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Title	**Enhanced gene delivery by chitosan-disulfide-conjugated LMW-PEI for facilitating osteogenic differentiation**
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Enhanced gene delivery by chitosan-disulfide-conjugated LMW-PEI for facilitating osteogenic differentiation

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Abstract

Chitosan-disulfide-conjugated LMW-PEI (CS-ss-PEI) was designed to combine the biocompatibility of chitosan and the gene delivery ability of polyethylenimine (PEI) utilizing bio-reducible disulfide for bone morphogenetic protein (BMP2) gene delivery in mediating osteogenic differentiation. It was prepared by conjugating low molecular weight PEI (LMW-PEI) to chitosan through oxidization of thiols introduced for the formation of disulfide linkage. The structure, molecular weight and buffer capacity were characterized by Fourier transform infrared (FTIR), light scattering and acid-base titration respectively. The reduction of the molecular weight of CS-ss-PEI by reducing agent indicated its bio-reducible property. With the increment in LMW-PEI component, the copolymer showed increased DNA binding ability and formed denser nano-complexes. CS-ss-PEI exhibited low cytotoxicity in COS-1, HepG2 and 293T cells over the different weight ratios. The transfection efficiency of CS-ss-PEI4 was significantly higher than that of PEI 25k and comparable to Lipofectamine in mediating luciferase expression. Its application for BMP2 gene delivery was confirmed in C2C12 cells by BMP2 expression. For inducing *in vitro* osteogenic differentiation, CS-ss-PEI4 mediated BMP2 gene delivery showed stronger effect in MG-63 osteoblast cells and stem cells in terms of alkaline phosphatase (ALP) activity and mineralization compared with PEI25k and Lipofectamine. This study provides a potential gene delivery system for orthopaedic related disease.

Key words: Non-viral vector; Chitosan; Polyethylenimine; Disulfide; BMP2; Osteogenesis

1. Introduction

Gene therapy has received significant attention for its possible clinical application. By delivering therapeutic gene, it could show the advantages in the treatment of human genetic and acquired diseases over the conventional treatment.[1] However, the progress has been quite slow due to the difficulties in developing satisfactory gene carriers. Concerns regarding the immune and genetic toxicity of viral vectors have driven extensive attention to the development of nano-scale non-viral vectors. This kind of vector is superior in terms of simple usage, ease of producing, no specific immune response and high genetic material carrying capacity, whereas more effort is needed in improving its gene transfer ability.[2]

Non-viral vectors could expand the application of gene therapy from cancer and monogenic diseases treatments to more prevalent disease such as orthopaedic disease.[3] Bone morphogenetic protein (BMP2) has been widely used for bone regeneration.[4] Gene therapy provides a promising way to sustainably express the BMP2 at the regeneration site, which could overcome the problems of protein treatment including short half-life, large dose requirement and high cost.[5] Furthermore, the property of transient gene expression possessed by non-viral vector is especially suitable for bone regeneration with the benefit in avoiding the adverse effect of BMP2 over expression.[6]

Among the non-viral vectors, chitosan has been widely investigated for its excellent biocompatibility and biodegradability. Its low cytotoxicity has been demonstrated in experimental animals as well as clinical trials.[7] However, its application has been limited by its low transfection efficiency. Alternatively, polyethylenimine (PEI), known for the "proton sponge effect", has been proven to be one of the most powerful and versatile members of non-viral vector both *in vitro* and *in vivo*.[8, 9] Nonetheless, the high cytotoxicity and the non-degradability hampered its clinical application. Although low molecular weight PEI (LMW-PEI) showed lower cytotoxicity, its transfection efficiency also decreased.[10] For developing satisfactory gene carrier, various modifications have been done to PEI and chitosan respectively.

Taking into account the respective advantages of PEI and chitosan, their copolymer is expected to act as satisfactory gene carrier. Relevant studies showed that the copolymers of

chitosan and PEI (chitosan-g-PEI) exhibited enhanced transfection efficiency comparing to chitosan and improved biocompatibility relative to PEI.[11-14] Nonetheless, the non-degradable bonding in the copolymers may affect the further improvement of their properties.

Disulfide bond with the property of reversible cross-linking has attracted extensive attentions recently. As it is reduction-sensitive, it could be cleaved by the high concentration of glutathione within the reductive intracellular environment.[15] This property could not only attenuate the overly condensation of DNA to facilitate DNA dissociating, but also further decrease the cytotoxicity.[16] Various biodegradable poly(disulfide amine)s containing repetitive disulfide bonds showed significantly increased transfection efficiency and decreased cytotoxicity.[17] Similarly, disulfide containing PEI derivatives constructed by LMW-PEI showed efficient gene transfection.[18-20] However, few studies could drive the goal of constructing biodegradable copolymer of chitosan and PEI utilizing disulfide linkage.[21]

The aim of this study is to develop bio-reducible chitosan-disulfide-conjugated LMW-PEI (CS-ss-PEI) as novel non-viral vector and evaluate its efficiency. The optimal formula will be used to deliver BMP2 gene for osteogenic differentiation to provide a potential candidate for orthopaedic gene therapy.

2. Materials and methods

2.1. Materials

Chitosan (CS, MW = 10 kDa) with 92% degree of deacetylation was supplied by AK Biotech Ltd. (Shandong, China). PEI (MW = 1.8 kDa and 25 kDa), dithiobis (2-nitrobenzoic acid) (DTNB) DL-Dithiothreitol (DTT) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was provided by Thermo. PEI 25 kDa was purified by dialysis in deionized water and lyophilization before using for transfection. Lipofectamine^{TM} 2000 was obtained from Invitrogen. Luciferase assay system and reporter lysis buffer were provided by Promega. BCA^{TM} protein assay kit was obtained from Thermo. Alizarin Red S, cetylpyridinium chloride (CPC) and Alkaline phosphatase yellow (pNPP) liquid substrate were got from Sigma. Phosphate-buffered

saline (PBS), ethylenediaminetetraacetate (EDTA) sodium acetate (NaAc) and acetic acid (HAc) were obtained from Sigmal-Aldrich. Other chemicals were used directly without further purification.

2.2. Synthesis of CS-ss-PEI

2.2.1. Synthesis of thiolated LMW-PEI and chitosan

SPDP (2.5 mg) was firstly dissolved in DMSO, and then added to 1 mL PBS with EDTA buffer (PBS, 1mM EDTA, 0.02% sodium azide, pH 7). LMW-PEI (5 mg, 1.8 kDa) was dissolved in the reaction solution containing SPDP, and the reaction was performed at room temperature under stirring for 24 h to form thiolated LMW-PEI. The obtained product was purified by dialysis against deionized water (MW cutoff of 1000 Da) for 1 day to remove the unreacted SPDP.

Thiolated chitosan was prepared with the similar procedure. Specifically, chitosan was firstly dissolved in 50 mM NaAc/HAc buffer, and then added to reaction solution containing SPDP with the final volume of 1 mL. After reaction with SPDP, the product was purified by dialysis (MW cutoff of 3500 Da) for 1 day.

The thiolated degree was determined through monitoring the generation of pyridine-2-thione (PDP) groups at 343 nm (molar absorptivity=8080 $cm^{-1}M^{-1}$) upon the treatment of 20 mM DTT. Then the reduced products containing thiols were purified by dialysis in nitrogen atmosphere. The synthetic scheme was shown in Fig. 1A.

2.2.2. Preparation of CS-ss-PEI

Chitosan-disulfide-conjugated LMW-PEI (CS-ss-PEI) was prepared by oxidization of reduced thiols on PEI and chitosan at room temperature in the air to form disulfide linkage. Copolymers with different component were prepared at the different weight ratio (chitosan : LMW-PEI) of 2:1, 1:1, 0.5:1 and 0.25:1 (Table 1). The course of reaction was traced by monitoring the remaining content of free thiols using Ellman's assay. Specifically, at each time point during the oxidation, a small amount of the reaction solution was incubated with dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) working solution for 15min at room temperature. Absorbance values were obtained on microplate reader at 405nm (Spectra Max 340), and thiol contents were determined using cysteine standards of known concentrations. The final products

were purified by dialysis (MW cutoff of 12 kDa) for 3 days.

2.3. Characterization of CS-ss-PEIs

The structure of CS-ss-PEIs was characterized by Fourier transform infrared (FTIR) spectroscopy on Perkin-Elmer spectrum BX FT-IR system. The molecular weight was estimated on static light scattering using Zetasizer Nano series by measuring the aqueous solution at different concentrations and calculated by Zimm Plot Software. The elemental compositions (C, N, O and S) of the copolymers were analyzed by energy dispersive X-ray spectrometer (EDS microanalysis, Oxford) equipped on the scanning electron microscopy (Hitachi S-3400N Variable Pressure SEM).

The buffering capacity of CS-ss-PEIs was determined by acid-base titration. 1 mg of each sample was dissolved in 2 mL of deionized water and the pH value was initially adjusted to 11 by 0.1 M NaOH. Then the pH value of solution was titrated to 3 by adding aliquots of 0.1 M HCl monitored by a microprocessor pH meter (PHM240).

2.4   Characterization of CS-ss-PEI/DNA complexes

2.4.1. Plasmid DNA preparation

Plasmid used included pGL3-control (Promega) encoding luciferase, pEGFP-N1 (Clontech) encoding a red-shifted variant of wild-type green fluorescent protein (GFP) and pCI-neo-BMP2 maintained in the lab encoding BMP2 gene. These plasmids were transformed into DH5α Escherichia coli, propagated in LB broth (Fluka) supplemented with 100 μg/mL ampicillin or kanamycin (Sigma), purified by Pure Yield^{TM} Plasmid Midiprep System (Promega), and stored in Tris-EDTA (TE) buffer (pH, 8) at -20°C respectively. The purity of plasmid DNA was determined to be above 1.8 by measuring the absorbance ratio of OD260/OD280 on ultraviolet (UV) spectrophotometer.

2.4.2. Formation of CS-ss-PEI/DNA complexes

The complexes of CS-ss-PEI and DNA were freshly prepared by mixing the equal volume of sterilized aqueous solution of copolymer and diluted DNA stock by deionized water. After briefly vortexing, the mixture was incubated for 30 min to allow the complexes formation. Various

weight ratios of complexes were reached by manipulating the concentration of copolymer to the same amount of DNA.

2.4.3. Gel retardation of complexes

The DNA condensation ability of CS-ss-PEIs was investigated by gel retardation. Agarose gel (1.0%) containing ethidium bromide (0.5 μg/mL) was prepared in Tris-Acetate-EDTA (TAE) buffer. Complexes solutions (8 μL) with various weight ratios were loaded into gels running at 90 V for 20 min. Each well contained 0.1 μg of DNA. The results were visualized by irradiation under UV light.

2.4.4. Transmission electron microscopy (TEM)

The morphology of the complexes was observed by TEM (Philips Tecnai G2 20 S-TEM). One drop of each complex solution was carefully dropped on a clean copper grid and negatively stained by 1.5 wt.% phosphotungstic acid (pH 6.7). The samples were dried at room temperature before imaging.

2.4.5. Particle size and zeta-potential

The particle size and surface charge of CS-ss-PEI/DNA complexes were measured by Zetasizer Nano ZS instrument (MALVERN Instrument) in triplicates at 25 ^{o}C. The complexes containing 2 μg of DNA at various weight ratios (0.5:1–25:1) were diluted by deionized water to 1 mL. The size was presented as the average value of five runs.

2.5. Transfection efficiency and cytotoxicity

2.5.1. Cell culture

African green monkey kidney cells (COS-1), human kidney cells (293T) and human hepatoblastoma cells (HepG2) were cultured in Dulbecco's Modified Eagles Medium (DMEM, GIBCO), supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin (Invitrogen) at 37°C in a humidified atmosphere.

2.5.2. Transfection efficiency

Cells were seeded in 24-well plate at an initial density of 6×10^4 cell/well and allowed to reach 70%~80% confluence. Before transfection, the cells were washed with PBS and refreshed with antibiotics free medium. Then the cells were treated with complexes containing 1 μg of

pGL3-control plasmid at various weight ratios and incubated for 24 h. Before examination, cells were refreshed with complete medium and cultured for another 48 h. The transfection efficiency was evaluated by the expressed luciferase activity normalized by the protein content. The luciferase activity was determined by luciferase assay on the cell lysate using a microplate luminometer LB96v according to the manufacturer's instruction. The protein content of the cell lysate was determined by BCA protein assay kit. All the experiments were carried out in triplicate. The GFP gene transfection was also conducted in 6-well plate with the similar procedure and observed under fluorescence microscopy.

2.5.3. Cytotoxicity

In vitro cytotoxicity of CS-ss-PEIs was evaluated by MTT assay. Cells were seeded in 96-well plates at an initial density of 1×10^4 cells/well and cultured in 100 μL medium containing cationic copolymers with different concentrations. The procedure was similar to the course of transfection for evaluating their cytotoxicity during transfection. The cytotoxicity of CS-ss-PEI/DNA complexes was also examined after transfection and at an early time point of 24h incubation. The MTT assay was performed according to the manufacturer's instructions, and the results were showed as the percentage of cell viability. Each value was averaged from six independent experiments.

2.6. Osteogenic differentiation

2.6.1. Cell culture

C2C12 myoblast cells were maintained in DMEM supplemented with 10% FBS and antibiotics. MG63 osteoblast-like cells were maintained in α-minimal essential medium (α-MEM) with 10% FBS and antibiotics. Murine bone marrow stromal cells (BMSC) were collected by flushing bone marrow and cultured in α-MEM with 20% FBS and antibiotics. After 72 h of culture, non-adherent cells were removed by rinsing with PBS and adherent stromal cells were seeded into six-well plate at a density of 5.0×10^5 cells/well.[22] MSCs isolated from human umbilical cord

(hUCB-MSC) were kindly donated by Chinese Eastern Union Stem cell & Gene Engineering Company and maintained in DMEM/F12 with 20% FBS and antibiotics.[23]

2.6.2. Osteogenic differentiation of skeletal cells

Cells were transfected with pCI-neo-BMP2 by CS-ss-PEI and induced for osteogenic differentiation. For transfection, cells were seeded at 4×10^5 cells/well in six-well plate with the similar procedure to the reporter gene transfection. Then the cells were induced for osteogenic differentiation in α-MEM with 20 mM β-glycerol phosphate (Sigma), 1 nM dexamethasone (Sigma), and 0.5 μM ascorbate 2-phosphate (Sigma). The medium was changed every 2 days.

The expressed BMP2 mRNA and protein in C2C12 cells after transfection were examined by RT-PCR and Western blot. After extraction of total RNA by Trizol, RT-PCR was performed using High-Capacity cDNA reverse Transcription kit (Applied Biosystems) and Platinum PCR superMix High Fidelity (Invitrogen) according to the manufacturer's instructions. In western blot analysis, the cell lysates were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories). The proteins were analyzed with Anti Smad1 and Anti-phospho-Smad1/5/8 (Cell signaling) antibodies and visualized by SuperSignal West Femto Substrate system (Pierce). For osteogenic differentiation of osteoblast cells, the alkaline phosphatase (ALP) activity was stained by Sigma Fast BCIP/NBT Tablets (B5655) at the 10 days after transfection. The quantitative result of ALP activity was examined by determining p-nitrophenol content using ALP substrate solution (Sigma) normalized by total intracellular protein determined by BCA protein assay kit. Calcium mineralization of osteoblast and MSCs was stained by Alizarin Red S on 21 days of culture and quantitatively analyzed by destaining using 10% (w/v) cetylpyridinium chloride (CPC) on 550 nm absorbance.

2.7. Statistical analysis

All the data presented are expressed as mean ± SD. The statistical analysis was made using ANOVA and a multiple comparisons test. Normality and homogeneity of the variances were checked using the Shapiro-Wilk and Levene test before ANOVA. The difference was considered statistically significant when p value was less than 0.05.

3. Results and discussion

3.1. Synthesis and characterization of CS-ss-PEI

Chitosan-disulfide-conjugated LMW-PEI (CS-ss-PEI) was prepared utilizing SPDP heterobifunctional cross-linking reagent under mild condition. Firstly, SPDP was reacted with amine of chitosan or low molecular weight PEI (LMW-PEI) to prepare thiolated polymer respectively. Then, the reduced free thiol-containing LMW-PEI and chitosan underwent oxidation in air at room temperature to form disulfide bond with formation of the copolymer, as showed in Fig. 1A. Different copolymers were prepared by varying the molar ratio of chitosan to LMW-PEI. The proposed transfection mechanism of CS-ss-PEI mediated gene transfection was showed in Fig. 1B with the intracellular reductive sensitivity.

During the reaction, the thiolated degree was analyzed by the average number of thiols conjugated on every molecular chain utilizing the concentration of generated pyridine-2-thione (PDP). The results were 0.89, 1.74, 2.96 and 3.52 for four different thiolated chitosan as well as 1.15 for the thiolated LMW-PEI respectively (Table 1). For chitosan, as the molar amount decreased in the reaction, the thiolation degree increased. Comparing with the initial feed ratio, the higher thiolation reaction rate could be found in PEI (38%) comparing with chitosan (22%~11%) as showed in table 1. This may be attributed to the higher amino density of PEI and less solubility of chitosan in the neutral reaction solution in terms of molecular chain. During the oxidation, the formed disulfide bonds were monitored using Ellman's assay. As shown in Fig 2A, the remaining free thiols were expressed in the percentage of the initial concentration. The reaction rates were similar for the different copolymers, which may be attributed to the same amount of SPDP added in each system. After 4 days of oxidation, about 85% of the thiols were oxidized to form disulfide bonds. The average molecular weight of CS-ss-PEI was determined by static light scattering (SLS) (Table 1). It is an well-established analytical method and has been extensively used to determine the weight-average molecular weight of various polymers. CS-ss-PEI1 showed the largest molecular weight among the copolymers around 60 kDa and CS-ss-PEI4 showed the smallest one around 16 kDa. The molecular weight decreased with the increment of LMW-PEI component. When treated with DTT, the average molecular weight decreased because of the degradation of disulfide indicating its reducible property.

The molecular structure of CS-ss-PEI was characterized by FTIR spectroscopy and

elemental analysis as shown in Fig. 2B and Table 2 respectively. In FTIR spectra, chitosan showed the representative bands at 1640 cm^{-1} and 1563 cm^{-1}, which were attributed to C=O stretching (amide I band) and N-H deformation (amide II band). The band at 1077 cm^{-1} corresponded to C-O stretching vibration.[24] PEI showed the characteristic peaks at 1660 cm^{-1} for NH$_2$ vibration and 1139 cm^{-1} for C-N stretching.[25] For CS-ss-PEI copolymers, with the increment of LMW-PEI component, there was an increase in the signal at around 1660 cm^{-1} contributed by NH$_2$ vibration. In addition, the signals at 1563 cm^{-1} for the amide II band and 1077 cm^{-1} for C-O stretching decreased with reduction of chitosan component. Meanwhile, the appearance of the signals at 910 cm^{-1} and 840 cm^{-1} in the copolymers were corresponding to the C-S stretching vibration introduced by the SPDP cross-linking reaction.[26, 27] The elemental compositions (C, N, O and S) of chitosan and their copolymers were analyzed by the semi-quantitative SEM-EDS (Table 2). With the increment of PEI component in the copolymers, the mole fraction of oxygen atom brought by chitosan decreased. The fraction of nitrogen atom was correspondingly increase for its higher content in LMW-PEI than chitosan. Sulfur was introduced during the reaction, so it could only be found in the copolymers and showed increase with LMW-PEI component. The structure characterization confirmed the formation of CS-ss-PEI.

The buffering effect polycations was assumed to facilitate the escaping of the complexes from endosomes. PEI is the most representative one and known for "proton sponge effect". The buffering capacity of CS-ss-PEIs was investigated by the acid-base titration (Fig. 2C). Chitosan was not studied here because of the poor solubility in base solution. The buffering capacity of the CS-ss-PEIs increased with the component of the LMW-PEI contributed by its high amino density. The enlarged figure at the top right corner gave a clearer view of the titration curve in the range of pH 5.1-7.4 which is the pH change from the extracellular environment to the lower pH value of the endosomes. The buffering capacity of CS-ss-PEI4 is very close to that of PEI.

3.2. Characterization of CS-ss-PEI/DNA complexes

DNA condensation is one of the prerequisites for gene transfer. Examined by agarose gel electrophoresis, the migrations of DNA in agarose gel were completely retarded when the weight ratio was higher than 2:1 (polymer:DNA), indicating that all polymers could electrostatically

neutralize the plasmid DNA and deter electrophoretic mobility as shown in Fig. 3A. With the increment of LMW-PEI component, the critical complex ratio dropped to 1:1. This was attributed to the relative stronger DNA-binding ability of PEI when compared with chitosan.

The surface properties of CS-ss-PEI/DNA complexes were investigated in morphology, particle size and zeta potential. Fig. 3B showed the representative morphology of complexes at weight ratio of 10 obtained by TEM. These complexes had spherical shape and compacted structure. The size of the complexes decreased with the increment of LMW-PEI component, corresponding to the gel retardation results.

The particle size of complex is an important factor which influences cellular uptake. It has been reported that the typical size for cellular uptake ranges from 50 to several hundred nanometer.[28] As shown in Fig. 3C measured by SLS, above the weight ratio of 5:1 when the copolymer could completely condense DNA, the sizes of the complexes were less than 200nm. The relatively homogenous size distributions of complexes were unimodal as shown in Figure S1. The particle size of the complexes decreased with increasing weight ratio and approached a plateau around 100nm. Within the same weight ratio, it showed a decrease with the increment of LMW-PEI component, which was similar to the TEM results. The size of the complexes during transfection is influenced by many factors and the measurement in water is a limitation in this study.

The complexes are formed upon self-assembly of negatively charged DNA with cationic polymer through electrostatic interaction, and the excessive cationic polymer contributes to the surface positive charge. This is necessary for the complexes binding to anionic cell surfaces for cellular internalization.[29] Fig. 3D showed the zeta potentials of the complexes at various weight ratios. At weight ratio 0.5, when the polymer could not completely condense DNA, the zeta potential of CS-ss-PEI/DNA complexes was almost negative except for CS-ss-PEI4. With the increase of weight ratio, the zeta potentials of the complexes rapidly increased to positive value and approached a plateau around 30 mV due to the saturation of polycations complexed with DNA.

3.3. Cytotoxicity and transfection efficiency of CS-ss-PEI

The cytotoxicity of CS-ss-PEI investigated by MTT assay was much lower than that of PEI and comparable to chitosan in COS-1 cells as shown in Fig. 4A. With the increment of LMW-PEI component, it showed slightly increase. The cytotoxicity of the complexes was even lower than that of polymer (Figure S2). This may be because part of the positive charge of polymer could be counterbalanced by the negative charge of DNA to minimize the direct contact with cell membrane.[30] Higher cell viability at earlier time point indicated that CS-ss-PEI mediated gene transfer does not have significant immediate toxicity as shown in Figure S3. Its cytotoxicity was also investigated with 293T and HepG2 cells. The highest cell viability of the copolymers was found in HepG2 cells. Although different cells showed different values, the tendency was similar to the situation of COS-1 cells as shown in Fig. 4B and 4C. PEI showed obvious cytotoxicity in the three kinds of cells. When the concentration of PEI was above 10 μg/mL, cells were barely alive.

The gene transfer ability of CS-ss-PEI was investigated using the reporter genes of luciferase and GFP. Fig. 5A showed the gene transfection activity in COS-1 cell evaluated by luciferase assay. It could be observed that the gene expression increased with complex weight ratio and gradually leveled off. The optimum weight ratios for gene transfer were in the range of 15 to 25. CS-ss-PEI4 showed the highest transfection efficiency among the copolymers. At the weight ratios of 20 and 25, the gene transfection efficiency was superior to that of PEI and compatible to lipofectamine. The enhanced gene delivery ability was attributed to the incorporation of LMW-PEI component and increment of molecular weight, at the same time it may also include the contribution of bio-reducible disulfide bonding. It could be found that CS-ss-PEI3 and CS-ss-PEI4 showed relatively higher gene delivery ability among CS-ss-PEI copolymers, so further evaluation on 293T and HepG2 cells for these two copolymers were performed (Fig. 5B, 5C). The results showed a similar trend. The transfection efficiency of CS-ss-PEI4 was higher than that of PEI both in 293T and HepG2 cells. In 293T cells, CS-ss-PEI4 showed 10-fold higher efficiency than that of PEI and even higher efficiency than that of Lipofectamine. For CS-ss-PEI3, it was similar to that of PEI. When applying the CS-ss-PEI4 for mediating GFP gene transfer, the clearly GFP expression could be observed in Fig. 5D

Comparing the three cell lines, the transfection activity of the copolymers showed

dependence on cell lines, and the highest transfection activity was observed in COS-1 cells for both the copolymers and the control groups. CS-ss-PEIs showed significant enhancement in efficiency when compared with chitosan in all of the three cell lines, especially for CS-ss-PEI3 and CS-ss-PEI4 with several hundred-fold increase.

3.4. Osteogenic differentiation by BMP2 gene transfection

CS-ss-PEI4 had shown the optimal transfection efficiency among CS-ss-PEIs in mediating reporter gene delivery. Here it was applied to deliver BMP2 gene *in vitro* for osteogenic differentiation. Since BMP2 was mainly synthesized by skeletal cells, C2C12 myoblast cells were used for examining the expression of BMP2. The mRNA expression of BMP2 could only be observed in the group of BMP2 gene transfection 1 day post-transfection as shown in Fig. 6A. The control group and the GFP transfection group showed negative results. Similarly, apparent phosphorylation of Smad1/5/8 was observed in BMP2 gene transfection group 2 days post-transfection (Fig. 6B), indicated that the expressed BMP2 possesses the formal function. Smad1/5/8 is the well-known downstream signaling pathway of BMP2. BMPs exert their biologic effects via the Smad-independent signaling pathway during which Smad1/5/8 is phosphorylated and translocated into the nucleus.[31] These results confirmed the expression of BMP2 in C2C12 cells after transfection mediated by CS-ss-PEI4.

Bone formation involves the differentiation of immature osteoblast and MSC. Transfection of MG-63 osteoblast cells with GPF gene by CS-ss-PEI4 could show clear GFP expression (Fig. 6C). By delivering BMP2 gene and osteoinduction, the enhanced osteogenesis could be observed on CS-ss-PEI4 group with darker red staining of Alizarin red at 21 days post-transfection comparing with the group of PEI and Lipofectamine (Fig. 6D). The quantification of mineralization was conducted by CPC extraction and the osteogenic effect showed in the order of CS-ss-PEI4 > PEI >Lipofectamine >control (Fig. 6E). This result suggested BMP2 gene delivered by CS-ss-PEI4 showed the stronger promoting effect in osteoblast differentiation and function.

The mesenchymal stem cell has the multiple differentiation capacity and could be induced osteogenic differentiation by BMP2. The osteogenic differentiation of BMSC was investigated through BMP2 gene transfection mediated by CS-ss-PEI4. Significant enhanced ALP activity in

transfection group could be observed both in the ALP staining (Fig. 6F) and in the quantitative assay (Fig. 6G) 10 days post-transfection. For the mineralization ability, there was clearly bone modules formed in transfection group stained by Alizarin Red with dark red which could not be found in the untransfected group (Fig. 6H). When applying this system to other kind of MSC, hUCB-MSC could also be induced for osteogenic differentiation which showed apparent calcium deposits in transfected group.

4. Discussion

Chitosan-disulfide-conjugated LMW-PEI (CS-ss-PEI) was synthesized by conjugating chitosan with low molecular weight PEI (LMW-PEI) utilizing disulfide. Different copolymers were prepared by tuning the amount of chitosan to the same amount of LMW-PEI. From CS-ss-PEI1 to CS-ss-PEI4, the component of chitosan decreased and LMW-PEI increased. Since chitosan has much higher molecular weight than LWM-PEI, the higher chitosan content resulted in higher molecular weight of the copolymer as indicated by the SLS results. During the preparation, SPDP was used for introducing the disulfide bond and maintained at the same molar ratio to LMW-PEI. As a result, the amount of disulfide in the copolymers increased with LMW-PEI component as shown by the results of elemental analysis. The increased disulfide did not contribute much to the molecular weight of the copolymer which was mainly affected by the chitosan component. However, the disulfide provided the copolymer with bioreducibility demonstrated by the reduced molecular weight after DTT treatment. The disulfide may also form between the molecular chain of chitosan or LMW-PEI themselves. This may contribute to the high molecular weight of CS-ss-PEI1 which possessed the highest chitosan component among the copolymers. However, the synthesized copolymers containing just chitosan or PEI content can hardly exist. Without PEI component, chitosan isn't soluble in water which could be removed during the purification. With 1.15 average number of thiol group on every LMW-PEI molecular chain, it has low chance to form large-molecular weight PEI preserved during dialysis purification.

The increased LMW-PEI component in the copolymer not only increased the buffer capacity, but also enhanced the DNA condensation ability. This influence is stronger than the one brought

by molecular weight. Plasmid DNA was complexed into denser nano-particles with lower critical complex ratio and smaller particle size. Before the endosomes matures, the gene carrier should be able to facilitate DNA escape, avoiding its degradation and inactivation.[32] It is widely accepted that the strong buffering capacity of PEI could successfully avoid its trafficking to acidic lysosomes, resulting in its high transfection efficiency.[33] The high density of nitrogen atom on its molecular backbone with the protonation potential gives it effective buffer capacity through a wide pH range.[34] However, the high charge density also results in the overly tight condensation of DNA which may affect the DNA dissociation and the cytotoxicity property.[35] The component of chitosan and reduction sensitive disulfide bond in CS-ss-PEI copolymer contributed to the high gene transfection efficiency of CS-ss-PEI4 in mediating reporter genes to different cells, which were even higher than that of PEI. The proposed mechanism of CS-ss-PEI mediated gene delivery was depicted in Fig. 1B.

CS-ss-PEI was designed to balance the contributions of every component to maximize the collective effects on its gene transfer ability and biocompatibility. Although PEI is efficient in gene delivery, it usually showed high cytotoxicity with does- and molecular weight-dependent characteristic. It is mainly caused by its strong positive charge, which leads to strong electrostatic interaction with the plasma membrane and resulted in their damage.[36] Furthermore, PEI is a non-degradable polymer and will accumulate in the body leading to an unknown risk for long-term use. As shown in the results of the cytotoxicity, cells were barely alive in PEI group when its concentration was above 10 μg/mL. Since lower molecular weight PEI and chitosan showed relative low cytotoxicity, CS-ss-PEI showed good biocompatibility as expected. More than 80% of cell viability could be observed in three kinds of cells when the concentration of polymer reached 100 μg/mL. When forming the complexes with DNA, it showed even lower cytotoxicity. More effort in further improving its biocompatibility will be conducted for *in vivo* evaluation by turning the molecular weight and cross-linking degree.

Efficient gene carrier is required to exhibit general significance in multiple cell types, which is also crucial for the extended therapeutic application in bone generation. This course involves the maturation of osteoblast for bone matrix synthesis and differentiation of mesenchymal stem cell into osteoblast lineage.[37] CS-ss-PEI4 was investigated in delivering BMP2 gene to multiple

cells for osteogenic differentiation. The BMP2 mRNA expression and the activated downstream signaling were observed in the transfected C2C12 myblasts cells as the confirmation of BMP2 expression and function. Inducing osteogenic differentiation of mesenchymal stem cells and enhancing the function of the differentiated osteoblast is the fundamental function of BMPs.[38] In MG63 immature osteoblast cells, CS-ss-PEI4 group showed stronger effect in inducing mineralization comparing with PEI and Lipofectamine groups. The cytotoxicity of PEI did negatively affect its effect. The stronger osteogenic effect for CS-ss-PEI4 mediated BMP2 gene transfer compared with Lipofectamine may benefit from the PEI component, which has been reported to show much higher transfection level than any other non-viral vector in mammalian cells.[39] Apart from its "proton-sponge" effect, the superiority of PEI in these cells has also been suggested to associate with the different mechanism of cellular internalization of polyplexes comparing with lipoplexes.[40] The primary cells such as MSC are relative more difficult to transfect comparing with usually used transfected cell lines. It has been observed that most gene carriers work effectively for immortal cells but failed in transfecting primary cells[41]. Bone marrow stem cell (BMSC) and human umbilical cord blood mesenchymal stem cells (hUCB-MSC) isolated from mammalian were induced for osteogenic differentiation through BMP2 gene transfer mediated by CS-ss-PEI4 and showed significant stronger mineralization ability comparing with untransfected cells. More studies in osteogenic differentiation of MSC and *in vivo* bone regeneration should be performed to further elucidate the effects of CS-ss-PEI mediated gene delivery for osteogenesis in future study.

5. Conclusions

Chitosan-disulfide-conjugated LMW-PEI (CS-ss-PEI) CS-ss-PEI was prepared by grafting chitosan with low molecular weight PEI utilizing disulfide bond. This copolymer showed good DNA binding ability. The size of the complex inversely decreased with the weight ratio and PEI component which is around 100 nm. It exhibited low cytotoxicity in maintaining above 80% of cell viability in different cells. In delivering luciferase gene, CS-ss-PEI4 showed the highest transfection efficiency among the copolymers which is higher than PEI and comparable to Lipofectamine in COS-1, 293T and HepG2 cells. With respect to osteogenesis, this copolymer

medicated BMP2 gene delivery showed significant effect on osteogenic differentiation of osteoblast and mesenchymal stem cells in vitro. This novel non-viral gene transfer method shows great potential in gene therapy for treating orthopaedic disease.

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Captions

Figure 1. CS-ss-PEI preparation and gene delivery mechanism. (A) Reaction scheme for the preparation of **CS-ss-PE**I; **(B)** Proposed scheme for the mechanism of CS-ss-PEI mediated gene delivery.

Figure 2. Characterization of CS-ss-PEI. (A) During the oxidation of free thiols to form disulfide bond, the reaction was traced by monitoring the remaining content of thiols using Ellman's assay; **(B)** Structure characterization by FTIR spectra: with the increase of LMW-PEI component, the copolymer showed increase signal at around 1640 cm^{-1}(◀), decreased signal at 1077 cm^{-1} (*) and appearance of the signal at 910 cm^{-1} and 840 cm^{-1}(☆); **(C)** Buffering capacity of the polymers by acid-base titration.

Figure 3. Characterization of CS-ss-PEI/DNA complexes. (A) Gel retardation of complexes for their critical complex ratio, Lane 0, pDNA only; Lane 1-9, complex at weight ratio of polymer to pDNA=1:10, 1:6, 1:4, 1:1, 2:1, 4:1, 6:1, 10:1, scale bar: 500 nm; **(B)** TEM images of complexes at weight ratio of 10:1; **(C)** Particle size and **(D)** zeta-potential of complexes at various weight ratios ranging from 0.5:1 to 25:1.

Figure 4. Cytotoxicity of copolymers at various concentrations in **(A)** COS-1, **(B)** 293T and **(C)** HepG2 cells by MTT assay.

Fiture 5. Transfection efficiency of CS-ss-PEI in mediating luciferase gene delivery in **(A)** COS-1, **(B)** 293T and **(C)** HepG2 cells. **(D)** GFP expression in COS-1 cells transfected by pEGFP-N1 mediated by chitosan, CS-ss-PEI4, PEI and Lipofectamine. Scale bar, 200 um.

Figure 6. Application of CS-ss-PEI4 to BMP2 gene delivery for osteogenic differentiation. (A) BMP2 mRNA expression in C2C12 cells; **(B)** Activation of Smad1/5/8 as the downstream signaling pathway of BMP2 in C2C12 cells; **(C)** GFP expression in MG-63 osteoblast cells transfected by pEGFP-N1 mediated by CS-ss-PEI4, PEI and Lipofectamine. Scale bar, 200 um; **(D)** Mineralization of MG-63 stained by Alizarin Red S on 21 day, and **(E)** semi-quantificational analysis; **(F)** Alkaline phosphatase (ALP) activity in BMSC and **(G)** the quantitative analysis; **(H)** Mineralization of BMSC and hUCB-MSC stained by Alizarin Red S on 21 day. Scale bar, 100 um.

Table 1. Preparation and characterization of CS-ss-PEI

Table 2. SEM-EDS analysis of the elemental compositions (C, N, O and S) of chitosan and CS-ss-PEIs (n=3)

Graphical Abstract (for review)

1

Figure 1.

(A)

(B)

Figure 2.

Figure 3.

(A)

(B)

(C)

(D)

Figure 4.

Figure 5.

(A)

COS-1

Legend: Chitosan, CS-ss-PEI1, CS-ss-PEI2, CS-ss-PEI3, CS-ss-PEI4, LMW-PEI

Y-axis: RLU/mg protein

X-axis: Cell, pDNA, 5:1, 10:1, 15:1, 20:1, 25:1, PEI, Lipo

Weight ratio (polymer:DNA)

(B) 293T

Legend: Chitosan, CS-ss-PEI3, CS-ss-PEI4

Y-axis: RLU/mg protein

X-axis: Cell, pDNA, 10:1, 15:1, 20:1, PEI, Lipo

Weight ratio (polymer:DNA)

(C) HepG2

Legend: Chitosan, CS-ss-PEI3, CS-ss-PEI4

Y-axis: RLU/mg protein

X-axis: Cell, pDNA, 10:1, 15:1, 20:1, PEI, Lipo

Weight ratio (polymer:DNA)

(D) COS-1

Chitosan   CS-ss-PEI4   PEI   Lipo

5

Figure 6

(A) C2C12
(B) C2C12
(F) BMSC
(C) MG-63
(D) MG-63
(E) MG-63
(G) BMSC
(H)
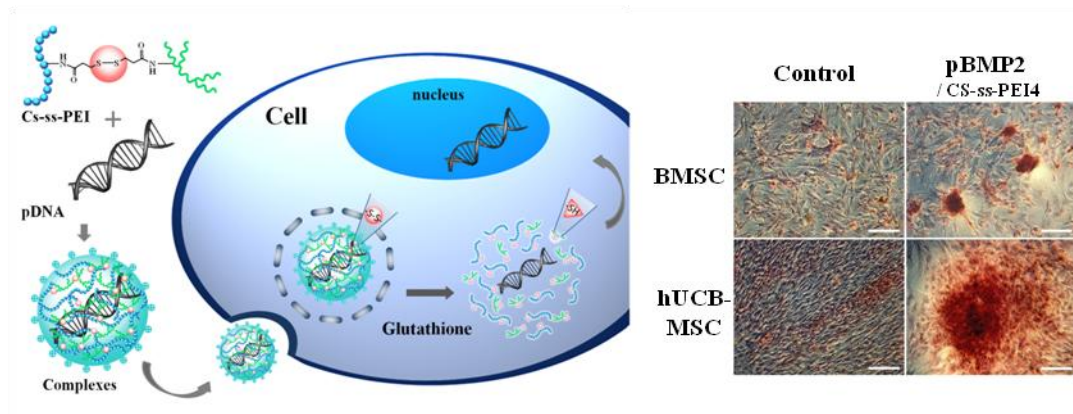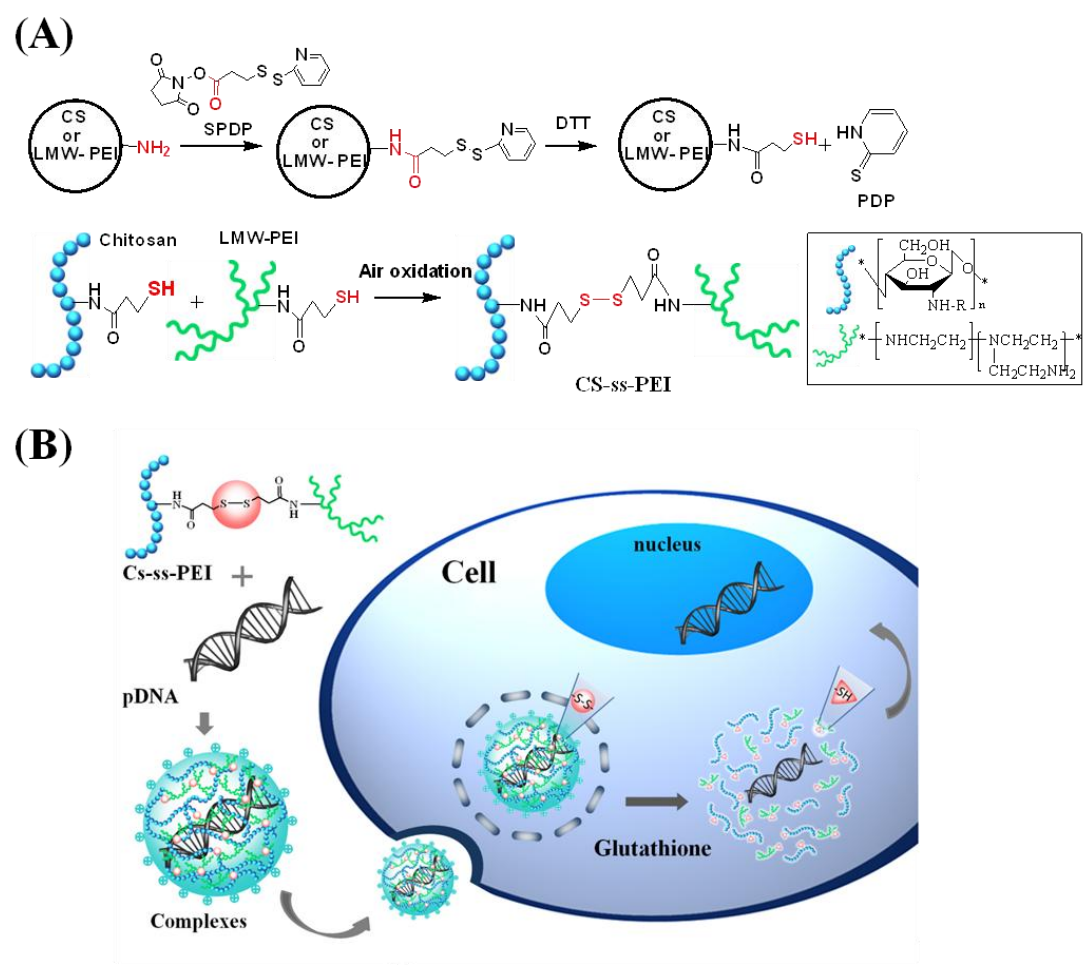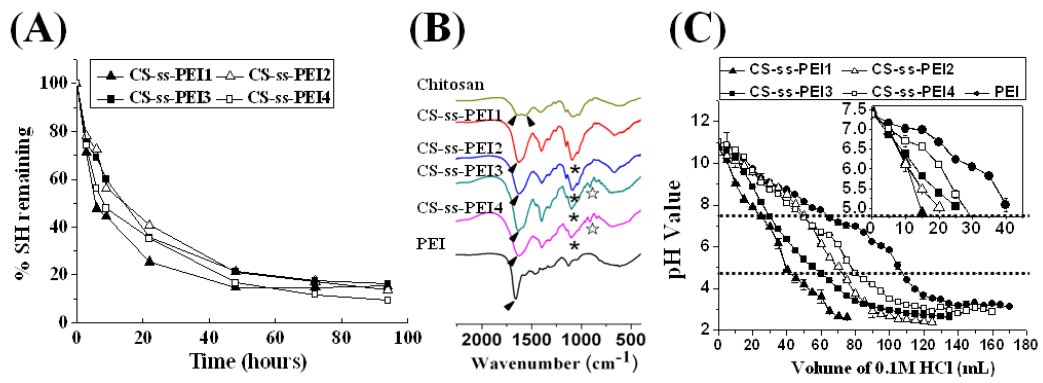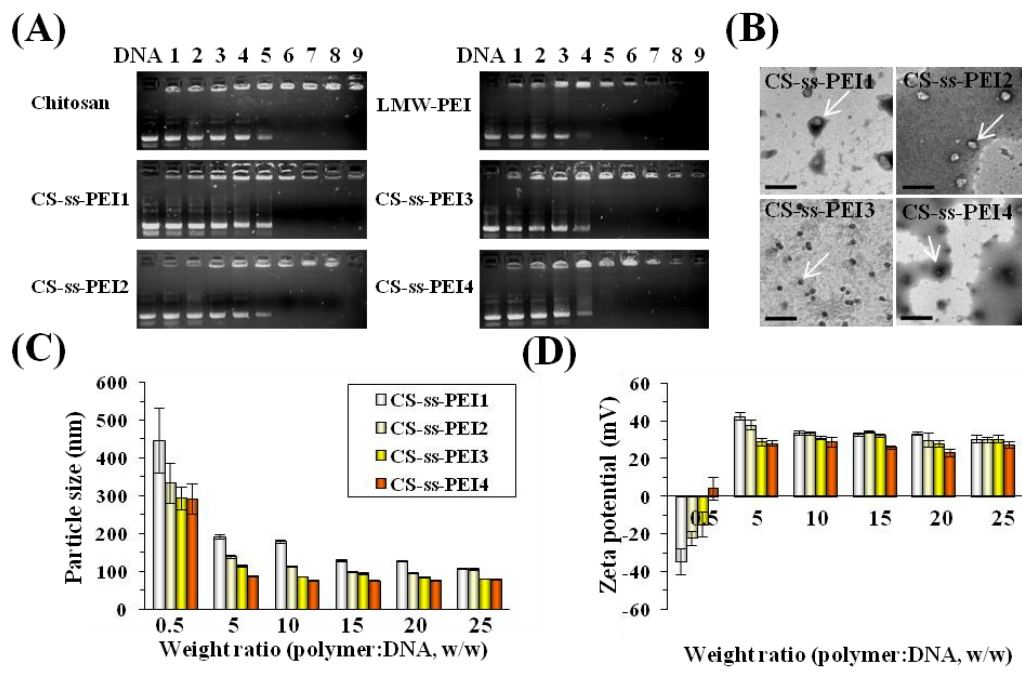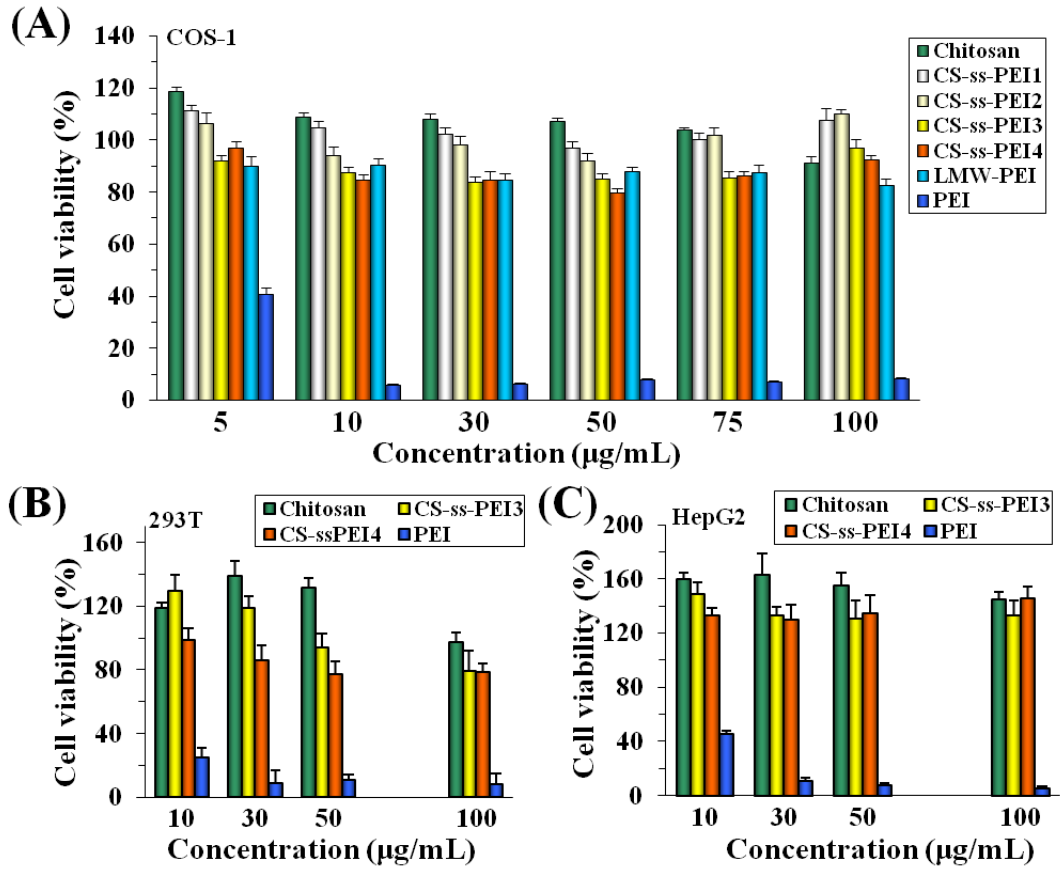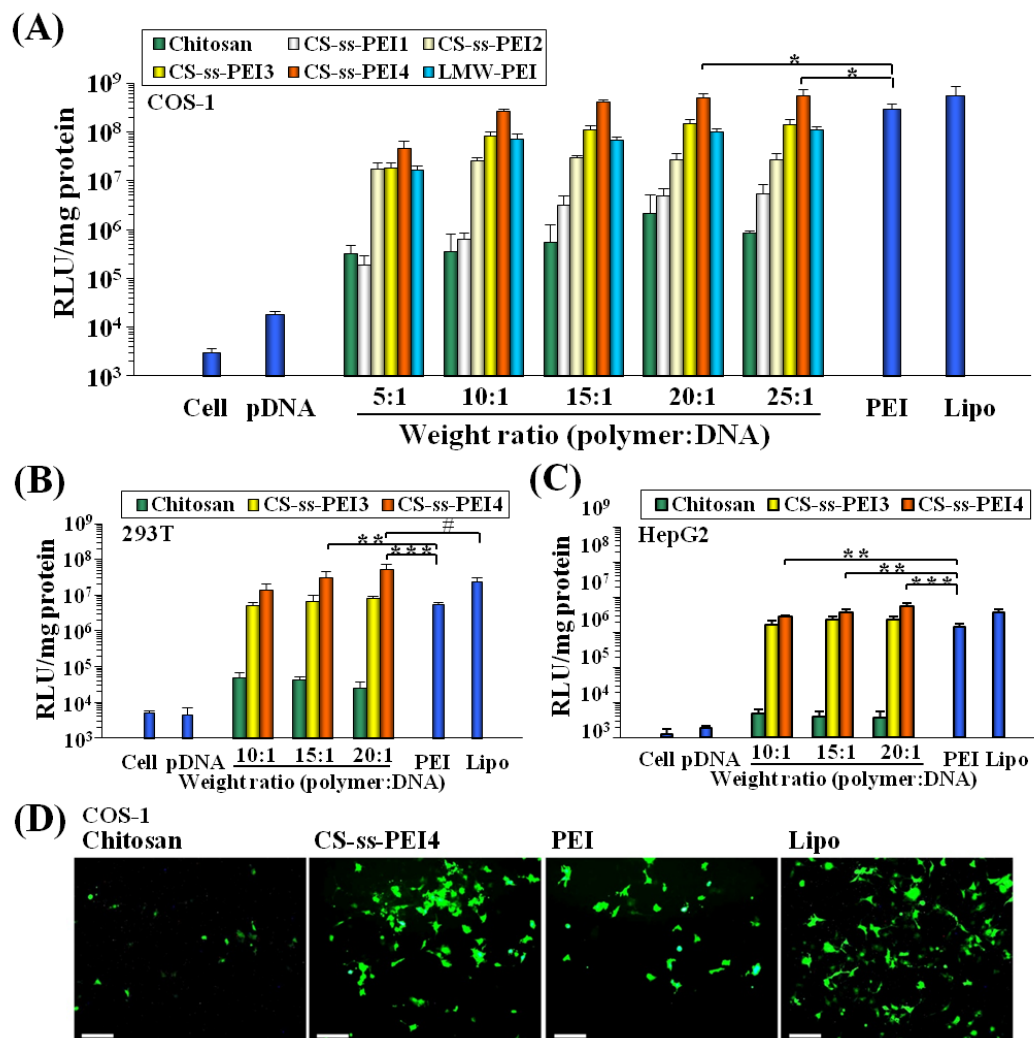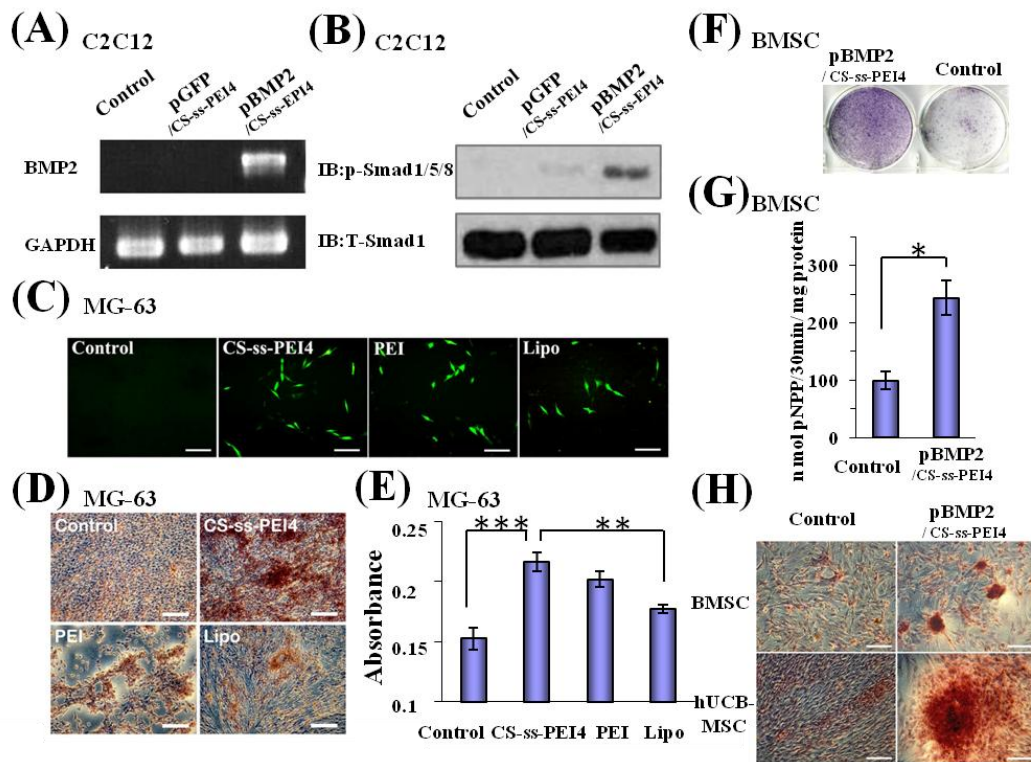
Table(s)

Table 1.

Samples	Preparation			Thiolation		Products		DTT treat
	Feed (mg, nM)	SPDP Feed (mg, nM)	Feed rate	Thiol[1] /Chain	Thiolation rate (%)		MW[2] (kDa)	MW[2] (kDa)
Chitosan-1	10, 1	1.25, 4	4	0.89	22	CsP1	59.5	50.0
Chitosan-2	5, 0.5	1.25, 4	8	1.74	22	CsP2	19.75	16.8
Chitosan-3	2.5, 0.25	1.25, 4	16	2.96	19	CsP3	17.2	12.5
Chitosan-4	1.25, 0.125	1.25, 4	32	3.52	11	CsP4	16.6	8.6
PEI	5, 2.8	2.5, 8	3	1.15	38	Chitosan	11.35	12

1, The average number of thiol groups on every molecular chain is calculated by monitoring the generation of PDP group at 343 nm upon treatment of 20mM DTT (molar absorptivity=8080 $cm^{-1}M^{-1}$).

2, Molecular weight determined by static light scattering.

Table 2.

Sample	C mol%	N mol%	O mol%	S mol%
Chitosan	53.97±0.72	7.40±0.57	38.66±0.57	0.01±0.01
CS-ss-PEI1	50.39±0.47	9.92±0.8	39.58±0.48	0.11±0.02
CS-ss-PEI2	49.15±0.52	11.27±1.10	39.37±1.08	0.22±0.04
CS-ss-PEI3	50.88±1.02	12.78±1.16	35.99±2.19	0.343±0.1
CS-ss-PEI4	50.01±1.89	16.33±1.18	33.20±2.17	0.45±0.09

Supplementary Material