Carbohydrate Polymers 86 (2011) 1509–1518

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Efficient gene transfer into rat mesenchymal stem cells with cationized *Lycium barbarum* polysaccharides nanoparticles

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ARTICLE INFO

Article history:
Received 29 March 2011
Received in revised form 10 June 2011
Accepted 17 June 2011
Available online 18 July 2011

Keywords:
Lycium barbarum
Cationized polysaccharide nanoparticle
Gene delivery
TGFβ-1
Mesenchymal stem cell

ABSTRACT

A new gene vector, cationized *Lycium barbarum* polysaccharides (cLBP) nanoparticles, prepared with different amine compounds for non-viral delivery of plasmid DNA encoding for transforming growth factor beta 1 (TGFβ-1) was developed and the efficiency of these nanoparticles to transfect rat mesenchymal stem cells *in vitro* was determined. The spherical cLBP-plasmid TGFβ-1 nanoparticles were 160–330 nm in diameter and can retard the migration of plasmid TGFβ-1 in electrophoresis. Ethylenediamine-LBP nanoparticles manifested better transfection efficiency compared with spermine-LBP and polyethylenimine-LBP nanostructures. At $M_{cLBP}/M_{pTGF\beta-1}$ ratio 80, maximum TGFβ-1 expression mediated by Ethylenediamine-LBP was much higher than both PEI ($p < 0.001$) and Lipofectamine™2000 ($p < 0.001$) in serum or serum-free media, and the cellular uptake with live cell imaging was in good agreement with the gene transducing effect. Transfected cells were of outstanding cell viability, indicating the low cytotoxicity of cLBP. The results suggested cLBP could be a promising carrier for gene delivery.

1. Introduction

Gene therapy has been proposed as a potential strategy for genetically based and infectious diseases such as hemophilia, cystic fibrosis, gynecological diseases, etc. (Griesenbach & Boyd, 2005; Hassan, Othman, Hornung, & Al-Hendy, 2009; Lozier, 2004). Successful gene therapy depends on the efficient delivery of nucleic acids into the cell nucleus and its effective expression within the cell (Yudovin-Farber & Domb, 2007). Several techniques have been developed for gene delivery; intervention of genetic material by cell electroporation, microinjection and incorporation of the gene using viral and synthetic carriers (Dezawa et al., 2002; Hosokawa et al., 2009; Wunderbaldinger, Bogdanov, & Weissleder, 2000). Although non-viral vectors have lower expression levels of the delivered genes than viral ones *in vivo*, these vehicles present more important advantages such as safety, low immunogenicity, specificity and long lasting in gene expression.

Non-viral vehicles involve cationic polyplexes and cationic lipid based carriers (Brown, Schatzlein, & Uchegbu, 2001). Nucleic acids are incorporated into complexes by electrostatic interactions between anionic phosphate groups of nucleic acids and cationic groups of lipids or polycations under physiologic conditions. Cationic polymers used for gene delivery acquire their charge from primary, secondary, tertiary, or quaternary amino groups, which are capable of forming electrostatic complexes with DNA (Eliyahu, Siani, Azzam, Domb, & Barenholz, 2006). A large number of polycations have been studied as non-viral vehicles, such as poly-L-lysine and its derivatives (Kang, Kim, Lee, & Bae, 2005), polyethylenimine (PEI) (Deng et al., 2009; Godbey & Mikos, 2001; Werth et al., 2006), polymers cross-linked with ethylenediamine and its derivatives, spermine modified nanopaticles (Christensen et al., 2007; Hosseinkhani & Tabata, 2003; Kushibiki, Nagata-Nakajima, Sugai, Shimizu, & Tabata, 2006a), etc. These polyplexes condensed DNA can be taken up by cells through adsorptive endocytosis due to their positive charged character; leading to considerable expression of exogenous genes (Persiani & Shen, 1989; Weiss et al., 2006). The major problems with these polymers are the noticeable toxicity due to cationic charges and nonbiodegradable properties, while polymers based on amino acids such as poly-L-lysines, are immunogenic (Gao et al., 2009; Vanderkerken et al., 2000).

Among the various polycations used in gene transfection, cationic polysaccharides are considered to be the most attractive candidates. They are natural, non-toxic, biocompatible, and biodegradable materials and can be modified easily for improved physicochemical properties (Dergunova et al., 2009; Lee, 1987; Li,

Abbreviations: LBP, *Lycium barbarum* polysaccharides; cLBP, cationized *Lycium barbarum* polysaccharides; TGFβ-1, transforming growth factor beta 1; pTGFβ-1, plasmid DNA encoding for transforming growth factor beta 1; MSC, mesenchymal stem cell; PEI, polyethylenimine; PEI-LBP, polyethylenimine-LBP; Ed-LBP, ethylenediamine-LBP; Sm-LBP, spermine-LBP; PEI-LBP, polyethylenimine-LBP; CLPTN, cationized LBP-plasmid TGFβ-1 nanoparticles.

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doi:10.1016/j.carbpol.2011.06.073

Ma, & Liu, 2007; Schepetkin & Quinn, 2006). Chitosan is a naturally occurring cationic polysaccharide. Chitosan-mediated gene transfection systems have been shown to excel in transcellular transport (Alatorre-Meda et al., 2010; Guang Liu & De Yao, 2002; Jayakumar et al., 2010; Kean, Roth, & Thanou, 2005). Dextran–spermine conjugate and DEAE-dextran transfection method have been developed to mediate the reproducible transfection of primary cultured adherent mammalian cells (Azzam, Eliyahu, Makovitzki, Linial, & Domb, 2004; Eliyahu et al., 2006; Mack, Wei, Elbagarri, Abbey, & McGrath, 1998). Cationic pullulan has shown great potential as a non-viral vector for liver cell targeted gene delivery because of its blood compatibility and low toxicity (Rekha & Sharma, 2009).

Chitosan is widely acknowledged as one of the most attractive cationic vectors, but the main drawback of chitosan is the poor water solubility at physiological pH and low transfection efficiency (Li et al., 2010; Opanasopit et al., 2009). Several chitosan derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics (Rakkhithawatthana et al., 2010; Xu et al., 2009). Compared with chitosan, cationized polysaccharides extracted from Chinese herb have the advantage of better water solubility, while as for the transfection efficiency it is barely reported. Until recently, few studies of gene transfection have been published on gene delivery system mediated with polysaccharide extracted from Chinese herb, not mentioning the particular one, *Lycium barbarum*.

The objective of the present study is to develop a new vector, cationized *L. barbarum* polysaccharide (cLBP) nanoparticles for non-viral delivery of plasmid DNA encoding for transforming growth factor beta 1 (TGFβ-1) into mesenchymal stem cells (MSCs) *in vitro*, and to determine the release of TGFβ-1 in transfected MSCs over time and the effect on biosynthesis. The ultimate goal is to use the nanoparticles to achieve a prolonged and localized release of TGFβ-1 whose robust transduced effect on MSCs chondrogenic differentiation have been demonstrated (Kawamura et al., 2005). Three systems for efficient delivery of DNA were designed by preparing LBPs modified with ethylenediamine, low weight PEI (1300 Da) and spermine respectively. The ratio of LBP to amino groups in the complexes was optimized to obtain the maximum transfection efficiency with low cytotoxicity and particles of nanometer level size. The experiment of MSC transfection was performed in presence/absence of 10% FBS to evaluate the influence of serum on transfection efficiency. It was speculated that nanoparticles containing plasmids would be taken up by cells. Once entering cells, the nanoparticles would release plasmids, which might gain entry into the nucleus and enable the over expression of TGFβ-1. The cellular uptake of prepared complex was characterized by Live Cell Image.

The novelty of cationizing polysaccharides extracted from *L. barbarum*, a traditional Chinese medicine, is worth mentioning. Besides, it is innovative to transfect MSCs with these cationized polysaccharides. This approach was evaluated in order to determine the suitability of the potential use of cationized *L. barbarum* polysaccharides as a novel gene vector, with promising minimal toxicity associated and salient transfection efficiency compared with lipofectamine.

2. Materials and methods

2.1. Materials

Fresh *L. barbarum* was obtained from Zhenjiang Zhilin Pharmacy (Zhenjiang, China). LBP was isolated from *L. barbarum* by homogenizing dry *L. barbarum* and dispersed twice in 60 °C double distilled water (DDW) for 2 h each time, and then filtered to remove impurities. Polysaccharides were concentrated under reduced pressure at 50 °C, precipitated by addition of three vol. 95% (v/v) ethanol (final concentration 75% (v/v)) and left to stand overnight. The resultant precipitate was collected by centrifugation, washed with absolute ethyl alcohol, and resuspended in DDW. Then 1/4 vol. chloroform/n-butanol (5:1, v/v) was added to precipitate proteins and the aqueous phase was subsequently collected, dialyzed via a 3000–4000 MW cut-off filter, and lyophilized to obtain a crude extract. The crude was then dissolved in DDW and purified by a DEAE-cellulose (1.6 × 50 cm) column. Fractions were obtained and subjected to a Sephadex G-100 (1.6 × 50 cm) column for further purification. Target fractions monitored by the phenol–sulfuric acid assay were combined and lyophilized to give pure LBP (Cuesta, Suarez, Bessio, Ferreira, & Massaldi, 2003). The purified LBP had a molecular weight of 35,000 Da (determined by HPLC, TSK-GEL G4000PW, Φ 7.5 × 300 mm, 5 μm, Dalian, China).

Ethylenediamine, polyethylenimine (PEI, 1300 Da and 25 kDa), spermine, N,N′-carbonyldiimidazole (CDI) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture products were purchased from Gibco-BRL-Life Technologies (Burlington, ON, Canada). Rat TGFβ-1 ELISA Kit was obtained from Yantai Science & Biotechnology Co. Ltd (Yantai, China). EndoFree Plasimad Maxi Kit was purchased from CWBIO Co. Ltd (Beijing, China). Other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China) and used without further purification.

The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals.

2.2. Preparation of plasmid DNA

The plasmid encoding for TGFβ-1 (pTGFβ-1) was amplified in Escherichia coli (strain DH5α) and purified using EndoFree Plasimad Maxi Kit (CWBIO, Co. Ltd, Beijing, China) according to the manufacturer's instructions. The antibiotic used to select pTGFβ-1 transformed cells was ampicilin. The yield, purity, and integrity of the plasmid DNA were evaluated with an ultraviolet spectrophotometer and by gel electrophoresis. The absorbance ratio at wavelengths of 260–280 nm for the plasmid DNA solution was measured to be between 1.8 and 2.0 (BioSpec-mini, Shimadzu Co, Japan).

2.3. Preparation of cationized LBP derivatives

2.3.1. Preparation of oxidized LBP

200 mg LBP was dissolved in 20 ml DDW, to which was added 285 mg potassium periodate. Following agitation with magnetic stirrer in darkroom at room temperature for 40 h, the reaction mixture was dialyzed against DDW for 2 days with a dialysis membrane (3000–4000 MW cut-off filter), and then freeze-dried to obtain the oxidized LBP.

2.3.2. Preparation of spermine-LBP

100 mg oxidized LBP prepared in previous work was dissolved in 20 ml DDW, to which was added 5 ml of 0.1 M borate-buffered solution (pH 9.0) containing 190 mg of spermine. The mixture was agitated with magnetic stirrer at room temperature for 24 h, and then 100 mg sodium borohydride was added to the reaction mixture for another 48 h. Following agitation 100 mg sodium borohydride was again added to the system with magnetic stirrer at room temperature for 24 h. The reaction mixture was dialyzed against DDW for 2 days with a dialysis membrane (3000–4000 MW cut-off filter), and then freeze-dried to obtain the sample of spermine-introduced LBP (Sm-LBP).

2.3.3. Preparation of ethylenediamine-LBP

100 mg oxidized LBP was dissolved in 20 ml DDW, to which was added 5 ml of 0.1 M borate-buffered solution (pH 9.0) containing 0.13 ml of ethylenediamine. The mixture was agitated with magnetic stirrer at room temperature for 24 h, and then 100 mg sodium borohydride was added to the reaction mixture for another 48 h. Following agitation 100 mg sodium borohydride was again added to the system with magnetic stirrer at room temperature for 24 h, and the reaction mixture was dialyzed against DDW for 2 days with a dialysis membrane (3000–4000 MW cut-off filter). It was then freeze-dried to obtain the sample of ethylenediamine-introduced LBP (Ed-LBP).

2.3.4. Preparation of PEI-LBP

100 mg LBP was dissolved in 10 ml phosphate-buffered solution (PBS, pH 7.0) in a round bottomed flask which was degassed with N_2. 200 mg CDI was dissolved in 5 ml methylene chloride. After adding 0.1 ml triethylamine (Et_3N) into the reaction system, 5 ml methylene chloride containing 200 mg of CDI was added into the flask slowly and gently with mechanical agitation. The reaction lasted for 2 h, and then the PEI (MW 1300 Da) solution thus, 3.2 g PEI dissolved in 10 ml PBS (pH 7.0), was slowly added into the reaction mixture by continual agitation in a dark room for 24 h at room temperature. The reaction mixture was dialyzed against DDW for 2 days with a dialysis membrane (3000–4000 MW cut-off filter), and then freeze-dried to obtain the sample of PEI modified LBP (PEI-LBP).

The content of primary amine groups in cLBP were determined by the conventional elemental analysis and expressed by the molar percentage of amine groups introduced to LBP. Additionally, LBP and cLBP were characterized by FT-IR (Nicolet Avatar-370, Thermal Fisher Scientific, USA) to retrieve the structure information.

2.4. Incorporation of plasmid TGFβ-1 into cLBP nanoparticles

Cationized LBP-plasmid TGFβ-1 nanoparticles (CLPTN) were prepared by the mechanism of complex electrostatic adsorption, which involves separation by the interaction of two oppositely charged groups. The cLBP stock solution (40 mg/ml) was made by dissolving 0.12 g cLBP in 3 ml distilled water. The solution was then filtered for sterilization. Working solutions with different cLBP concentrations were diluted with sterilized water. The plasmid TGFβ-1 (pTGFβ-1) working solution (100 μg/ml) was prepared with sterile filtered 10 mM phosphate-buffered saline solution (PBS, pH 7.4). Aliquots (50–100 μl) of cLBP and pTGFβ-1 solution were heated separately at 55 °C for 30 min. Equal volume of the two kinds of solution were mixed immediately and vortexed for 30 s, followed by staying at room temperature for 60 min to obtain CLPTN. Nanoparticles prepared with pTGFβ-1 were used without further purification. The weight ratios of cLBP to plasmid were 100:1, 80:1 and 10:1 respectively for Sm-LBP, Ed-LBP and PEI-LBP. This conclusion was drawn after a series of ratios were investigated when preparing nanoparticles encapsulating pTGFβ-1. As a control group, nanoparticles were also synthesized using the original (non cationized) LBP (Xu, Capito, & Spector, 2008a,b).

2.5. Cell culture

MSCs were isolated from SD rats (30–50 g, Experimental Animal Center at Jiangsu University, China) by flushing femurs and tibias with phosphate-buffered saline (PBS, pH 7.4) under sterile conditions. Bone marrow aspirates were passed through a density gradient (Percoll gradient being 1.074 g/ml) to derive boundary layer cells by centrifugation (2000 rpm, 20 min). After being washed (1500 rpm, 10 min, two times) with 10 ml PBS to remove the Percoll, the cells were suspended in 10 ml Dulbecco's modified Eagle's medium (DMEM, low glucose, Invitrogen Co, Carlsbad, CA) containing of 10% fetal bovine serum (FBS, Invitrogen Co, Carlsbad, CA), and then plated in two culture flask and incubated at 37 °C in 5% humidified CO_2. Nutrient liquid was totally substituted after 48 h to wash away hematopoietic cells, fibroblasts, and other nonadherent cells during medium changes, and then the remaining purified MSCs population was further expanded in culture.

2.6. Electrophoresis of cLBP-plasmid TGFβ-1 nanoparticles

CLPTN were studied using agarose gel electrophoresis. For each gel, naked pTGFβ-1 (0.6 μg per well) was used as the control. Increasing amount of cLBP were mixed with a single quantity of pTGFβ-1 (0.6 μg) to yield the following weight ratios: 80:1 (weight ratio of purified LBP to plasmid, lane 2), Ed-LBP to plasmid: 30:1; 50:1; 80:1; 100:1; 120:1; 150:1 (lanes 3–8); PEI-LBP to plasmid: 0.5:1; 1:1; 2:1; 5:1; 10:1; 20:1; 30:1 (lanes 2–8) and Sm-LBP to plasmid: 10:1; 20:1; 40:1; 60:1; 80:1; 120:1; 150:1 (lanes 2–8), respectively. 2 μl loading buffer (6×, Takara, Japan) was then added to 10 μl of each complex solution, and then applied on a 1% agarose gel in tris-borate–ethylenediamine tetraacetic acid EDTA buffer solution (TBE, pH 8.0) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the nanoparticle was carried out in TBE solution at 100 V for 90 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, BIO RAD laboratories, Hercules, CA).

2.7. Transmission electron microscopy

Transmission electron microscopy (TEM) (JEM2100, JEOL, Tokyo, Japan) was used to investigate the size and shape of the nanoparticles. Briefly, the samples (1 μl of the nanoparticle suspension) were applied to carbon-coated copper grids, blotted, washed, negatively stained with 2% (w/v) of phosphotungstic S5 acid, air dried, and then observed directly under TEM. Nanoparticles prepared with different amine reagents with different weight ratios of cLBP to plasmid were examined by TEM.

2.8. Determination of nanoparticle size distribution

The distribution of nanoparticle size was determined by the dynamic light scattering technique performed at 25 °C with a BI-90plus instrument (Brookhaven Instruments Corporation, Holtsville, NY). The measured scattering intensities were analyzed by the software provided by Brookhaven Instruments Corporation. The detection limits for dynamic light scattering generally ranged from 2–3 nm to 2–3 μm. CLPTN with different weight ratios were evaluated for their particle size distribution.

2.9. Gene transfection

Twenty-four hours prior to adding the nanoparticles to the cultures, MSCs (Passage 0) were seeded onto a 96-well format at a density of 4×10^4 cells per well. The cells were cultured in growth medium (DMEM containing 10% FBS without antibiotics). At 80–90% confluence, the medium was removed and replaced with 100 μl of serum-free medium (DMEM, low glucose, without antibiotics). The cells were incubated at 37 °C in a 5% CO_2 incubator for 12 h before transfection. After removing the medium, 100 μl suspension of CLPTN (pTGFβ-1 at the amount of 200 ng per well) in serum-free medium described above was added to the well. It was gently mixed by rocking the plate back and forth. Four hours later, the medium was changed with fresh growth medium (DMEM containing 10% FBS without antibiotics). The cells were incubated at 37 °C in 5% humidified CO_2 for 72 h in order to obtain considerable efficiency of gene transfection.

In control cultures, TGFβ-1 plasmid alone (not incorporated into nanoparticles) combined with PEI (25 kDa) or Lipofectamine™2000 (Invitrogen Co, Carlsbad, CA) according to the protocol was added directly to the cultures; and as a negative control group, some cultures were not treated with any transfection reagent or plasmid.

For cultures transfected with TGFβ-1 plasmid nanoparticles, the medium was collected 3 days after transfection and assessed for TGFβ-1 protein using a Rat TGFβ-1 ELISA Kit (Yantai Science & Biotechnology Co., Ltd, Yantai, China).

To evaluate the influence of FBS on transfection, for the CLPTN with greatest transfection efficiency, gene transfection was also conducted in absence of serum with the same method mentioned above.

2.10. Cytotoxicity

Cytotoxicity of CLPTN was measured using the MTT assay. MSCs seeded in a 96-well plate were transfected as described above. 72 h later, MTT solution (5 mg/ml, 20 μl per well) was added to the cells and incubated for 4 h. The supernatant was then aspirated, and the formazan crystals were suspended in dimethylsulfoxide. Aliquots were drawn from each well and the intensity of color was measured spectrophotometrically at 570 nm based on a microplate spectrophotometer (Spectra Max 190, Molecular Devices, USA). Cells without treatment were taken as control with 100% viability. The relative cell viability (%) compared to control cells was calculated using the formula $[Abs]_{sample}/[Abs]_{control} \times 100$ (Patnaik et al., 2006).

2.11. Live cell imaging

To observe the processing of CLPTN in cells, propidium iodide (PI) was used as the marker of pDNA. PI was added into the plasmid during the preparation of nanoparticles. Work on cells was the same as described in Section 2.9. Time-lapse images of cell morphology and movement were captured with 400 mm z-stacks (15 mm slices) every 30 min for an 18 h period. The pictures were taken under a Nikon TE 2000 PFS fluorescent microscope equipped with a motorized stage and environmental sample chamber.

2.12. Statistical analyses

Data were analyzed by one or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc tests to determine the significance in the difference between selected groups using SPSS statistical software (SPSS company, USA). Data were presented as means and ±standard error of the mean (SEM).

3. Result

3.1. Characterization of LBP and cLBP

In the present study, LBP was isolated from *L. barbarum*. According to the determination of thin-layer chromatography (TLC), the monosaccharide content of LBP is arabinose, galactose, glucose, mannose, rhamnose and xylose with relative ratios of 13.2:19:10:10.5:3.4:2.9 respectively. The molecular weight of Ed-LBP (45,000 Da) was found to be larger than purified LBP (35,000 Da) (determined by HPLC, TSK-GEL G4000PW, Φ 7.5 × 300 mm, 5 μm, Dalian, China).

The qualitative functional group analysis in Ed-LBP by FTIR (KBr) yielded the peaks at 3400, 1650, 1535, 1060 cm^{-1}. Fig. 1 shows the FTIR spectra of LBP and Ed-LBP. Compared with the spectrum of LBP, obvious changes at 3400 cm^{-1} (amino stretching), 1650, 1535 cm^{-1} (indicating the existence of β-NH group), 1060 cm^{-1} (–C–O–C– stretching) could be observed in the spectrum of cLBP, demonstrating that amino groups were grafted onto polysaccharides successfully.

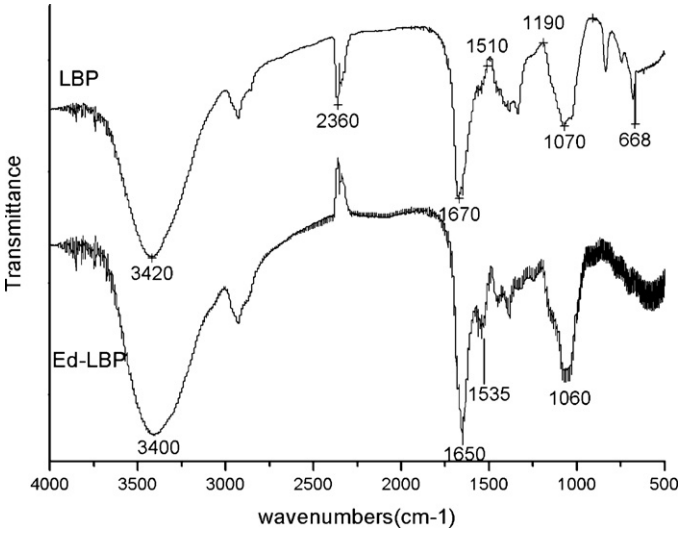

Fig. 1. FT-IR spectra of LBP and Ed-LBP.

3.2. Electrophoresis of the DNA

Fig. 2 shows the electrophoretic patterns of nanoparticles prepared with pTGFβ-1 and cLBP. In each gel, naked pTGFβ-1 (0.6 μg per well) was used as control and loaded to lane 1. Migration of DNA was retarded with the increase of $M_{cLBP}/M_{pTGFβ-1}$ ratio. In Fig. 2(A), the complex made of purified LBP and plasmid TGFβ-1 at the $M_{cLBP}/M_{pTGFβ-1}$ ratio 80 was loaded to lane 2. Lanes 3–8 shows the migration of nanoparticles consisting of plasmid and progressively increasing amount of Ed-LBP with the ratio of M_{cLBP} to $M_{pTGFβ-1}$ being 30:1, 50:1, 80:1, 100:1, 120:1 and 150:1, respectively. Ed-LBP was able to retard pTGFβ-1 migration partially at $M_{cLBP}/M_{pTGFβ-1}$ ratio of 30 (Fig. 2(A), lane 3). Increasing amount of Ed-LBP nanocomposites in plasmid complexes tended to decrease electrophoretic mobility, and completely inhibited pTGFβ-1 migration at $M_{cLBP}/M_{pTGFβ-1}$ ratio of 80 (Fig. 2(A) lane 5). As a control group, purified LBP could not retard pTGFβ-1 migration at the same ratio (Fig. 2(A), lane 2). As shown in Fig. 2(B), the PEI-LBP nanocomposite completely retarded 0.6 μg plasmid at $M_{cLBP}/M_{pTGFβ-1}$ ratio of 1:1 (lane 3, Fig. 2(B)). With the increasing of cLBP:pTGFβ-1 weight ratio (lanes 3–8, Fig. 2(B)), there was no sign of migration of pTGFβ-1, and the orange fluorescence was barely seen from the 4th lane on the agarose gel, indicating that it remained within the nanoparticles. At the ratio of $M_{cLBP}/M_{pTGFβ-1}$ being 5:1 (lane 5, Fig. 2(B)), it was observed that there was sufficient retention of most of the plasmid in the polymer nanoparticles. Increasing content of PEI-LBP (lanes 6–8, Fig. 2(B)) in cLBP–pTGFβ-1 nanoparticles resulted in the sample migration towards the negative electrode, indicating the positive charge of PEI-LBP. As for Fig. 2(C), to lanes 2–8 were loaded nanoparticles made up of plasmid accompanied with progressively increasing amount of Sm-LBP with the ratio of $M_{cLBP}/M_{pTGFβ-1}$ being 10:1, 20:1, 40:1, 60:1, 80:1, 120:1 and 150:1 respectively. Sm-LBP was not able to completely retard plasmid even at $M_{cLBP}/M_{pTGFβ-1}$ ratio of 60 (lane 5, Fig. 2(C)). The migration to negative electrode was at a state of dispersion, rather than distinct belts which could be clearly recognized.

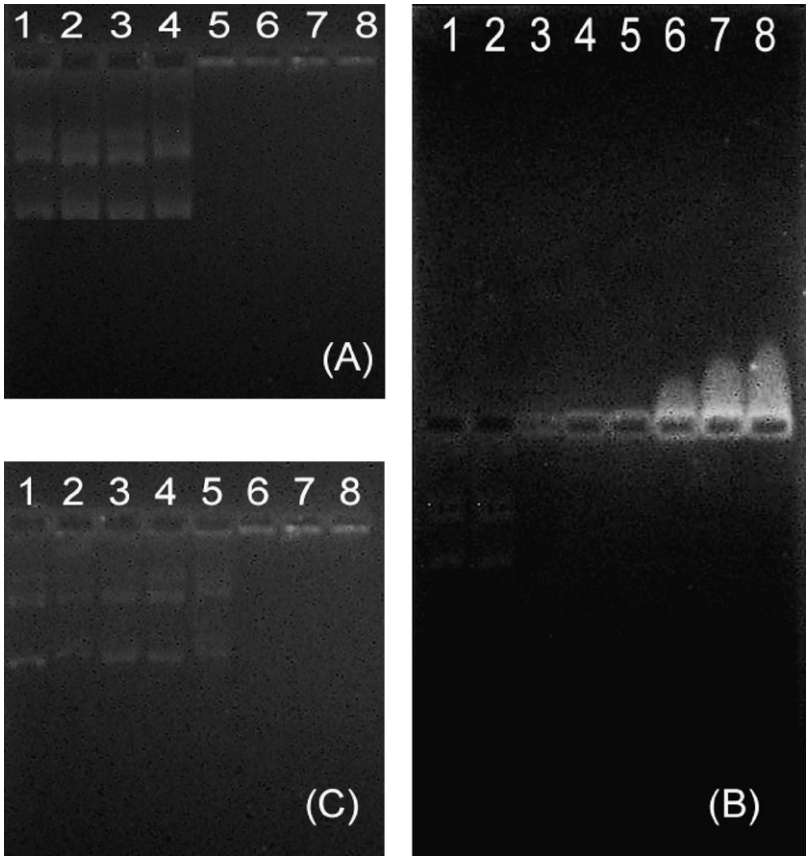

Fig. 2. Electrophoretic mobility of plasmid in cLBP–pTGFβ-1 nanoparticles at various ratios. (A) Ed-LBP–pTGFβ-1 nanoparticles, (B) PEI-LBP–pTGFβ-1 nanoparticles, (C) Sm-LBP–pTGFβ-1 nanoparticles.

3.3. Morphology and size distribution

TEM revealed the sizes and morphologies of Ed-LBP nanoparticles using the $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio of 80, 120, 150 (Fig. 3(A)–(C)), PEI-LBP nanoparticles using the $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio of 2, 5, 10 (Fig. 3(D)–(F)). The small size of the nanoparticles precluded definitive measurement of their diameter by TEM.

The nanoparticles generally appeared to be of monodisperse spherical shape, and were generally uniformed in size less than 200 nm. The result was consistent with the measurements made

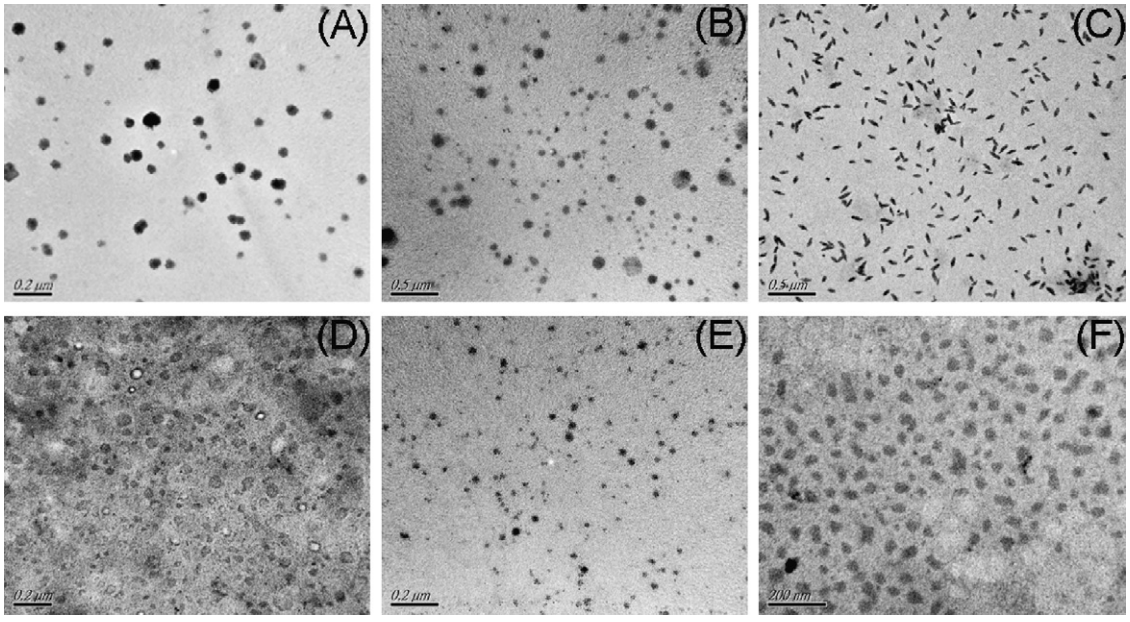

Fig. 3. Transmission electron microscopy images of nanoparticles incorporating pTGFβ-1 prepared with different cLBP at various cLBP/pTGFβ-1 weight ratios. (A) Ed-LBP/pTGFβ-1 weight ratio: 80:1. (B) Ed-LBP/pTGFβ-1 weight ratio: 120:1. (C) Ed-LBP/pTGFβ-1 weight ratio:150:1. (D) PEI-LBP/pTGFβ-1 weight ratio: 2:1. (E) PEI-LBP/pTGFβ-1 weight ratio: 5:1. (F) PEI-LBP/pTGFβ-1 weight ratio: 10:1.

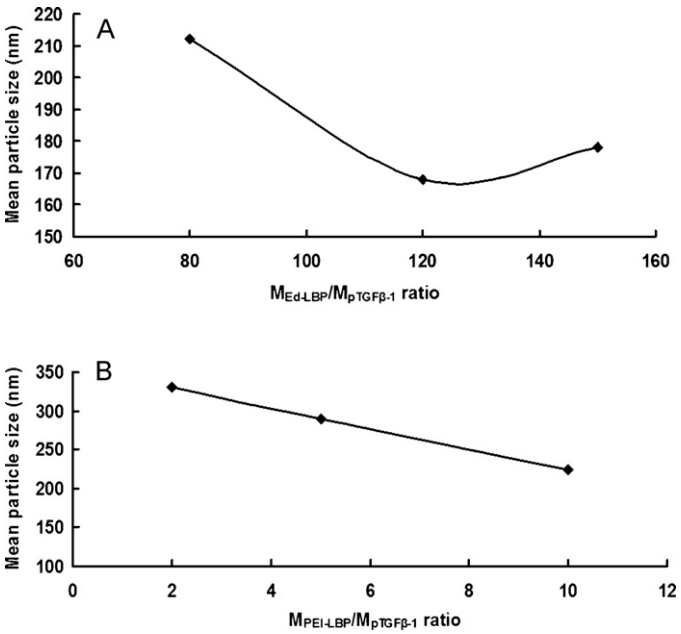

Fig. 4. (A) Particle size of Ed-LBP–pTGFβ-1 nanoparticles with $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratios ranging from 80 to 120. (B) Particle size of PEI-LBP–pTGFβ-1 nanoparticles with $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratios ranging from 2 to 10.

by dynamic light scattering. It revealed that the particle size of Ed-LBP–pTGFβ-1 complex ranged from 168 to 212 nm, with an average diameter of 190 nm (Fig. 4(A)). While the particle size of PEI-LBP–pTGFβ-1 nanostructures ranges from 224 to 311 nm, the average diameter was 267 nm (Fig. 4(B)).

3.4. TGFβ-1 gene expression in transfected MSCs

Transfection experiment was performed on MSCs using TGFβ-1 as a reporter gene. The weight ratios, higher than the ratio at which complete DNA retardation was observed, were used for transfection (weight ratios of Sm-LBP/pTGFβ-1: 80:1; 120:1; 150:1, Ed-LBP/pTGFβ-1: 80:1; 120:1; 150:1, PEI-LBP/pTGFβ-1: 2:1; 5:1; 10:1). The transfected cells treated with CLPTN appeared to be healthy on microscopic examination and had a fairly high level of reporter gene expression, which was found to be 19.6–21.6 fold higher compared to free plasmid. As revealed in Fig. 5, gene packed with Ed-LBP in transfected MSCs gave more considerable expression compared with PEI-LBP and Sm-LBP nanocomplexes. Transfection efficiency depended on $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio. Maximum transfection efficiency was recorded in the case of Ed-LBP at $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio of 80 (1084.3 pg/ml), much higher than that of PEI (926.5 pg/ml, $p < 0.001$) and even more than that of Lipofectamine™2000 (1001.6 pg/ml, $p < 0.001$). Weight ratio resulting in optimal transfection was much lower in the case of PEI-LBP nanocomposites, however, TGFβ-1 gene expression (1015 pg/ml) in transfected MSCs was higher than that of PEI ($p < 0.001$) and similar to that of Lipofectamine™2000. Sm-LBP nanoparticles did not show added appreciable transfection efficiency than Lipofectamine™2000.

Ed-LBP–pTGFβ-1 nanoparticles prepared with $M_{Ed\text{-}LBP}/M_{pTGF\beta\text{-}1}$ ratio of 80:1, 120:1 and 150:1 were used to transfect cells both in presence of 10% FBS and in absence of FBS. As shown in Fig. 6, the expression of TGFβ-1 manifested no obvious change for blank and naked plasmid group with or without 10% FBS. For Lipofectamine™2000 group the expression was slightly higher in absence of FBS than in presence of 10% presence. However, the transfection efficiency of Ed-LBP–pTGFβ-1 nanoparticles (80:1) in absence of FBS was significantly higher than Lipofectamine™2000 ($p < 0.001$), basically equal to that in presence of 10% FBS, indicating whether in presence of FBS or not had no obvious influence on transfection efficiency of prepared nanoparticles. Even there was no FBS to stimulate cell growth and proliferation, Ed-LBP–pTGFβ-1 nanoparticles (80:1) still had great transfection efficiency.

3.5. Cell viability

The cytotoxicity associated with cLBP nanoparticles has always been a concern in their use as plasmid carriers. The toxicity of Ed-LBP–pTGFβ-1, Sm-LBP–pTGFβ-1 and PEI-LBP–pTGFβ-1 nanoparticles was evaluated on MSCs. Cells treated with PEI-LBP yielded an almost similar viability as controls (untreated cells). Furthermore, an increasing weight ratio of Sm-LBP to plasmid improved cell viability and also supported cell growth in some cases. Cells treated with Sm-LBP nanoparticles at $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio of 150 had a fairly high level of cell viability, and had improved 1.18 fold compared with the control group. Likewise, Ed-LBP promoted cellular growth and cell viability of 107.1%, much higher than that of Lipofectamine™2000 (79%, $p < 0.001$) (Fig. 7). However, there was a gradual decrease in cell viability with the increasing load of Ed-LBP.

3.6. Live cell imaging

As shown in Fig. 8, at 6 h, 8 h and 18 h, there was nearly no free plasmid entering the nuclear. The number of cells stained by PI in Ed-LBP–pTGFβ-1 (80:1) group were much greater compared with cells treated with Lipofectamine™2000, at both 8 h and 18 h, indicating that Ed-LBP can efficiently carry plasmid into cells, faster than positive control Lipofectamine™2000.

4. Discussion

L. barbarum plays multiple roles in pharmacological and biological functions as a well-known Chinese traditional medicine and is also a kind of food (Gan, Zhang, Liu, & Xu, 2003). The biological activities of the polysaccharides have attracted more attention recently in biochemical and medical fields (Zhu et al., 2010) because of their immunomodulatory, antitumor effects, antioxidant, and blood lipid-lowering properties. We purified a polysaccharide from *L. barbarum*, analyzed its monosaccharide components and investigated its chemical structure. We found that the main monosaccharide composition was arabinose, galactose and glucose. Our previous work demonstrated that LBP significantly enhanced MSCs adhesion. RGD has been reported to modify polymeric nanoparticles to improve the cellular uptake of gene vectors and enhanced gene transfection efficiency because they selectively recognize and bind integrins (Sakae et al., 2008; Suk et al., 2006; Sun et al., 2008). The specific adhesive property of the polysaccharides may act in the same way to facilitate the entry of cLBP nanoparticles into cells.

In the present study, the efficacy of cLBP prepared with different amine compounds (ethylenediamine, PEI (1300 Da), spermine) as a non-viral gene delivery system was evaluated. The result of gel chromatography informed us of the acquisition of purified LBP and Ed-LBP with stable and uniform molecular weight. The molecular weight of Ed-LBP was slightly larger than that of purified LBP, which was probably attributed to the molecular bond fracture in purified LBP caused by the joining of amine groups.

The gel retardation assay revealed that the electrostatic interaction between cLBP and the plasmid DNA. At higher $M_{cLBP}/M_{pTGF\beta\text{-}1}$ mixing ratios, the plasmid DNA band did not migrate in electrophoresis any more (Fig. 2). Probably the negative charge of plasmid DNA was neutralized by cLBP and the molecular size

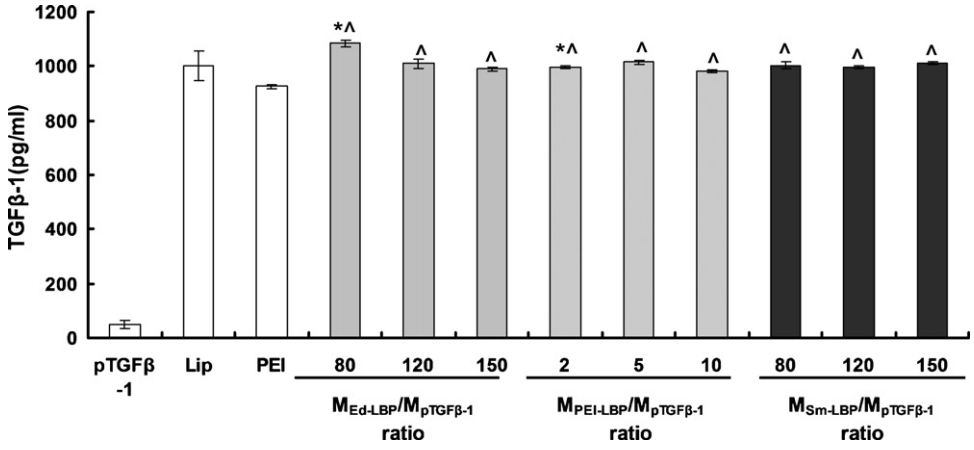

Fig. 5. TGFβ-1 protein release into the medium from rat mesenchymal stem cells 72 h after treatment with different cLBP–pTGFβ-1 nanoparticles at different $M_{cLBP}/M_{pTGFβ-1}$ ratios with a 200 ng plasmid load. Cells treated with naked pTGFβ-1, Lipofectamine™2000 and PEI (25 kDa) were set as control. $n = 3$, mean ± SEM. $^{*}p < 0.001$ compared with Lipofectamine™2000. $^{\wedge}p < 0.001$ compared with PEI (25 kDa).

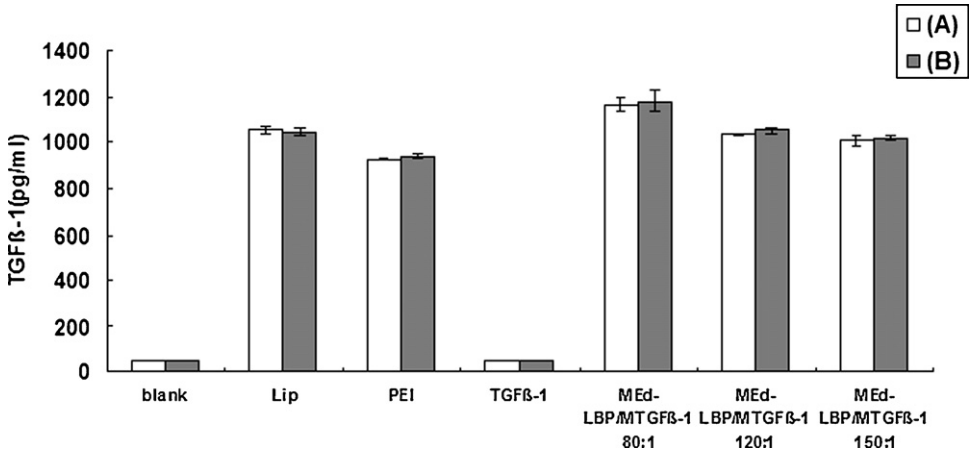

Fig. 6. TGFβ-1 protein release into the medium from rat mesenchymal stem cells 72 h after treatment with Ed-LBP–pTGFβ-1 nanoparticles at different $M_{Ed-LBP}/M_{pTGFβ-1}$ ratios with a 200 ng plasmid load. Blank group, cells treated with naked pTGFβ-1, Lipofectamine™2000 and PEI (25 kDa) were set as control. (A) In absence of FBS. (B) In presence of 10% FBS.

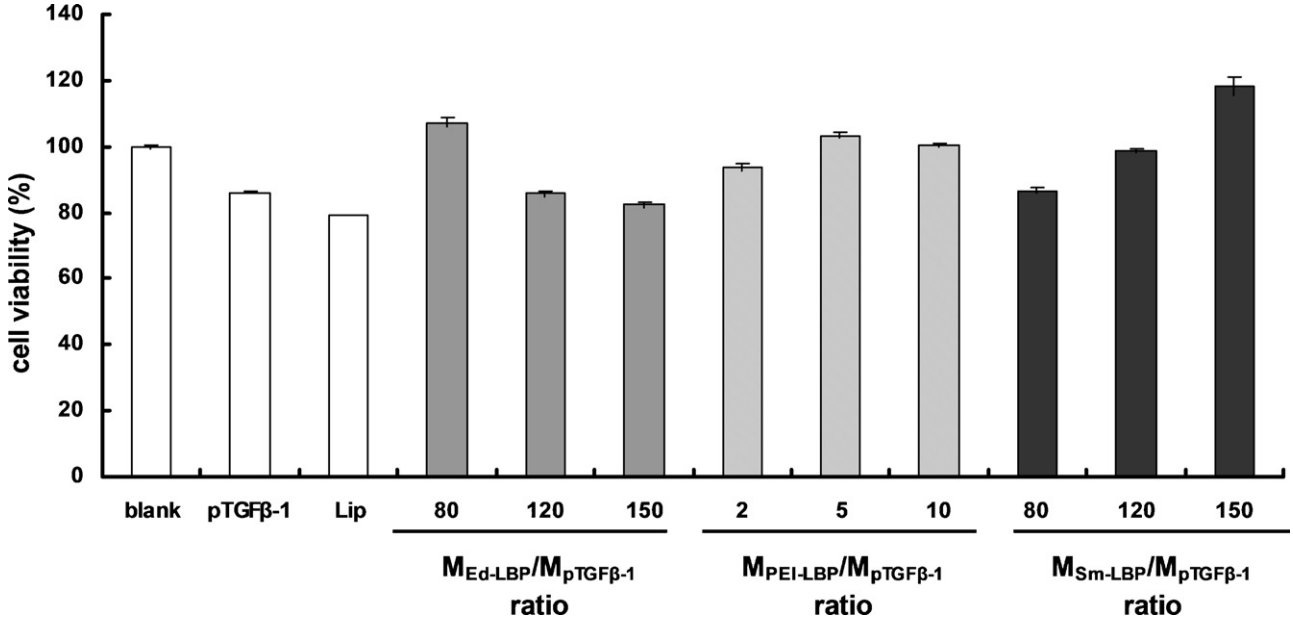

Fig. 7. Viability % (percentage of blank group (untreated cells, set as control)) of rat mesenchymal stem cells after 72 h of treatment with different cLBP–pTGFβ-1 nanoparticles at different $M_{cLBP}/M_{pTGFβ-1}$ ratios using a 200 ng plasmid load. Cells treated with naked pTGFβ-1, Lipofectamine™2000 are set as control. $n = 3$, mean ± SEM.

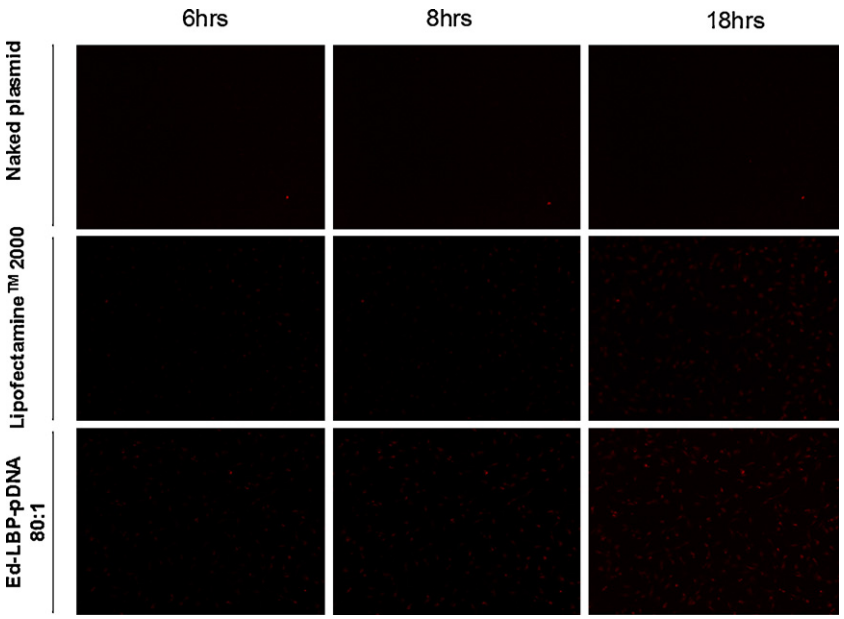

Fig. 8. Live cell imaging.

of plasmid DNA increased with the joining of cLBP, resulting in reduced electrophoretic migration of plasmid DNA. The results demonstrated that the interaction of plasmid DNA with PEI-LBP was explicitly greater than with Ed-LBP or Sm-LBP. Once the weight ratio of PEI-LBP to pTGFβ-1 reached 10:1, the nanoparticles migrated to the negative electrode, illustrating the positive charge of the plasmid nanoparticles encapsulated by PEI-LBP. However, the migration to the negative electrode was at a state of dispersion, instead of clearly recognizable belts. This was probably because of the migration of the excessive PEI-LBP, which failed to be connected to pTGFβ-1.

The cationized LBP–pTGFβ-1 nanoparticles generally appeared to be of monodisperse spherical shape, and showed an apparent molecular size in the nanometer range that could be favorably taken up by cells. It is an advantageous feature of plasmid TGFβ-1 complex prepared from cLBP to enhance the transfection efficiency for gene expression in terms of efficient plasmid condensing to a nano-order size (Kushibiki, Tomoshige, Iwanaga, Kakemi, & Tabata, 2006b). On the basis of the result, it is obvious that molecules with smaller particle diameter may not result in higher transfection efficiency, nor is particle diameter the decisive factor influencing transfection efficiency. It has been proven that nanoparticles with a particle diameter of 200 nm can be better taken up by cells, release more plasmid in cells, and demonstrate higher transfection efficiency.

The degree of cationization was reflected in the zeta potential measurements. In the present study, Ed-LBP alone was found to have a potential of +17 mV, and Ed-LBP–pTGFβ-1 nanoparticles displayed the potential of about +11 mV (weight ratio of LBP to pTGFβ-1 being 80:1) compared with −42 mV for naked plasmid TGFβ-1. In addition, the surface charge of Ed-LBP–pTGFβ-1 nanoparticles tended to increase with the increasing $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio, indicating that the surface of the nanoparticles is covered with cLBP molecules.

Of interest were the findings demonstrating the difference in the functionality of Ed-LBP, Sm-LBP and PEI-LBP nanoparticles as transfection agents for plasmid TGFβ-1. Gene packed with Ed-LBP in transfected MSCs gave more considerable expression than PEI-LBP and Sm-LBP nanocomplexes. Even the weight ratio resulting in optimal transfection was much lower than in the case of PEI-LBP and Sm-LBP nanocomposites. The result also revealed an optimal Ed-LBP: pTGFβ-1 weight ratio for transfecting cells, with the maximum TGFβ-1 expression recorded at weight ratio of 80:1. The transfection efficiency was found to be much higher than that of both PEI and Lipofectamine™2000. Interestingly, low cytotoxicity was found at test concentrations. Cells treated with PEI-LBP nanoparticles yielded an almost same viability as controls. Furthermore, an increased weight ratio of Sm-LBP:plasmid in the nanoparticles improved the cell viability and also supported cell growth in some cases (118%, $M_{cLBP}/M_{pTGF\beta\text{-}1}$ ratio of 150). Likewise, cells treated with Ed-LBP nanoparticles promoted cellular growth and had a fairly high level of cell viability of 107.1% with the weight ratio recorded for optimal transfection efficiency compared to control groups (100%, untreated cells; 79%, Lipofectamine™2000).

The surface charge of the nanoparticles prepared at various weight ratios, however, may not have been a significant factor influencing gene transfer to MSCs as the surface charge did not change significantly using different weight ratios. A smaller particle diameter may increase the probable entry of cLBP into cell, resulting in enhanced gene expression (Xu et al., 2008a). The specific adhesive property of LBP may improve the cellular uptake of exogenous gene and enhance gene transfection efficiency probably because of the receptor-mediated internalization of plasmid DNA. The result of transfection experiments indicated that there was no significant difference in transfection efficiency with/without the existence of FBS. Interestingly, the cLBP nanoparticle had no cytotoxicity; it could even improve cell viability and support cell growth in suitable weight ratio range. Prepared nanoparticles can be quickly taken by cells, faster than positive control Lipofectamine™2000.

Additional studies are very necessary to gain deeper understanding of the transfection mechanism that is responsible for the difference in the behaviors of Ed-LBP, Sm-LBP and PEI-LBP nanoparticles, cell binding and endocytosis, release of the contained plasmid, and cell nucleus incorporation.

5. Conclusions

As a novel idea for developing adaptable gene carriers, the study demonstrates that polysaccharide isolated from *L. barbarum* can be chemically modified by different amine reagents to obtain cationized vectors to incorporate plasmid containing the reporter gene TGFβ-1. Despite the relatively low reproduction speed com-

pared with tumor cells, MSCs were finally successfully transfected *in vitro*, which indicated the prospective use of cationic polysaccharide in gene delivery. Ed-LBP/DNA showed optimal transfection efficiency compared with PEI-LBP and Sm-LBP nanoparticles. Maximum gene expression of Ed-LBP nanoparticles was recorded at the ratio of M_{cLBP} to $M_{pTGF\beta\text{-}1}$ being 80, which was found to be significantly much higher than that of both PEI ($p < 0.001$) and Lipofectamine^{TM}2000 ($p < 0.001$), representing a fairly high transfection efficiency level. There was no significant difference in transfection efficiency with/without the presence of 10% FBS. The transfected cells appeared to be of low cytotoxicity, outstanding cell viability and excellent cell morphous. Live Cell Image was performed to confirm the nuclear uptake of cationized LBP/plasmid TGFβ-1 nanoparticles. The complex was quickly taken by cells, indicating the possibility of high transfection efficiency. These accomplished studies will provide valuable information for further development of polysaccharides extracted from Chinese herb on further application of safe and efficient non-viral vectors.

Acknowledgements

This work was supported by National Natural Science Foundation of China (30772661 and 81072586), special funds for 333 projects (BRA2010138) and Industry-University-Research Institution Cooperation (BY2009141) in Jiangsu Province.

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