

Delivery of a transforming growth factor β -1 plasmid to mesenchymal stem cells via cationized *Pleurotus eryngii* polysaccharide nanoparticles

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Abstract: The objective of this study was to investigate the use of cationized *Pleurotus eryngii* polysaccharide (CPEPS) as a nonviral gene delivery vehicle to transfer plasmid DNA encoding transforming growth factor beta-1 (pTGF- β 1) into mesenchymal stem cells (MSCs) in vitro. Crude *P. eryngii* polysaccharide was purified, and then cationized by grafting spermine onto the backbone of the polysaccharide. Agarose gel electrophoresis, transmission electron microscopy, and a Nano Sense Zetasizer (Malvern Instruments, Malvern, UK) were used to characterize the CPEPS-pTGF- β 1 nanoparticles. The findings of cytotoxicity analysis showed that when the nanoparticles were formulated with a CPEPS/pTGF- β 1 weight ratio $\geq 10:1$, a greater gel retardation effect was observed during agarose gel electrophoresis. The CPEPS-pTGF- β 1 nanoparticles with a weight ratio of 20:1, respectively, possessed an average particle size of 80.8 nm in diameter and a zeta potential of $+17.4 \pm 0.1$ mV. Significantly, these CPEPS-pTGF- β 1 nanoparticles showed lower cytotoxicity and higher transfection efficiency than both polyethylenimine (25 kDa) ($P = 0.006$, Student's *t*-test) and Lipofectamine™ 2000 ($P = 0.002$, Student's *t*-test). Additionally, the messenger RNA expression level of TGF- β 1 in MSCs transfected with CPEPS-pTGF- β 1 nanoparticles was significantly higher than that of free plasmid DNA-transfected MSCs and slightly elevated compared with that of Lipofectamine 2000-transfected MSCs. Flow cytometry analysis demonstrated that 92.38% of MSCs were arrested in the G1 phase after being transfected with CPEPS-pTGF- β 1 nanoparticles, indicating a tendency toward differentiation. In summary, the findings of this study suggest that the CPEPS-pTGF- β 1 nanoparticles prepared in this work exhibited excellent transfection efficiency and low toxicity. Therefore, they could be developed into a promising nonviral vector for gene delivery in vitro.

Keywords: nonviral gene vector, transfection, plasmid DNA, spermine

Introduction

Gene therapy has been well defined as a strategy for transferring nucleic acids, such as therapeutic DNA, antisense oligonucleotides, and small interfering RNA, to the target tissues or cells to correct or supplement the defective genes that are responsible for disease development.¹⁻⁴ The ideal gene delivery system must be able to protect the nucleic acid from many types of degrading systems such as DNA-degrading enzymes and lysosomes,⁵ in order to penetrate the cell membrane and gain entry into the target cells, and to promote efficient gene expression. The system must also possess specific properties including biocompatibility, biodegradability, nontoxicity, nonimmunogenicity, and stability, during storage and treatment.^{1,6} However, the lack of safe and efficient vectors for DNA delivery is currently a major hurdle for the success of gene therapy.

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Gene delivery systems can be classified into viral and non-viral systems.⁷ Despite the fact that viral gene carriers are widely employed in clinical treatments due to their high transfection efficiency and long-term gene expression,^{8,9} there are several limitations that impede their applications, including immunogenic properties and the potential to cause mutational infection and toxic side effects.¹⁰ Nonviral gene vectors have emerged as a promising alternative to viral vectors because they offer such advantages as low immunogenicity, increased biological safety, the ability to deliver large genes, excellent flexibility in their building block structures, and the possibility of large-scale production at a reasonable cost.^{11,12}

As the leading nonviral gene vectors, cationic polymers can form complexes with the negatively charged nucleic acids so that large pieces of these nucleic acids can be condensed to nanometer-sized particles. This promotes the interaction of cationic polymers with the negatively charged cell membrane, which protects the incorporated nucleic acids in the complex from many types of degrading systems.^{13,14} A large number of cationic polymers have been designed as gene carriers, such as cationized gelatin,¹⁵ poly(L-lysine) (PLL) polymers,^{16–18} polyethylenimine (PEI),^{19–21} and so on. However, these polymers suffer several inherent disadvantages including high toxicity, poor biodegradability, and low transfection efficiency.

In recent years, cationic polysaccharide, a type of natural, water-soluble, nontoxic, biocompatible and biodegradable cationic polymer, has been receiving increasing scientific attention and is considered to be the most attractive candidate for gene transfer.²² Compared with other cationic polymers, cationic polysaccharide presents such advantages as the presence of groups that can be easily modified to improve the physicochemical properties and cellular uptake, which can be facilitated by a sugar-recognition receptor on the cell surface.²³ Chitosan, a naturally occurring linear amino polysaccharide, has been reported as a vector for gene delivery.²⁴ Researchers have found that apart from its biocompatibility, biodegradability, and low toxicity, chitosan and its derivatives showed excellent ability to transfer genes into cells.^{25–27} However, chitosan has demonstrated lower transfection efficiency compared with conventional nonviral gene carriers such as liposomes,²⁸ PEI,²⁹ and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA).³⁰ Other leading cationic polysaccharides that have been investigated as gene vectors include dextran,^{7,31–33} pullulan,^{34,35} pectin,³⁶ and schizophyllan.^{22,37}

The cationization of polysaccharide is a promising strategy to develop nonviral gene vectors for use in gene therapy.^{38,39} Polysaccharides represent a structurally diverse

class of macromolecules of widespread occurrence in nature and offer a high capacity for carrying biological information because they have great potential for structural variability.⁴⁰ In support of the above findings, the aim of this study was to develop a nonviral gene vector by grafting spermine residues onto a polysaccharide extracted from *Pleurotus eryngii*. *P. eryngii* is an edible mushroom which is very common in China. Mushrooms are known for their nutritional and medicinal values as well as for the diversity of their bioactive components such as nucleases,⁴¹ proteases,⁴² and polysaccharides.^{43,44} They also contain polysaccharide–peptide and polysaccharide–protein complexes.⁴⁵ So far, most of the research regarding *P. eryngii* has focused on its enzymes.^{46–48} One of the major components of *P. eryngii*, the polysaccharide of *P. eryngii* (PEPS), has been studied because of its positive health effects, which include cholesterol-lowering, antioxidant, and anticancer activities; however, little investigation into its application as a gene vector has been carried out.⁴⁹ Previous studies have indicated that the polysaccharides isolated from *P. eryngii* were mostly β -glucans, which exhibited potential prebiotic activities.⁵⁰ Therefore, to construct a cationic polysaccharide for use as a gene carrier, this study chemically modified PEPS by the reductive amination method. Cationized PEPS (CPEPS) enjoys the merits of both the positive charge of amine compounds and the bioactivity of PEPS. This advantage is favorable for delivering plasmid DNA into cells in vitro.

Nanoparticles are a desirable vehicle for gene delivery because of their nanoscale particle size, good stability, and excellent diffusion properties, which may facilitate cellular uptake.^{51,52} The plasmid encoding transforming growth factor beta-1 (TGF- β 1) was used in the study. TGF- β 1 is a widely used cytokine which can participate in and regulate many kinds of biochemical processes, including cell growth and differentiation, the formation of extracellular matrices, cytoadherence, immunoregulation, embryonic development, and wound healing.^{53,54} It has been reported that TGF- β 1 was able to induce mesenchymal stem cells (MSCs) to differentiate into chondrocytes in three-dimensional scaffolds, and this study is exactly aimed at this direction. Therefore, plasmid TGF- β 1 (pTGF- β 1) was combined with CPEPS, resulting in CPEPS-p TGF- β 1 nanoparticles that would have the ability to condense large plasmid DNA into nanoscaled supermolecular assemblies. The driving force for the complexation comes from the attraction between the two oppositely charged ions of each polyelectrolyte. Such properties as particle size distribution, zeta potential, and gel retardation

effect were rigorously tested to characterize the CPEPS. Most importantly, the *in vitro* transfection efficiency of the CPEPS/plasmid DNA nanoparticles was investigated, and cytotoxicity was also tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.⁵⁵ It is expected that CPEPS could be developed into a promising nonviral gene vector for gene therapy.

Materials and methods

Materials

P. eryngii were kindly provided by the Zhenjiang edible mushroom growth base (Zhenjiang, China). Spermine and branched PEI (molecular weight 25 kDa) were purchased from Sigma-Aldrich (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, trypsin, MTT, fetal bovine serum (FBS), and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). All other chemicals and reagents were of analytical or even higher grade and were used without further purification. All solutions were prepared with double distilled water (DDW). The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the *Guide for the Care and Use of Laboratory Animals*.⁵⁶

Polysaccharide extraction and purification

The polysaccharide was extracted from the fruiting bodies of *P. eryngii* by purification, as illustrated in Figure 1. The milled fruiting bodies of *P. eryngii* (2000 g) were extracted with 2 L of distilled water at 90°C for 3 hours (repeated three times). The aqueous extracts were concentrated under reduced pressure at 50°C and precipitated with absolute ethanol (with a final ethanol concentration of 75% (v/v)). The precipitate was washed twice with absolute ethyl alcohol, and further dissolved in distilled water. This was dialyzed for 48 hours against distilled water (molecular weight cutoff [MWCO] 8000–14,400, Biosharp).³⁹ The retentate portion was centrifuged to remove insoluble material. The supernatant was lyophilized, and then primrose-yellow crude PEPS was obtained.

The crude PEPS was purified by anion–exchange chromatography on a column (*D* 2.6 cm × 40 cm, Shanghai Huxi Analysis Instrument Factory Co, Ltd, China) filled with DEAE-52 cellulose resin (Whatman, UK). Then, the fractions that were collected from the DEAE-52 cellulose resin chromatographic column were loaded onto the SephadexG-100 (Shanghai Richu Bioscience Co, Ltd, China) gel chromatographic column (*D* 2.6 cm × 40 cm, Shanghai Huxi Analysis Instrument Factory). Fractions were collected and monitored

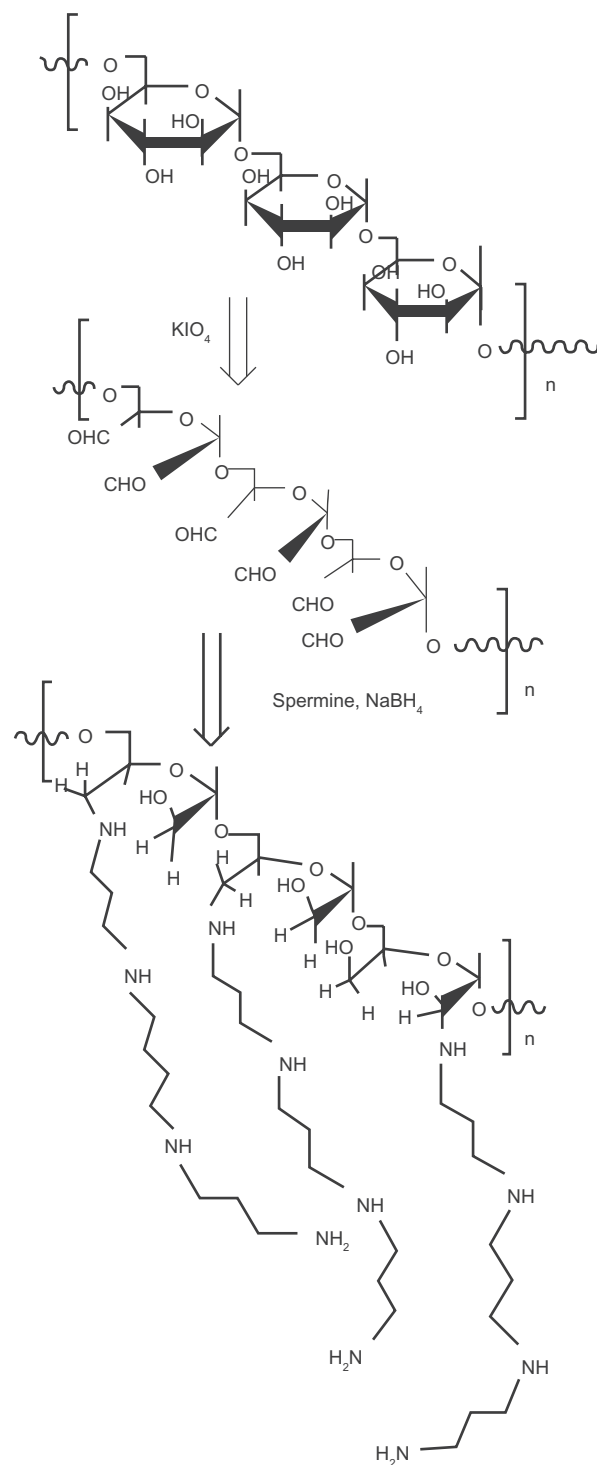


Figure 1 The process of polysaccharide of *Pleurotus eryngii* extraction and purification.

for the presence of carbohydrate with a phenol–sulfuric acid assay.⁵⁷ A single peak was observed with an ultraviolet (UV) absorption photometry instrument (UV2401PC, Shimadzu, Tokyo, Japan). The target fractions were combined and dialyzed against distilled water (MWCO 3500, Biosharp).³⁹ After lyophilization, purified PEPS was obtained.

Preparation of CPEPS

The CPEPS was prepared by the reductive amination method (Figure 2) according to a previous study,³² with slight modifications. The process is shown in Figure 3. The PEPS (0.5 g, 3.125 mmol of glucose units) was dissolved in 50 mL of DDW which was followed by the addition of potassium periodate (0.716 g, with an IO_4^- /saccharide molar ratio of 1:1). The mixture was quickly placed in a darkroom to allow it to react at room temperature with vigorous magnetic stirring for 72 hours. The resulting polyaldehyde derivative was dialyzed (MWCO 3500, Biosharp)³⁹ for another 48 hours against DDW. After freeze-drying, oxidized PEPS was obtained.

The aldehyde content was determined according to Jun-ichiro et al.⁵⁸ Oxidized PEPS (0.3 g) was dissolved in 20 mL of freshly prepared hydroxylamine hydrochloride water solution (0.25 M, pH 4). The resulting mixture was then gently stirred overnight at room temperature, followed by titration with standardized sodium hydroxide solution (0.1 M) till the end point as recorded on a digital pH meter (model PHS-3TC, Shanghai Tainda, Shanghai, China) was reached.

A solution of oxidized PEPS (containing 6.77 mmol of aldehyde groups) in 50 mL of DDW was slowly added (using a Sage Metering pump model 365 [Sage Metering,

Monterey, CA]) over 2 hours to a basic solution containing a 1.25 equimolar amount of spermine (8.46 mmol) dissolved in 30 mL of borate buffer (0.1 M, pH 11). The mixture was then gently stirred at room temperature for 24 hours. Then, NaBH_4 (0.3 g) was added to the mixture to react for 48 hours and the process was repeated with an additional portion of NaBH_4 (0.3 g) for another 24 hours under the same conditions. The resulting light-yellow solution was dialyzed (MWCO 3500, Biosharp) for 48 hours against DDW. After freeze-drying, cationized PEPS (CPEPS) was obtained.

Characterization of PEPS and CPEPS

Based on the literature and the limited experimental conditions in our laboratory, in this study, the average molecular weights of PEPS and CPEPS were analyzed with a Shimadzu gel permeation chromatography (GPC) system-equipped Shimadzu RID-10A refractive index detector (RID) (LC-10 AVP, Shimadzu). Analysis of the monosaccharide composition of PEPS was preliminarily determined by thin layer chromatography. After the spermine modification, the quantity of spermine residues that were grafted onto the PEPS was reflected by the amount of primary amino groups determined by the trinitrobenzene sulfonic acid

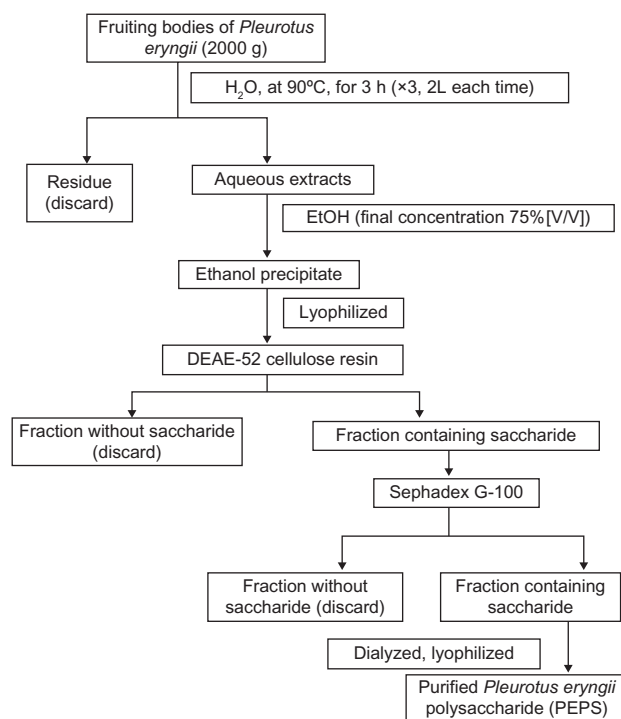


Figure 2 The process of spermine grafting to the polysaccharide (dextran is used as an example).

Notes: The reductive amination method is used to produce the cationized polysaccharide: the polysaccharide is first oxidized by KIO_4 ; after the addition of spermine, NaBH_4 is used to reduce the reactants.

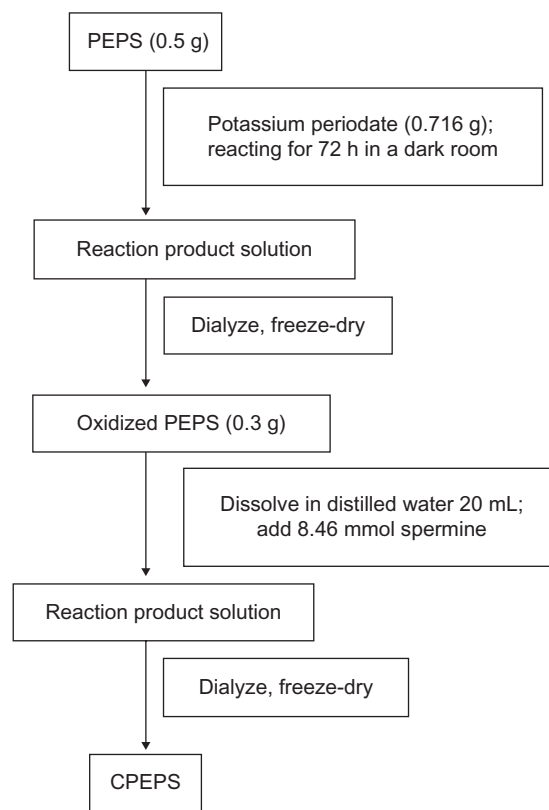


Figure 3 The process of cationized *Pleurotus eryngii* polysaccharide (CPEPS) preparation.

method. The quantity of total nitrogen per unit weight of polymer was determined with a Euro EA elemental analyzer. A Fourier transform infrared spectrometer (KBr) (Nicolet 170SX, Thermo Fisher Scientific, Waltham, MA) was used to characterize the PEPS and CPEPS and to retrieve their structural information.

Preparation of plasmid DNA

The TGF- β 1 was amplified in *Escherichia coli* host strain DH5 α and purified by column chromatography with the PureYield™ Plasmid Maxiprep Start-Up Kit (Promega, Madison, WI) according to the manufacturer's protocol. Ampicilin was used to select for the pTGF- β 1-transformed cells. The DNA concentration was quantified by measuring the UV absorbance at 260 nm with a UV spectrophotometer (DU 530 Life Science UV/Vis spectrophotometer; Beckman Coulter, Fullerton, CA).

Preparation of CPEPS-pTGF- β 1 nanoparticles

The CPEPS-pTGF- β 1 nanoparticles were prepared by complex coacervation. A number of CPEPS-pTGF- β 1 nanoparticle samples that differed in their CPEPS/pTGF- β 1 weight ratios were prepared. The CPEPS stock solution (8 mg/mL) was made by dissolving 8 mg CPEPS in 1 mL distilled water, which was then sterilized by heating at 80°C for 4 hours. The required solutions for the different CPEPS concentrations and that of the pTGF- β 1 concentration (400 μ g/mL) were prepared with sterilized DDW. Meanwhile, aliquots (100 μ L) of each of the CPEPS and pTGF- β 1 working solutions were heated separately at 55°C for 30–45 minutes. Equal volumes of each solution were then quickly mixed and vortexed for 60 seconds to obtain the CPEPS-pTGF- β 1 nanoparticles.

Analysis of gel retardation effect of CPEPS-pTGF- β 1 nanoparticles

The CPEPS-pTGF- β 1 retention effect was analyzed using gel electrophoresis. CPEPS-pTGF- β 1 nanoparticle solutions (10 μ L) with different weight ratios were mixed with 1 μ L loading buffer (0.1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue) and put into 1% agarose gel in tris-borate-ethylenediaminetetraacetic acid buffer solution (pH 8.0) containing 1 μ g/mL ethidium bromide. The diluted free pTGF- β 1 solution (10 μ L) was used as a control. Electrophoretic evaluation of the complex was carried out in tris-borate-ethylenediaminetetraacetic acid buffer solution at 80 V for 90 minutes. A photograph of the gel was

taken with a UV transilluminator (Gel Doc 2000, Bio-Rad, Hercules, CA).

Zeta potential

The zeta potentials of the nanoparticle suspensions, which had different weight ratios of CPEPS to pTGF- β 1 (10:1, 20:1, and 30:1), were measured with a ZEN3600 Nano Series Zetasizer (Malvern Instruments, Malvern, UK). The zeta potentials of the free plasmid and original CPEPS were determined under the same conditions. This measurement would reflect the degree of cationization.

Determination of the nanoparticle size distribution

The nanoparticle size distribution was determined by a dynamic light scattering (DLS) technique, performed at 25°C with a Brookhaven BI-90plus instrument (Brookhaven Instruments Corporation, Holtsville, NY). The measured scattering intensities were then analyzed by the software provided by Brookhaven. The determination limits of the size distribution generally ranged from 1 nm to 6 μ m, with high sensitivity and reproducibility.

Transmission electron microscopy (TEM)

TEM (JEM-2100; JEOL, Tokyo, Japan) was used to detect the size and shape of the nanoparticles. Samples were prepared by placing 1 μ L nanoparticle suspension onto a copper screen and allowing them to air-dry. Then, the air-dried samples were observed directly under TEM without the need to coat the samples with a conducting layer as is required for conventional scanning electron microscopy.

Isolation and culture of MSCs

The method of isolation and culture of MSCs was as described by Alhadlaq and Mao.⁵⁹ The MSCs were isolated from 1-month-old rats by flushing the femurs and tibias with phosphate-buffered solution (PBS, pH 7.4). This was followed by the collection of 5 mL bone marrow suspensions into centrifuge tubes. Lymphocyte cell separation buffer (5 mL) was gently added into each tube along the walls of the tube. After centrifugation, a mist-like layer containing most of the MSCs had formed. The mist-like layer was then carefully collected into new tubes and washed twice with PBS. After centrifugation, the precipitate was suspended in cell culture medium containing DMEM, 10% FBS, low glucose (1%), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The resulting suspension was transferred into cell culture flasks. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cytotoxicity assay

The *in vitro* cytotoxicity of the different CPEPS-pTGF- β 1 nanoparticles (weight ratios 10:1; 20:1; 30:1) was examined by MTT dye reduction assay. The second-passage MSCs were seeded in a 96-well plate at a cell density of 2.5×10^4 cells/well and incubated at 37°C for 24 hours in 100 μ L of DMEM containing 10% FBS. After that, the medium was removed and replaced with a 100 μ L suspension of CPEPS-pTGF- β 1 nanoparticles (pTGF- β 1 200 ng/well) which had been diluted in a serum-free medium. Lipofectamine 2000/pTGF- β 1 and branched PEI (25 kDa)/pTGF- β 1 were employed as control groups according to each manufacturer's protocol, under the same conditions. This was to ensure that the optimal ratios for transfection were used. The concentration of pTGF- β 1 in each group was 2 ng/ μ L. The same concentration of pTGF- β 1 was used in the subsequent experiments.

Cell viability was tested after the addition of the CPEPS-pTGF- β 1 complexes for 48 hours at 37°C in 5% CO₂. After that, 10 μ L of MTT solution (5 mg/mL) was added to each well for an additional 4-hour incubation under the same conditions. The MTT-containing medium was then removed, and 100 μ L dimethyl sulfoxide was added. Dimethyl sulfoxide was used to dissolve the formazan crystals that were formed by the living cells. The absorbance was measured at 570 nm using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA). The measured absorbance was normalized with the absorbance of nontreated control cells.

In vitro transfection experiments

In the transfection experiment, second-passage MSCs were seeded in 96-well plates at a density of 2.5×10^4 cells/well in 100 μ L complete culture medium (DMEM, containing 10% FBS) and incubated for a period of 24 to 48 hours to obtain a confluence of 80% before transfection. The medium was then removed and replaced with a 100 μ L suspension of CPEPS-pTGF- β 1 nanoparticles (pTGF- β 1 200 ng/well) in a serum-free medium, which contained DMEM, 100 U/mL, and 100 μ g/mL streptomycin glutamate. In the positive control groups, Lipofectamine 2000/pTGF- β 1 and branched PEI (25 kDa)/pTGF- β 1 were handled rigorously according to the protocols provided by the manufacturers and naked pTGF- β 1 (200 ng/well) in serum-free medium was used for the negative control group. Four hours later, the medium was replaced with 100 μ L fresh complete medium and the cells were incubated for 72 hours. A rat TGF- β 1 enzyme-linked immunosorbent assay kit (Yantai Addcare Biotech, Shandong, China) was used to detect cell transfection efficiency, according to the protocol.

RNA isolation and reverse transcriptase polymerase chain reaction

Similar to the enzyme-linked immunosorbent assay test, second-passage MSCs were seeded in six-well plastic culture plates at a density of 2×10^6 cells/well in 2 mL complete culture medium (DMEM, containing 10% FBS) and incubated for 24–48 hours to obtain a confluence of 80%. This was followed by transfection as described above. After 24 hours of transfection, total RNA was extracted using TRIzol[®] Reagent (Invitrogen), thus following the instructions provided by the manufacturer. RNA concentration and purity were measured by a spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Reverse transcriptase polymerase chain reaction analysis was carried out to determine the messenger RNA (mRNA) expression level of TGF- β 1. The reverse transcriptase reaction was conducted with 1.0 μ g total RNA using a RevertAid[™] cDNA First Strand Synthesis Kit (K1622; Thermo Fisher Scientific, Shenzhen, China). The following polymerase chain reaction (PCR) amplification reaction utilized the *Taq* polymerase and specific primers. The specific sequences of the primers for PCR were 5'-TGGTGGACCGCAACAACGCA-3' (forward primer) and 5'-TGCACGGGACAGCAATGGGG-3' (reverse primer) (GenBank Accession No NM 021578.2). In this study, glyceraldehyde 3-phosphate dehydrogenase was used as an internal standard. The PCR was run in an iCycler (Bio-Rad) using a Brilliant II SYBR[®] Green QPCR Master Mix (Stratagene, La Jolla, CA). The PCR conditions were as follows: 95°C for 10 minutes (for initial denaturation), followed by 26 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at 57°C for TGF- β 1 and at 58°C for glyceraldehyde 3-phosphate dehydrogenase, extension at 72°C for 30 seconds. The PCR products were visualized on a 2% (w/v) agarose gel containing 1 μ g/mL ethidium bromide with a UV transilluminator (Gel Doc 2000, Bio-Rad).

Flow cytometry analysis

Cell cycling signals were analyzed using a Gallios[™] Flow Cytometer (Beckman Coulter, Miami, FL). In this study, classic procedures were followed to evaluate propidium iodide (Beyotime Institute of Biotechnology, Shanghai, China), staining of DNA content. After 72 hours of transfection, cells were washed twice with PBS (pH 7.4) and detached from the wells with trypsin. The suspended cells were placed in sterilized centrifuge tubes and centrifuged at 1500 rpm for 5 minutes using an Eppendorf centrifuge (Eppendorf GA, Hamburg, Germany), removing the supernatant. The cell

pellet was then washed with PBS and resuspended with 0.5 mL of PBS (pH 7.4). The cell suspension was added into 5 mL of 75% ethanol with precooling at -20°C (very slowly, drop by drop) and then fixed at 4°C overnight, followed by centrifuge at 1000 rpm for 5 minutes at 4°C to remove the ethanol. The cell pellet was washed twice with PBS + 1% bovine serum albumin and resuspended in 400 μL PBS + 1% bovine serum albumin. After that, 50 μL of 500 $\mu\text{g}/\text{mL}$ propidium iodide was added into the cell suspension, followed by incubation at 37°C for 30 minutes before analysis by flow cytometer.

The cells were divided into four groups: group 1, treated with free pTGF- β 1; group 2, treated with Lipofectamine 2000/pTGF- β 1; group 3, treated with CPEPS-pTGF- β 1 nanoparticles, and group 4, treated with medium containing pTGF- β 1 protein.

Statistical analysis

The data were analyzed with both one-factor and two-factor analyses of variance. Student's *t*-test and the Fisher's protected least significance difference post hoc test were used to determine the significance (significance accepted at $P < 0.01$) of the difference between selected groups with SPSS statistics software (v15.0; SPSS Inc, Chicago, IL). The data were presented as the mean \pm standard error of the mean.

Results

Characteristics of the CPEPS

The GPC findings showed that the average molecular weights of the PEPS and CPEPS were 549 kDa and 333 kDa, respectively. The amount of primary and secondary amino groups derived from spermine and the percentage of cross-linked spermine were as shown in Table 1. The findings showed that CPEPS was not cross-linked.

Qualitative functional group analysis of the CPEPS by Fourier transform infrared spectroscopy (KBr) yielded peaks at 3410, 1650, 1460, 1270, and 1030 cm^{-1} (see Figure 4). Comparatively, the spectrum of the CPEPS revealed an obvious change at 3410 cm^{-1} (the presence of amine and $-\text{OH}$ groups),

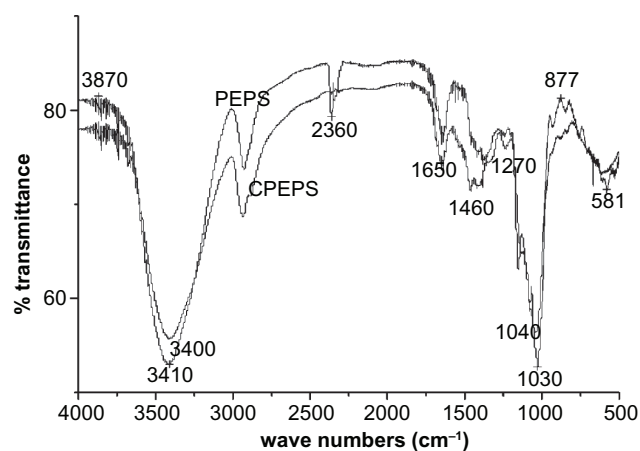


Figure 4 Fourier transform infrared spectra of polysaccharide of *Pleurotus eryngii* (PEPS) and cationized *P. eryngii* polysaccharide (CPEPS).

and peaks at 1650 cm^{-1} (indicating the existence of $-\text{NH}_2$),³² 1270 cm^{-1} (indicating the C–O group in an ester bond),³⁹ and 1030 cm^{-1} ($-\text{OH}$ stretching) as against the spectrum of PEPS. This demonstrated that the amino groups were successfully grafted onto the backbone of the polysaccharide.

Thin layer chromatography revealed that the monosaccharides in the PEPS mainly consisted of glucose, galactose, and mannose.

Gel retardation effect of the CPEPS-pTGF- β 1 nanoparticles

Figure 5 shows the electrophoretic pattern of the tested CPEPS-pTGF- β 1 nanoparticles with different CPEPS/pTGF- β 1 weight ratios. The nanoparticles with the lowest CPEPS/pTGF- β 1 weight ratio (1:5; well 2 of Figure 5) showed that a portion of the TGF- β 1 plasmids had detached itself from the nanoparticles and migrated into the gel, as demonstrated by a faint band corresponding to the location of the naked TGF- β 1 plasmid (well 1 of Figure 5). When the CPEPS/pTGF- β 1 weight ratio was increased to 10:1 (well 3 of Figure 5), there was no sign of plasmid migration across the agarose gel. This indicated that the complexation of pTGF- β 1 and CPEPS was strong enough to completely retard DNA migration. Accordingly, the CPEPS-pTGF- β 1 complexes with weight ratios of 10:1, 20:1, and 30:1 were used in the subsequent transfection study.

Table 1 Amino group content and cross-linking of CPEPS

Polycation	Nitrogen ^a ($\mu\text{mol}/\text{mg}$)	Primary amines ^b ($\mu\text{mol}/\text{mg}$)	Spermine ^c ($\mu\text{mol}/\text{mg}$)	Secondary amines ^d ($\mu\text{mol}/\text{mg}$)	Secondary to primary amine ratio	Cross-linked spermine ^e (%)
CPEPS	3.92 ± 0.23	1.24 ± 0.09	0.98 ± 0.06	2.94 ± 0.18	2.40 ± 0.30	0

Notes: ^aDetermined by elemental analysis ($n = 3$); ^bdetermined by the trinitrobenzene sulfonic acid method ($n = 3$); ^ccalculated from elemental analysis (total nitrogen divided by 4); ^dtheoretical secondary amine content (in case no cross-linking occurred: spermine content multiplied by 3); ^ecalculated for secondary to primary amine ratio ≥ 3 .

Abbreviation: CPEPS, cationized *Pleurotus eryngii* polysaccharide.

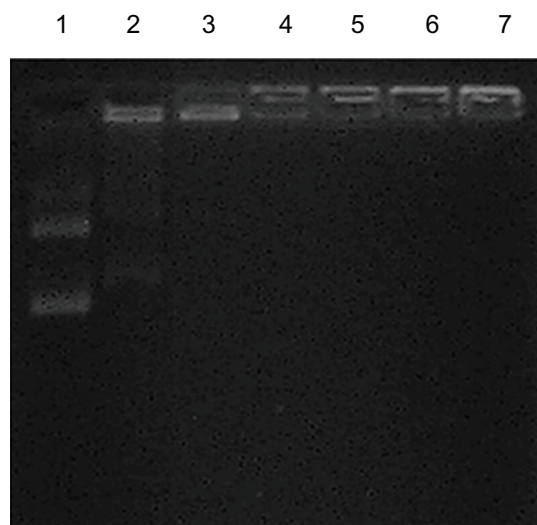


Figure 5 Agarose gel electrophoresis of the CPEPS-pTGF- β 1 nanoparticles.
Notes: Well 1, free pTGF- β 1; wells 2–7, CPEPS-pTGF- β 1 nanoparticles with various weight ratios of CPEPS/pTGF- β 1 (from left to right: 5:1, 10:1, 20:1, 30:1, 60:1, and 80:1).
Abbreviations: CPEPS, cationized *Pleurotus eryngii* polysaccharide; pTGF- β 1, plasmid encoding transforming growth factor beta-1.

Zeta potential

The zeta potential of the original CPEPS was $+19.4 \pm 0.2$ mV (mean \pm standard error for 15 runs of the same sample; see Figure 6). In contrast, the zeta potential of the naked pTGF- β 1 was -42.1 ± 0.3 mV (Figure 6). At the three different ratios of CPEPS/pTGF- β 1 (10:1, 20:1 and 30:1), the zeta potential changed from a negative value to a positive

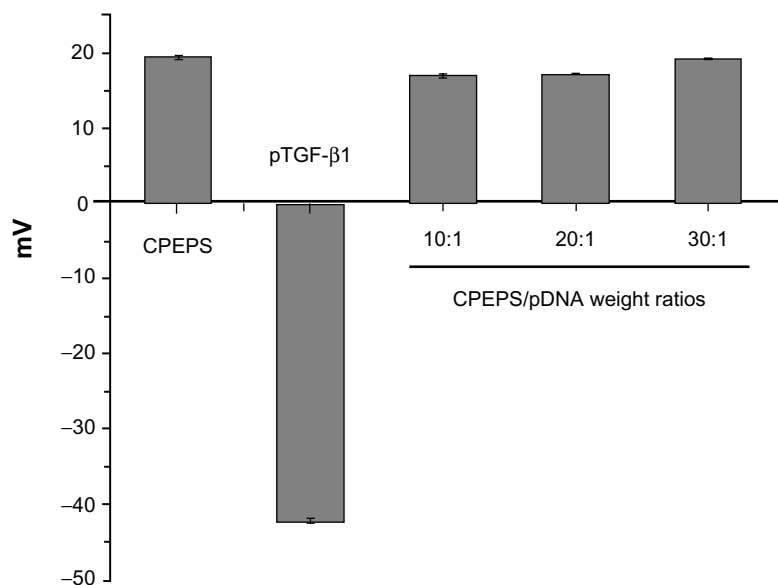


Figure 6 The zeta potentials of free pTGF- β 1, CPEPS, and CPEPS combined with different amounts of pTGF- β 1 to yield the following CPEPS/pTGF- β 1 weight ratios: 10:1, 20:1, and 30:1.

Note: The values are the means \pm standard error of the mean of three experiments.

Abbreviations: TGF- β 1, transforming growth factor beta-1; pTGF- β 1, plasmid encoding TGF- β 1; CPEPS, cationized *P. eryngii* polysaccharide.

value and increased to $+17.0 \pm 0.2$ mV, $+17.4 \pm 0.1$ mV, and $+19.2 \pm 0.2$ mV, respectively.

Particle size distribution and morphology

DLS demonstrated the various sizes of the nanoparticles with CPEPS/pTGF- β 1 weight ratios of 10:1, 20:1, and 30:1. The size distribution of the nanoparticles with a CPEPS/pTGF- β 1 weight ratio of 20:1 ranged from 29.2 nm to 168.5 nm, with an average diameter of 80.8 nm (Figure 7A), whereas the average sizes of the other two nanoparticle groups were 248.3 nm (10:1) and 151.1 nm (30:1), respectively (Table 2).

The TEM measurement showed that the monodispersed nanoparticles (CPEPS/pTGF- β 1 weight ratio of 20:1) had a spherical shape and a size distribution within a relatively narrow range (30–50 nm) (Figure 7B).

Cytotoxicity of the CPEPS-pTGF- β 1 nanoparticles

As shown in Figure 8, the result demonstrated that the CPEPS-pTGF- β 1 nanoparticles yielded comparative or even higher cell viability than that of Lipofectamine 2000 and branched PEI (25 kDa), indicating that the CPEPS-pTGF- β 1 nanoparticles were safe for MSCs.

In vitro gene transfection

The TEM images of MSCs before and after transfection are shown in Figure 9.

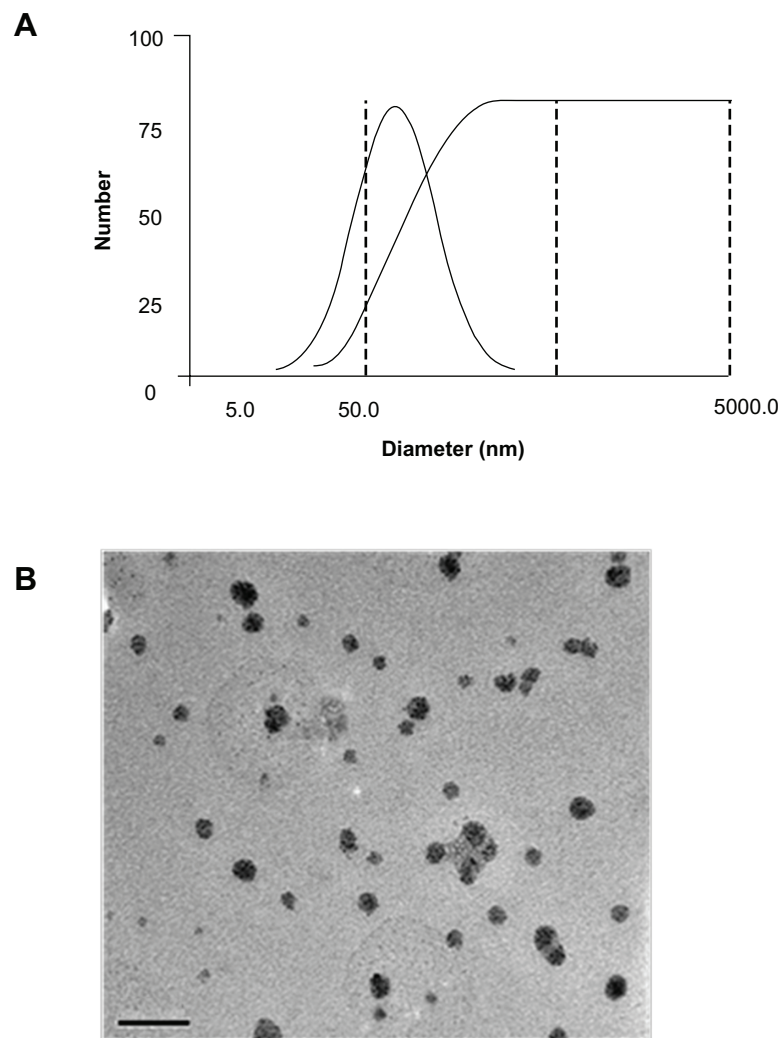


Figure 7 Particle size distribution and morphology. (A) The particle size distribution of the CPEPS/pTGF- β 1 nanoparticles with a weight ratio of 20:1; (B) transmission electron microscopy image of the CPEPS/pTGF- β 1 nanoparticles with a weight ratio of 20:1.

Abbreviations: CPEPS, cationized *Pleurotus eryngii* polysaccharide; pTGF- β 1, plasmid encoding transforming growth factor beta-1.

Among the three groups of different CPEPS/pTGF- β 1 weight ratios (10:1, 20:1, and 30:1), the optimal TGF- β 1 expression was recorded for a CPEPS/pTGF- β 1 weight ratio of 20:1 (Figure 10A). Statistically, the CPEPS-pTGF- β 1 nanoparticles with a CPEPS/pTGF- β 1 weight ratio of 20:1 revealed a significantly higher transfection effect than that of PEI (25 kDa) ($P = 0.002$, Student's *t*-test) and Lipofectamine 2000 ($P = 0.006$, Student's *t*-test), the

Table 2 The size distribution of nanoparticles with CPEPS/pTGF- β 1 weight ratios of 10:1, 20:1, and 30:1

CPEPS/pTGF- β 1 weight ratios	10:1	20:1	30:1
Particle size (means \pm SEM, nm)	248.3 \pm 7.4	80.8 \pm 6.8	151.1 \pm 7.2

Abbreviations: CPEPS, cationized *Pleurotus eryngii* polysaccharide; pTGF- β 1, plasmid encoding transforming growth factor beta-1; SEM, standard error of the mean.

current gold standard of commercial transfection reagents (Figure 10B).

mRNA expression level of TGF- β 1

As shown in Figure 11, the mRNA expression level of TGF- β 1 in CPEPS-pTGF- β 1 nanoparticle-transfected MSCs (well 1 in Figure 11) was notably higher than that of MSCs transfected with free pTGF- β 1 (well 2 in Figure 11) and also slightly higher than that of MSCs transfected with Lipofectamine 2000 (well 3 in Figure 11).

Flow cytometry analysis

As shown in Figure 12, the results of the flow cytometry of propidium iodide-stained cells demonstrated that the percentage of cells in the G1 phase for group 1 (85.20%, Figure 12A) was the lowest with the highest percentage being that of group 3 (92.38%, Figure 12C). However, the percentage of cells in the

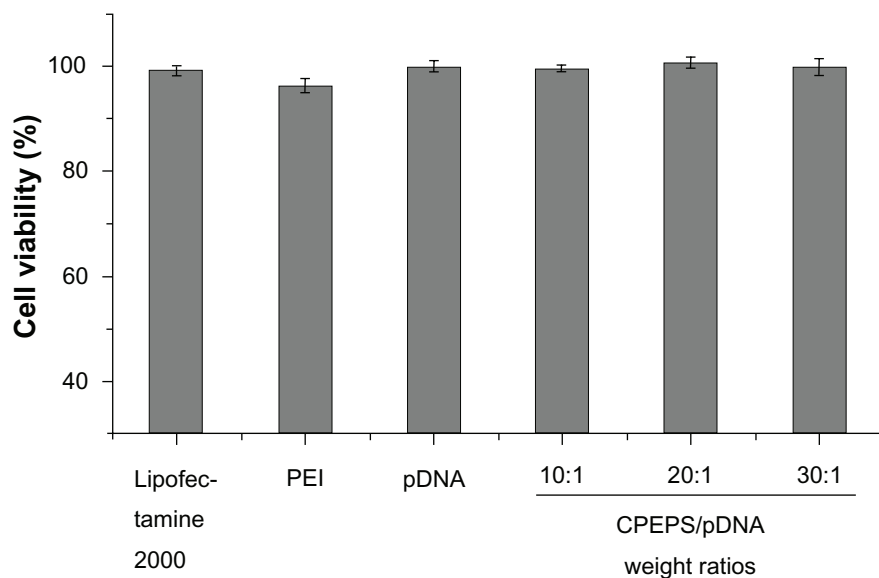


Figure 8 Cytotoxicity assay. From left to right: Lipofectamine™ 2000; polyethylenimine (25 kDa); free plasmid; CPEPS-pTGF-β1 nanoparticles with CPEPS/pTGF-β1 weight ratios of 10:1, 20:1, and 30:1.

Note: The values are the means ± standard error of the mean of three experiments.

Abbreviations: CPEPS, cationized *Pleurotus eryngii* polysaccharide; PEI, polyethylenimine; pTGF-β1, plasmid encoding transforming growth factor beta-1.

S phase for group 1 (12.35%) was higher than that of the other three groups (group 2 [Figure 12B]: 7.55%; group 3: 7.62%, and group 4: 8.4% [Figure 12D]). Interestingly, there were no cells in the G2 phase for group 3.

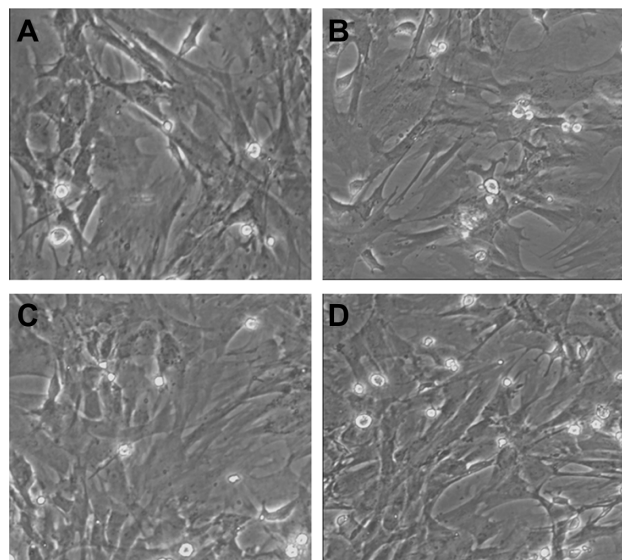


Figure 9 Transmission electron microscopy (TEM) images of MSCs before and after transfection. (A) TEM image of mesenchymal stem cells before transfection; (B–D) TEM images of mesenchymal stem cells after transfection by CPEPS-pTGF-β1 nanoparticles with CPEPS/pTGF-β1 weight ratios of 10:1, 20:1, and 30:1, respectively.

Abbreviations: CPEPS, cationized *Pleurotus eryngii* polysaccharide; pTGF-β1, plasmid encoding transforming growth factor beta-1.

Discussion

In this study, PEPS was used as a gene delivery material for the first time. PEPS was a promising candidate for gene delivery after proper modification due to its advantages, including biocompatibility, biodegradability, nontoxicity, and ease of chemical modification.

Generally, the molecular weights of polymers are determined by GPC, which is also known as size-exclusion chromatography, equipped with either an RID^{32,60} or a light-scattering detector.^{20,24} Despite the increasingly widespread application of DLS, GPC equipped with RID has equally also been used to measure the molecular weights of polymers in many recent studies.^{32,60} Based on other studies and the limited experimental conditions in the laboratory, a RID (Shimadzu GPC system-equipped Shimadzu RID-10 A) was used to determine the molecular weights of PEPS and CPEPS. The findings revealed a drastic decrease in the molecular weight of CPEPS (549 kDa) as compared to that of PEPS (333 kDa). This could possibly be due to the extensive aminolysis of PEPS during the conjugation reaction.^{38,58}

In previous studies,^{38,58} nuclear magnetic resonance (NMR) was employed to further characterize the structure of the polysaccharide. In this work, ¹H NMR spectra were recorded on a 400 MHz Bruker AV-400 NMR spectrometer (Bruker, Madison, WI) to characterize the structure of PEPS and CPEPS. Unfortunately, no valuable information could be obtained (data not shown). In some published reports^{58,61,62} the

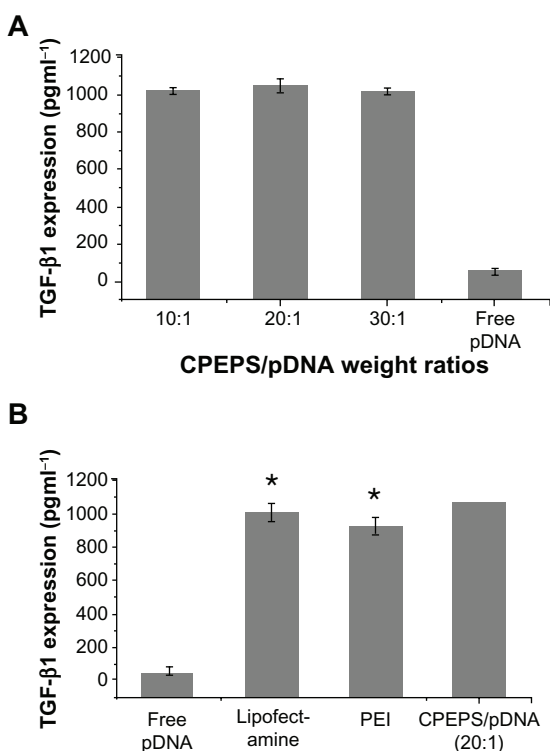


Figure 10 TGF- β 1 protein released into the medium from mesenchymal stem cells 72 hours after treatment with CPEPS-pTGF- β 1 nanoparticles. **(A)** TGF- β 1 protein concentrations in the medium with various weight ratios of CPEPS to pTGF- β 1: 10:1 (first bar), 20:1 (second bar), and 30:1 (third bar), as well as in free plasmid DNA (fourth bar). **(B)** A comparison of TGF- β 1 expression levels: free plasmid DNA (first bar); Lipofectamine™ 2000 (second bar); polyethylenimine (25 kDa, third bar); and CPEPS-pTGF- β 1 nanoparticles with a CPEPS/pTGF- β 1 weight ratio of 20:1 (fourth bar).

Note: The values are the means \pm standard error of the mean of three experiments. **Abbreviations:** TGF- β 1, transforming growth factor beta-1; pTGF- β 1, plasmid encoding TGF- β 1; CPEPS, cationized *Pleurotus eryngii* polysaccharide; PEI, polyethylenimine.

samples had molecular weights less than 100 kDa as against the results of this study (>100 kDa). It is possible that the molecular weights of the samples were beyond the sensitivity of the NMR spectrometer. The exact reason is still unknown, and it will be explored in our future study. According to some researchers,^{39,60} it was acceptable to combine the use of trinitrobenzene sulfonic acid, total nitrogen determination, and Fourier transform infrared spectroscopy to characterize the CPEPS.

Spermine, an amino compound with two primary amino groups and two secondary amino groups on each molecule, was chemically grafted onto PEPS to obtain CPEPS.⁵⁸ The quantity of the total amount of nitrogen per microgram of CPEPS indicated the amount of spermine grafted on the backbone of PEPS. As spermine was conjugated to the polysaccharide via one of its primary amino groups, the conjugated primary amino group

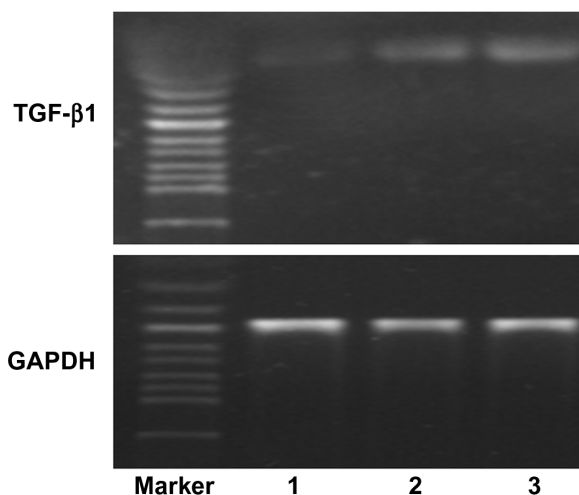


Figure 11 Messenger RNA expression of TGF- β 1 in mesenchymal stem cells (MSCs).

Notes: Well 1, MSCs transfected with free pTGF- β 1; well 2, MSCs transfected with Lipofectamine™ 2000/pTGF- β 1; well 3, MSCs transfected with CPEPS-pTGF- β 1 nanoparticles.

Abbreviations: TGF- β 1, transforming growth factor beta-1; CPEPS, cationized *Pleurotus eryngii* polysaccharide; pTGF- β 1, plasmid encoding TGF- β 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

became a secondary amine after conjugation.³⁸ Cross-linking was possible via the remaining primary amine of spermine. In this study, no cross-linking was observed (Table 1), indicating good control of the reaction conditions. Significantly, a low degree of cross-linking was desirable in this application.

The study investigated the monosaccharide content of the PEPS via preliminary thin layer chromatography analysis and found that D-glucose was the major monosaccharide component; the other components were galactose and mannose. The results were in agreement with previous studies.⁵⁰ It was speculated that because D-glucose was the major monosaccharide in PEPS, the monomer was more likely to be grafted with spermine. It was also likely that the spermine might be linked to the other monosaccharide components. In a related study, various polysaccharides (pullulan, dextran, and mannan) with different cationization degrees were used to transfect MSCs in order to examine the factors affecting transfection.⁶³ The findings showed that the gene expression level was closely related to the type of cationized polysaccharide. The exact composition of PEPS monosaccharides, which are the major contributors of cationization, will be addressed in future works.

Gel electrophoresis showed that the cationized polysaccharide could successfully incorporate TGF- β 1 plasmid. The complexation of CPEPS and TGF- β 1 plasmid was CPEPS-content dependent, with a greater CPEPS/pTGF- β 1 weight ratio resulting in better plasmid incorporation.

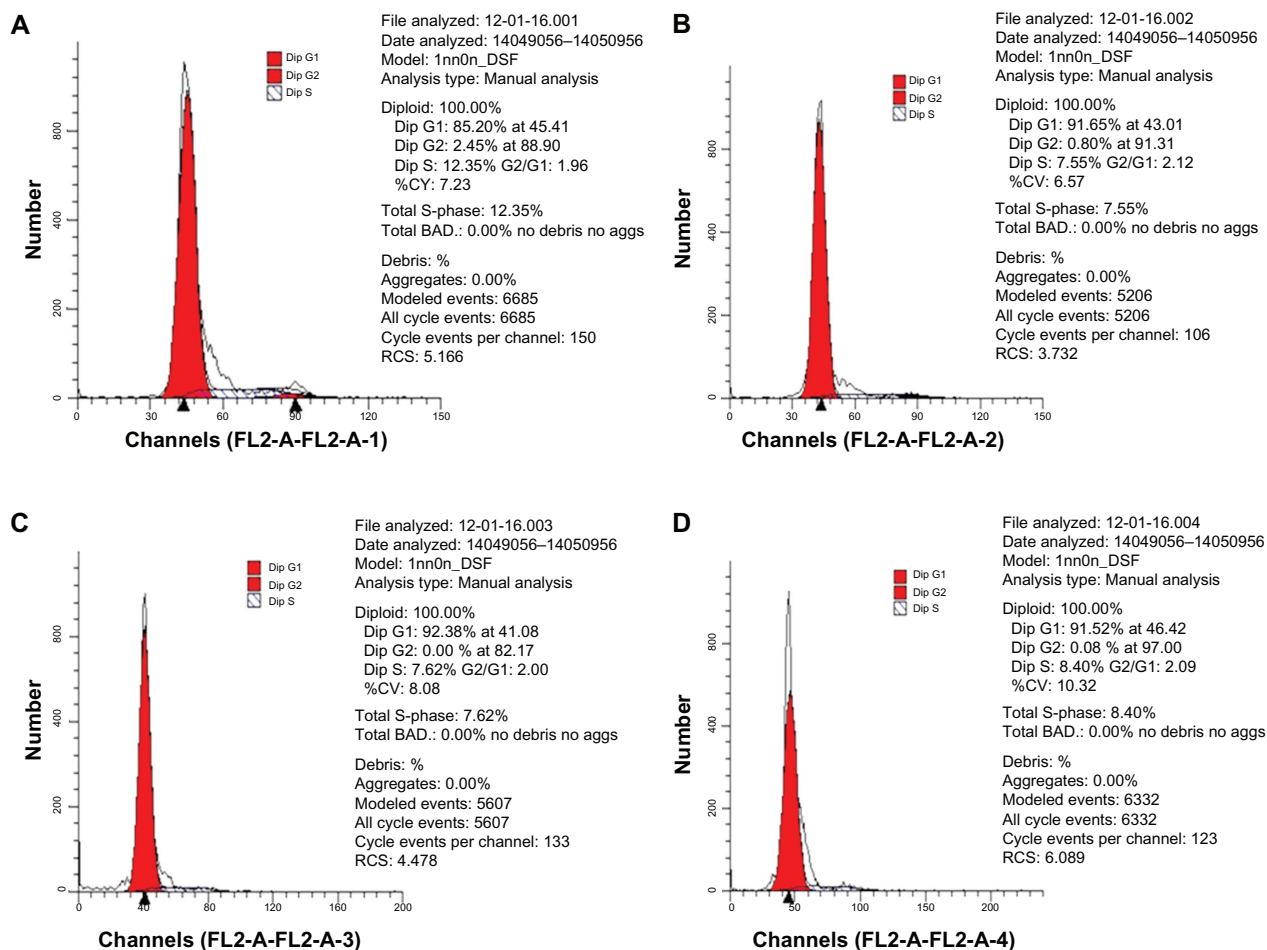


Figure 12 Flow cytometry of propidium iodide-stained mesenchymal stem cells (MSCs). **(A)** MSCs treated with plasmid TGF- β 1; **(B)** MSCs treated with Lipofectamine™ 2000/pTGF- β 1; **(C)** MSCs treated with CPEPS-pTGF- β 1 nanoparticles; **(D)** MSCs treated with medium containing pTGF- β 1 protein.

Abbreviations: TGF- β 1, transforming growth factor beta-1; pTGF- β 1, plasmid encoding TGF- β 1; CPEPS, cationized *Pleurotus eryngii* polysaccharide.

It is well known that DNA charge reversal is one of the basic requirements for the transfection of cells. Interestingly, the zeta potentials of the three different CPEPS-pTGF- β 1 nanoparticles were almost the same as that of the original polysaccharide ($+19.4 \pm 0.2$ mV) and statistically insignificant (see Figure 6.) This implies that the negatively charged plasmids were successfully combined with the positively charged polysaccharide to form nanoparticles of more positive charges on its outer surface. Therefore, the value of the positive charges on the surface of the nanoparticles will be comparable to that of the original CPEPS. This point will be further investigated in future studies.

The particle size values provided by TEM (30–50 nm, Figure 7B) and DLS (29.2–168.5 nm) were quite different (Table 2 and Figure 7A). A possible reason could be that the DLS and TEM had various determination conditions: samples measured by DLS were in suspended form, whereas dried particles were observed under TEM. In summary, the

CPEPS-pTGF- β 1 complex had a nanoscaled particle size. Interestingly, these findings showed that the average particle size of the formulated nanoparticles (CPEPS/pTGF- β 1 weight ratio of 20:1) was 80.8 nm in diameter, which was much smaller than the sizes reported in previous studies (cationized gelatin¹⁵ and chitosan²⁵ nanoparticles with an average size of approximately 172 nm in diameter and 250 nm in diameter were produced, respectively). However, the average size of the nanoparticles with a CPEPS/pTGF- β 1 weight ratio of 30:1 was 151.1 nm which is obviously larger than that of the 20:1 nanoparticles. This large particle size suggested that excess cationic polysaccharide might acquire a greater positive zeta potential, but it could also cause redundant CPEPS coacervation on the particle surface resulting in a particle size increase. This is unfavorable for cellular uptake.

Low cytotoxicity is one of the basic requirements for a safe and effective gene carrier. The outcome of the MTT test showed that the CPEPS-pTGF- β 1 nanoparticles exhibited

an exceptional safety profile (Figure 8). It is likely that the intracellular degradability of the polymer was responsible for the low cytotoxicity of CPEPS.⁴⁹ Inside the cells, the CPEPS could be degraded to different kinds of monosaccharides and oligoamines, which were easily metabolized, hence nontoxic.⁶⁴ Essentially, the CPEPS-pTGF- β 1 nanoparticles prepared in this study were safe for MSCs.

Cell transfection efficiency is the key index in evaluating the properties of nonviral gene carriers. The results of the CPEPS-pTGF- β 1 nanoparticles with a weight ratio of 20:1 (80.8 nm on average) showed the highest transfection efficiency (Figure 10A). This means that the smaller size of the nanoparticles facilitated the movement of the particles through membranes. In addition, when the CPEPS/pTGF- β 1 weight ratio was lower than 20:1, it could possibly lead to an incomplete encapsulation of the negatively charged naked pTGF- β 1, resulting in a relatively large particle size (248.3 nm) that probably hindered the process of cellular uptake. This assertion was also supported by the gel retardation assay (Figure 5). It was observed that there was less tendency for the plasmid to migrate as the CPEPS/pTGF- β 1 weight ratio increased. The free plasmid DNA showed the greatest migration towards the positive pole. This implies that the complex with a weight ratio of 10:1 had an inadequate amount of cationic polysaccharide. In the case of the nanoparticles with a CPEPS/pTGF- β 1 weight ratio greater than 20:1, the reduced TGF- β 1 expression could possibly be due to a larger particle size (151.1 nm), as well as to the strong bond of attraction between the CPEPS and pTGF- β 1. This would have prevented the release of pTGF- β 1 from the nanoparticles in a timely and complete manner once the nanoparticles entered the cells. Additionally, a high CPEPS/pTGF- β 1 weight ratio might lead to the state of free CPEPS that could competitively inhibit the interaction between CPEPS-pTGF- β 1 nanoparticles and cell membrane.³⁹ Further investigations would be needed to provide a deeper understanding of the MSC transfection mechanisms with respect to the CPEPS/pTGF- β 1 weight ratio.

Interestingly, the CPEPS-pTGF- β 1 nanoparticles (weight ratio of 20:1) showed a significantly enhanced transfection effect as compared to those of PEI (25 kDa), ($P = 0.002$, Student's *t*-test) and Lipofectamine 2000 ($P = 0.006$, Student's *t*-test). This was possible because CPEPS could condense the TGF- β 1 plasmid to a small size (80.8 nm on average), which facilitated the process of cellular uptake. At the same time, the positive charge on the surface of the nanoparticle ($+17.4 \pm 0.1$ mV) promoted the interaction with the negatively charged cell membranes. It is a well-known fact that polysaccharides can readily be transported to cells by

known biological processes, which might greatly contribute to the success of the transfection.³⁸

In the study, long-term (72-hour) expression of TGF- β 1 by MSCs was also investigated. In a related study,⁶⁴ immunofluorescence staining was conducted to check if the stem cells had differentiated after transfection. The findings of that study showed that the transfection was transient. In this study, the results supported that, and after transfection stem cells still maintained mesenchymal lineage properties (data not shown). This could also be supported by the TEM images of MSCs before and after transfection, which exhibited no obvious morphological changes after transfection. Longer-term (more than 72-hour) transfection investigation will be addressed in future works.

Reverse transcriptase polymerase chain reaction was carried out to reflect the transfection effect of CPEPS-pTGF- β 1 nanoparticles from the mRNA expression level of TGF- β 1, and the result was in agreement with that revealed by enzyme-linked immunosorbent assay, indicating the high transfection effect of CPEPS-pTGF- β 1 nanoparticles and their superiority over Lipofectamine 2000.

It is well known that the S phase is the cell cycle phase for DNA synthesis which prepares the cells for mitosis, and the G1 phase is the cell stage after mitosis to the beginning of the S phase. So the cells moving from the S to the G1 phase indicated a tendency of differentiation. Flow cytometry analysis revealed that cells transfected with CPEPS-pTGF- β 1 nanoparticles had low proliferation but showed a tendency of differentiation. This could possibly be due to the effect of TGF- β 1 on MSCs, because TGF- β 1 has the ability to induce chondrogenesis of MSCs.⁶⁵ Further investigation of this aspect will be addressed in future studies.

Conclusion

In this study, a polysaccharide isolated from the edible mushroom *P. eryngii* was chemically modified with spermine for the first time to obtain CPEPS, out of which CPEPS-pTGF- β 1 nanoparticles were then prepared. Gel retardation assay showed that CPEPS-pTGF- β 1 nanoparticles possessed the ability to prevent the plasmid DNA from migrating when the CPEPS/pTGF- β 1 weight ratio increased to 10:1. Varying the CPEPS to TGF- β 1 plasmid weight ratio affected the TGF- β 1 expression significantly, with the highest transfection efficiency noted at the CPEPS/pTGF- β 1 weight ratio of 20:1. In addition, the CPEPS-pTGF- β 1 nanoparticles (weight ratio of 20:1) showed significantly enhanced transfection efficiency compared with that of Lipofectamine 2000. Cytotoxicity assay revealed that the CPEPS/pTGF- β 1 nanoparticles were

less toxic than both PEI (25 kDa) and Lipofectamine 2000. Furthermore, the mRNA expression level of TGF- β 1 in MSCs transfected by CPEPS-pTGF- β 1 nanoparticles was significantly higher than that of free plasmid DNA-transfected MSCs and slightly elevated compared with that of Lipofectamine™ 2000-transfected MSCs. Flow cytometry analysis revealed that 92.38% of MSCs were arrested in the G1 phase after transfection with CPEPS-pTGF- β 1 nanoparticles, indicating a tendency of differentiation. All of these findings support the fact that the CPEPS-pTGF- β 1 nanoparticles could be developed into a promising gene delivery system in the near future.

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Disclosure

There was no financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper.

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