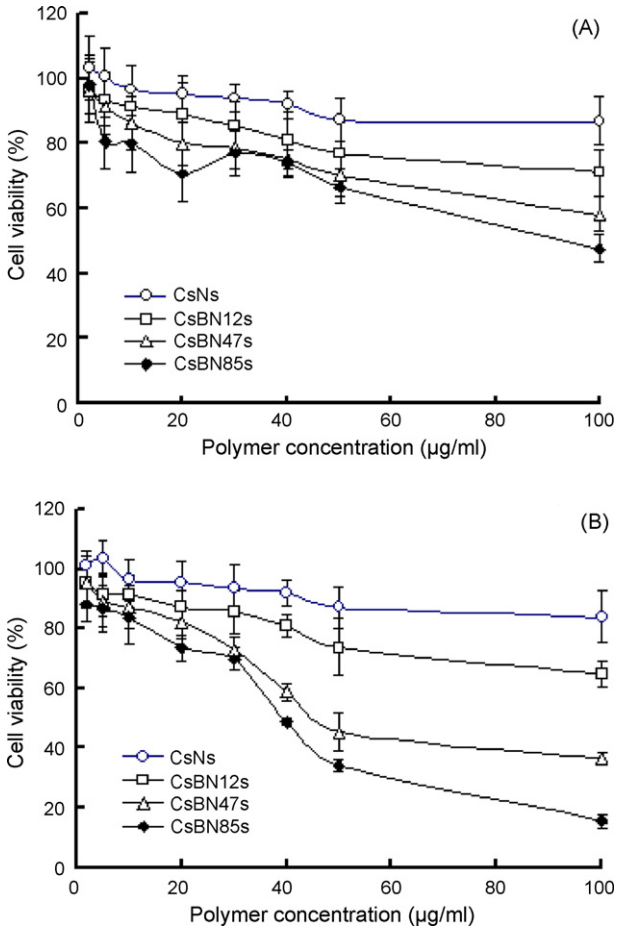

Fig. 6. Cytotoxicity of CsBNs by MTT assay. COS-7 cells (A) and MDA-MB-468 cells (B) were incubated with CsNs, CsBN12s, CsBN47s or CsBN85s at N/P ratio 10 for 8 h.

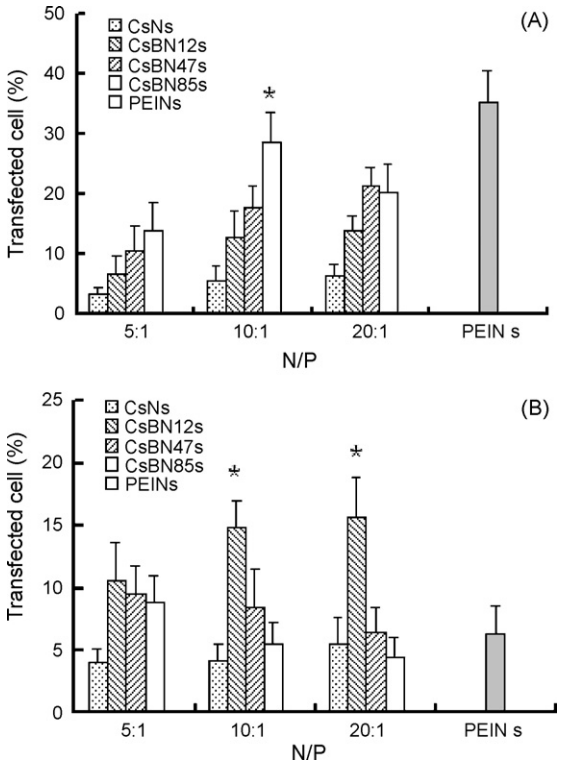

Fig. 7. Transfection efficiency of complexes nanoparticles in COS-7 (A) and MDA-MB-468 (B) cells. $^{*}p < 0.01$ indicated very significant difference between CsBN12s and CsBN85s.

enhanced green fluorescence protein (pEGFP). Cells were incubated with nanoparticles for 8 h to ensure sufficient cellular uptake, and further 48 h incubation was allowed to express the protein. Considering the severe cytotoxicity of PEI, PEINs were applied to cells for only 2 h.

The transfection efficiency of CsBNs in COS-7 cells increased with betaine substitution increasing, but the opposite result was observed in MDA-MB-468 cells, which showed that gene delivery of CsBNs was correlative with cellular kinds. Compared with CsNs, CsBNs showed that the transfection efficiencies in COS-7 cells increased (Fig. 7A). CsBN85s at N/P ratio 10 showed the highest transfection efficiency in this cell line with a little low transfection compared with that of PEINs. To CsBNs, the transfection efficiency increased with betaine substitution increasing at N/P ratio 5 or 10, which could be due to the increased cellular uptake of complex nanoparticles with substitution increasing (Fig. 5). The transfection efficiency of CsBN12s and CsBN47s increased with N/P ratio from 5 to 20, but the transfection efficiency of CsBN85s increased with N/P ratio only from 5 to 10, and the transfection efficiency of CsBN85s decreased at N/P ratio 20, which indicated that the N/P ratio also showed great influence on the gene transfection of CsBNs. For CsBNs, increasing N/P ratio might increase zeta potential of complex nanoparticles, thus increasing cellular uptake, which might lead to increased gene transfection. However, for polymers with high betaine substitution, the cellular toxicity was obvious (Fig. 6). The polymer with high concentration would conversely lead to decreased gene transfection due to severe cytotoxicity.

On the contrary, CsBN47s and CsBN85s showed low transfection efficiency in MDA-MB-468 cells, and the percentage of cells that

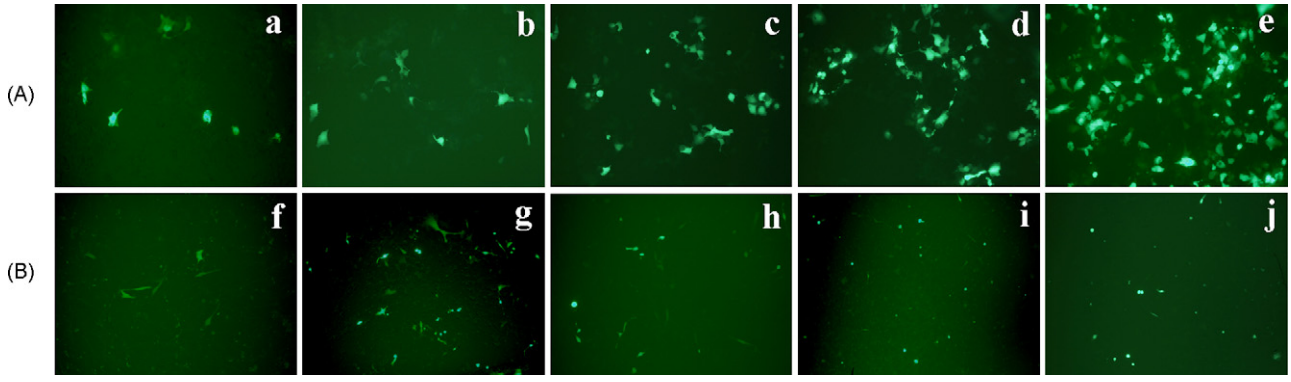

Fig. 8. Images of COS-7 (A) and MDA-MB-468 (B) cells transfected with CsNs, CsBN12s, CsBN47s and CsBN85s at N/P ratio 10 or PEINs at mass ratio 4 observed under fluorescent microscope (20× magnification). CsNs (a and f), CsBN12s (b and g), CsBN47s (c and h), CsBN85s (d and i) and PEINs (e and j).

expressed EGFP decreased with N/P ratio increasing. But CsBN12s showed higher transfection in this cell line compared with that of CsNs, CsBN47s or CsBN85s, and even higher than that of PEINs (Fig. 7B), and the transfection efficiency increased with N/P ratio increasing. Fig. 8 showed the fluorescent images of the two cell lines transfected with complex nanoparticles at N/P ratio 10, which also confirmed the FACS analysis above. During our experiments, we found that most of MDA-MB-468 cells exposed to PEINs were round and many had detached from the well surface after 2 h transfection (data not shown). These dead cells could not further produce EGFP. So, the transfection efficiency of PEINs in MDA-MB-468 cells was very low. After 8 h incubation, the cytomorphology of cells treated with CsNs or CsBN12s showed not any change, however, cells treated with CsBN47s or CsBN85s became round. From the fluorescent images (Fig. 8), we could see that many MDA-MB-468 cells treated with CsBN85s or PEINs were round with some of cells detached from the well, which indicated that the cells were dead. Toxicity in many non-viral vectors has been reported, and the relation between toxicity and transfection efficiency was often observed (Godbey et al., 2001; Florea et al., 2002). So we estimated that the low transfection efficiency of CsBN85s and PEINs on MDA-MB-468 cells could be due to their severe cytotoxicity, which had been confirmed by the cytotoxicity results (Fig. 6). Taking these results together, we could see that the role of cellular uptake or toxicity in affecting transfection efficiency was different in above two cell lines.

4. Conclusion

The CsBNs showed less pH dependency with smaller particle size and higher zeta potential than that of CsNs at neutral pH. CsBN85s showed stronger cellular uptake than that of CsBN47s or CsBN12s. CsBNs showed higher cytotoxicity than CsNs, and a trend increasing toxicity with substitution degree increasing. In COS-7 cells, the transfection efficiency increased with the substitution degree increasing, while the opposite result was observed in MDA-MB-468 cells. It could increase cellular uptake and transfection efficiency of complex nanoparticles in COS-7 cells to increase betaine substitution of CsB, however, the higher sensitivity of MDA-MB-468 cells to CsBs led to decreased transfection efficiency due to the increased cytotoxicity with betaine substitution increasing. These results provided an important guidepost for further development of chitosan derivatives/pDNA complexes as non-viral gene vectors.

Acknowledgements

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