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Transfection efficiency of depolymerized chitosan and epidermal growth factor conjugated to chitosan–DNA polyplexes

Sasamon Supaprutsakul · Wilaiwan Chotigeat · Supreya Wanichpakorn · Ureporn Kedjarune-Leggat

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Abstract An efficient non-viral gene delivery for varieties of cells has been considered essential for gene therapy and tissue engineering. This study evaluated transfection efficiency of chitosan (HW) with molecular weights (Mw) at 470 and degree of deacetylation (DDA) 80% and its depolymerization product (LW) with Mw at 16 kDa and DDA 54%, as well as epidermal growth factor (EGF) conjugated to chitosan–DNA microparticles of both HW and LW by using either disulfide linkage or NHS-PEO_4-Maleimide as a cross linker. The results revealed that the depolymerized LW at chitosan/DNA charge ratio 56:1 and pH 6.9 gave high transfection efficiency in both KB, a cancer cell line, and fibroblast cells at about the same level of Lipofectamine^{TM}, but the EGF-conjugated chitosan–DNA polyplexes from these methods did not improve transfection efficiency, which may come from the aggregation and fusing of the complexes as shown in scanning electron microscopy. However, this depolymerized LW chitosan showed the potential for further development as a safe and cost-effective non-viral gene delivery vehicle.

S. Supaprutsakul · W. Chotigeat
Center of Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

S. Supaprutsakul · S. Wanichpakorn · U. Kedjarune-Leggat (✉)
Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand
e-mail: ureporn.l@psu.ac.th

1 Introduction

An efficient gene delivery system into a variety of cells, which does not stimulate an immune response or a cytotoxic reaction, has been considered beneficial for gene therapy and tissue engineering. This has lead to a number of developments for non-viral gene delivery systems, although they remain less efficient than the virus system [1].

Chitosan (poly[β-(1-4)-2-amino-2deoxy-D-glucopyranose]), a nontoxic biodegradable biopolymer, has been widely studied as a promising non-viral vector for gene delivery [2]. This cationic polysaccharide was produced by partial deacetylation of chitin, a naturally polymer from crustacean shells. Chitosan-based gene delivery systems have been successfully applied to oral and nasal gene therapy systems [3]. Recently, a gene-activated matrix composed of chitosan/collagen, as a scaffold with embedded chitosan–DNA encoding platelet derived growth factor nanoparticles, has been developed to prolong plasmid release and transfection in periodontal ligament cells cultured, which had potential for periodontal tissue engineering [4]. However, the transfection efficiency of chitosan itself is not efficient enough and depends on many factors such as molecular weight, degree of deacetylation (DDA), DNA complexes charge ratio, pH and particle sizes [5, 6] as well as type of cells [7, 8].

Some studies revealed that low molecular weight chitosan, especially the product from oxidative depolymerization from higher molecular weight chitosan with $NaNO_2$, had low cytotoxicity and improved solubility properties [9], as well as having potential for gene delivery both in vitro [6] and in vivo [10]. However, some studies have reported decreased transfection efficiency with lower molecular weight chitosan [11, 12].

There have been many attempts at modifying chitosan in order to improve transfection efficiency [13–15], which

have included conjugation of chitosan–DNA complexes with ligands to target specific cell surface receptors; but results have been variable [16, 17].

Epidermal growth factor is a growth factor that has a mitogenic property in various kind of cells and a high EGF receptor expression frequently accompanies several tumor types such as squamous cell carcinoma [18], which may be suitable for target gene delivery because EGF can bind with its receptor with high affinity and internalized in the cell through receptor mediated endocytosis [19]. EGF conjugated with degradable polymer gene carriers, such as polyethylene glycol [20] and oligoethylenimine [21] can increase transfection efficiency in EGF-receptor rich cancer cells. It may be possible to increase transfection efficiency of chitosan nanoparticles especially in cancer cells via conjugation with EGF which will be beneficial especially for tumor-target gene delivery. Currently, DNAzyme and siRNA encapsulated into chitosan nanoparticles were studied as a new approach for cancer therapy (see review [22]).However, there are factors related to transfection efficiency of chitosan, including its molecular weight, DDA as well as the charge ratios (N/P), which could simply give rise to an increase in the transfection efficiency by adjusting for the appropriate condition. The purpose of this study was to improve the transfection efficiency of chitosan by using chitosan at 470 kDa and its depolymerization product with the adjusted N/P ratios for optimal transfection, as well as evaluating the transfection efficiency of EGF conjugated with chitosan–DNA polyplexes in order to investigate the feasibility to modify this DNA delivery system as a tumor-target gene delivery. The transfection was performed in KB cells, a cancer cell line which can over-express epidermal growth factor receptors (EGFR) [23], and primary human oral fibroblasts, as a normal cell line.

2 Materials and methods

Chitosan (Mw $\sim$ 470 kDa, DDA 80%) was obtained from Fluka (Japan). 2-Iminothiolane HCl (Traut's reagent) was purchased from Sigma-Aldrich (St. Louis, MO, USA) Dimethyl sulfoxide (DMSO), Ellman' reagent, disuccinimidyl suberate (DSS), *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), NHS-PEO_4-maleimide (succinimidyl-[*N*-maleidopionamido)-tetraethyleneglycol] ester), BCA assay kit and dialysis membranes with molecular weight cut off at 3.5 kDa were purchased from Pierce Chemical (Rockford, IL, USA). Lipofectamine™ 2000 reagent was purchased from Invitrogen Corporation (Singapore).The Steady-GloTM Luciferase Assay kit was obtained from Promega (Madison, USA). PD-10 column was purchased from Amersham (Amersham Biosciences, USA) All culture media were purchased from Gibco BRL Company (Invitrogen Corporation, USA).The purification and desalting of samples were performed by centrifugation using Amicon® Ultra 50 K and 30 K (Millipore corporation, Bedford, MA, USA.). All other chemicals were of the highest grade commercially available either from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

2.1 Plasmids and competent cells

Escherichia coli XLOLR cells (Stratagene, La Jolla, CA, USA) containing recombinant pBK-CMV (Stratagene, La Jolla, CA, USA) with chemically synthesized human EGF gene, plasmids pGL4.13-Luc (Promega, Madison, USA) and Escherichia coli Top 10 competent cells (Invitrogen corporation, USA), were used in this study.

2.2 Cell lines

Human epidermoid carcinoma cells (KB) were obtained from the American Type cell Culture Collection (ATCC number CCL-17, Manassas, USA). This cell line was cultured at 37°C with 5% CO_2 in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal calf serum and 100 units/ml penicillin, 100 μg/ml streptomycin and 1% amphotericin B. Primary fibroblast cells were cultured from normal gingival tissue using direct explant technique [24]. The gingival tissue came from patients undergoing dental surgery for crown lengthening at the Dental Hospital, Prince of Songkla University, Hat Yai, with the consent of the patients using protocols approved by the Ethics Committee of the Faculty of Dentistry, Prince of Songkla University, Thailand. Fibroblast cells were grown with the same conditions and media as KB cells.

2.3 Preparation of plasmid DNA

The plasmids pGL4.13-Luc with 4.641 kb cDNA encoding for luciferase from the Photinus pyralis driven by an SV40 promoter were used for transfection. They were propagated in *E. coli* Top 10 competent cells and purified by precipitation method [25]. The purified pDNA was then resolubilised in distilled water and its concentration/purity determined by UV spectrophotometry at 260 and 280 nm and by electrophoresis, respectively.

2.4 Preparation of depolymerized low molecular weight chitosan

The commercial chitosan (Mw $\sim$ 470 kDa, DDA = 80%) was depolymerized by chemical reaction with sodium nitrite to achieve low molecular weight chitosan [12]. Briefly, 2 g of chitosan was dissolved in 100 ml of acetic acid (6% v/v) under magnetic stirring at room temperature.

Then, 80 mg of sodium nitrite in 10 ml of water were added. After 1 h of incubation, the depolymerized chitosan was precipitated by raising the pH to 9 with 4 M NaOH. The resulting precipitate was filtered and further washed thoroughly with cold acetone. The residue was then dissolved in 100 ml of 0.1 M acetic acid and dialyzed against water before concentrated partially under vacuum. The samples were lyophilized at −80°C and 0.01 mbar (Christ Alpha 2-4, Osterode am Harz, Germany) prior to characterization and used in further experiments.

2.5 Preparation of recombinant human EGF protein

E. coli XLOLR cells carrying plasmids pBK-CMV encoding human EGF gene were grown in LB selective conditioned medium. The inclusion bodies containing human EGF protein (6.4 kDa) were extracted from bacteria pellets by sonication and centrifugation. before resuspended in 8 M urea, 25 mM Sorensen buffer pH 9 and the EGF protein was further purified and concentrated by centrifugation at 5000*g*, 4°C for 10 min through column of Amicon® Ultra 50 K and 30 K, respectively. The purified EGF was examined by SDS-PAGE. Only the single band at Mw 6.4 kDa was acceptable for further processing. The protein was dialyzed against 0.1 M sodium phosphate buffer with 5 mM EDTA pH 7.6 using dialysis membrane with a molecular weight cut off at 3.5 kDa (Piece, Rockford, IL, USA) and water was removed by Aquacide II with Mw 500 kDa (Calbiochem, Merk, Darmstadt, Germany) at 4°C. The concentrated sample was lyophilized and stored at −80°C until use. The amount of human EGF was measured using sandwich ELISA technique (R&D Systems, Minneapolis, USA).

2.6 Preparation of chitosan–DNA microparticles

The chitosan–DNA microparticles were prepared by using coacervation method, which was modified from Moa et al. [16] and Corsi et al. [7]. Briefly, chitosan of different molecular weights was dissolved in 0.2 M acetic acid at 45°C and diluted to 0.05% for high molecular weight chitosan (HW chitosan) and 0.02% of the depolymerized low molecular weight chitosan (LW chitosan) with 5 mM acetate buffer pH 5.5 and filtered sterile through 0.22 μm filters, 100 μg/ml of plasmid DNA was prepared in 50 mM of sodium sulfate solution. They were separately heated to 55°C for 30 min. The mixtures were subjected to intensive vortex for 2 min and left for 15 min at room temperature before transfection. The N/P ratios of chitosan/DNA of HW and LW chitosan were set at 7:1 and 56:1, respectively, and 5 μg of DNA was used for each well. The final volume of the mixture in each preparation was limited to below 500 μl in order to yield uniform particles.

2.7 Preparation of EGF conjugated with chitosan–DNA microparticles

EGF was conjugated via chitosan–DNA micropaticles that were performed by either HW or LW chitosan via disulfide linkage and using NHS-PEO_8-Maleimide as a cross linker.

2.7.1 Synthesis of EGF conjugation via a disulfide linkage

EGF was coupled with chitosan–DNA microparticles by ligation through disulfide bonds after modification with the coupling reagent 2-iminothiolane (Traut's reagent; Pierce, USA). A cold solution of 32–100 μg of EGF in 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.6, containing 5 mM EDTA) was mixed with 50 μl of 2-imminothiolane solution (5 mg/ml in water). The reaction was performed for 30 min at room temperature. Therefore, sulfhydryl group was introduced into EGF to form EGF-SH. To remove excess coupling reagent, 2-iminothiolane, the sample was separated by gel filtration using a PD-10 column and eluted with PBS containing 5 mM EDTA. The EGF-SH of the fractions was determined spectrophotometrically at 280 nm and degree of modification for the modified EGF was evaluated via Ellman's assay.

Chitosan–DNA nanoparticles of either high and low molecular weight were reacted with SPDP to yield a 2-pyridyldithiol-end group on the surface, which was reacted with EGF-SH to form a disulfide linkage between microparticles surface and EGF by following the method of Mao et al. [16]. Briefly, 1 ml of chitosan–DNA microparticles suspension was mixed with 5 μl of DSS solution (10 mM in DMSO), 10 μl of SPDP solution (10 mM in DMSO) and 100 μl of PBS (10×) buffer. The mixture was incubated at room temperature for 30 min before it was quenched by the addition of 50 μl of 1 M glycine solution. The mixture was further reacted with 250 μl of EGF-SH solution (1 mg/ml) for an hour at room temperature. The modified polyplexes were isolated by ultracentrifugation.

The amount of EGF conjugated on the chitosan–DNA microparticles was determined by using sandwich ELISA technique.

2.7.2 Synthesis of EGF conjugation to chitosan–DNA microparticles via NHS-PEO_4-Maleimide as a cross linker

EGF was coupled to chitosan–DNA microparticles by ligation through disulfide bonds after modification with the coupling reagent, 2-iminothiolane, with the same method that was described above. To synthesize chitosan–DNA microparticles-PEO_4-, NHS-PEO_4-Maleimide (succinimidyl-[*N*-maleidopionamido-tetraethyleneglycol] ester), as a cross linker with a molecular weight of 513.50 Da, was used

to modify the particle's surface. In brief, 1 ml of chitosan–DNA microparticles was incubated with 20 μl of NHS-PEO_4-Maleimide and 100 μl of PBS for 15 min at room temperature, followed by quenching with 50 μl of 1 M glycine solution. The PEO_4 modified polyplexes were purified by ultra-centrifugation or dialysis. The mixture was further incubated with 250 μl of EGF-SH in PBS (1 mg/ml) for 2 h at room temperature. The EGF-conjugated polyplexes were purified by centrifugation with Amicon® Ultra-4 centrifugal filter or dialysis.

So the methods used to conjugate EGF with chitosan–DNA microparticles can be postulated as following:

Method 1: EGF conjugation via a disulfide linkage by using HW chitosan
Method 2: EGF conjugation via a disulfide linkage by using LW chitosan
Method 3: EGF conjugation via NHS-PEO_8-Maleimide as a cross linker by using HW chitosan
Method 4: EGF conjugation via NHS-PEO_8-Maleimide as a cross linker by using LW chitosan

2.8 In vitro transfection

KB and oral fibroblasts were seeded in a 24-well culture plate at a density of 7×10^4 cells/well in 1 ml of DMEM containing 10% FBS, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and incubated for 24 h at 37°C in 5% CO_2. At the time of transfection, the media was aspirated and cells were washed with PBS pH 7.4. The amount of chitosan–DNA microparticles or polyplexes equivalent to 5 μg DNA was added to each well and incubated for 24 h in culture media at pH 6.9 without antibiotic. Non treated cells and cells transfected with naked plasmid DNA, cells with pDNA transfected with Lipofectamine™ 2000 reagent were acted as controls. Transfection with Lipofectamine™ 2000 reagent was followed the manufacture's protocol by using 1 μg of pDNA mixed with 2 μl of Lipofectamine™ 2000 reagent. After 24 h, cells were washed with PBS before replaced with 1 ml of fresh complete medium and incubated for another 24 h. All experiments were repeated independently at least three times.

2.9 Luciferase activity assay

The Luciferase assay was carried out according to manufacture's instruction (Promega, Madison, USA). Cells were harvested by removing the medium and then washed with PBS. Thereafter, 100 μl of 1 × Glo Lysis buffer was added (Promega, Madison, WI, USA). The cell lysate was centrifuged at 10,000 rpm for 3 min and the supernatant was collected. An aliquot of 40 μl of the supernatant was placed into 96-well white luminometer plates in which 40 μl of Steady-Glo Luciferase Assay System (Promega) reagent was added just prior to measurement on a luminometer (PerkinElmer, Wellesley, MA, USA). Transfection efficiency was reported as the relative light units (RLU) normalized to the protein concentration in the cell extracts of transfected cell which was measured using the BCA method (Pierce Biotechnology).

2.10 Characterization of chitosan

The number- and weight-average molecular weights (Mn and Mw) of two chitosans used in this study was determined by gel permeation chromatogram (GPC) (Waters 600E, water). The chromatographic system equipped with an Ultrahydrogel linear 1 column using 0.5 M CH_3COOH/0.5 M CH_3COONa (acetate buffer pH 4) as the mobile phase. The samples were then run at a flow-rate of 0.6 ml/min and a column temperature of 30°C. The degree of deacetylation of free amine groups of both chitosans was examined by 1H NMR spectra of chitosan. The NMR samples were characterized at room temperature (20 ± 1°C) with solid state 13C CP/MAS NMR.

2.11 Characterization of the physiochemical properties of the nanoparticles

2.11.1 Agarose gel electrophiresis

The DNA binding ability of the polyplexes and plasmid DNA were evaluated by electrophoretic mobility in 1.0% agarose gel in 1 × Tris-boric acid-EDTA buffer with 0.5 μg/ml ethidium bromide and elcetrophoresised at 100 V for 45 min. The resulting DNA dye migration pattern was examined under UV irradiation.

2.11.2 Morphology of chitosan–DNA nanoparticles

Samples of chitosan DNA microparticles as well as some of the EGF conjugated with chitosan DNA polyplexes from different methods were frozen in liquid nitrogen and freeze-dried. The samples were coated with gold/palladium. Subsequently, the morphological examination of these samples was performed by a JEOL JSM-5200 scanning electron microscope (SEM) (Tokyo, Japan).

3 Results

3.1 Transfection efficiency

Transfection efficiency of different chitosan–DNA polyplexes into KB and fibroblast cells were compared to naked

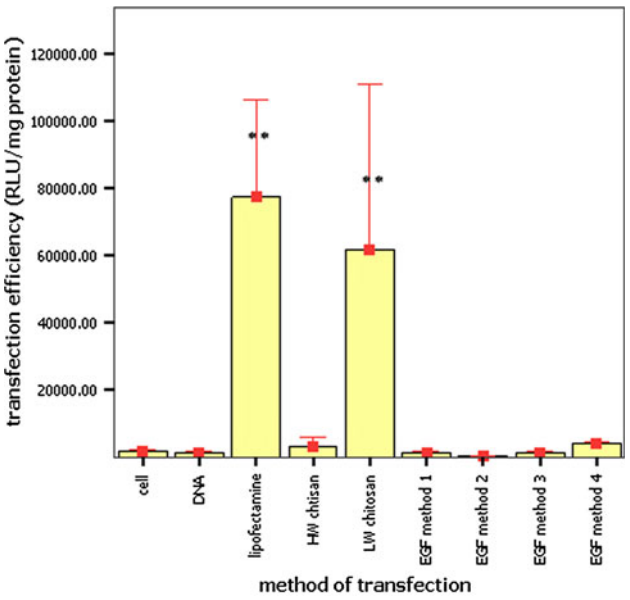

Fig. 1 Means and 95% confidence intervals of means of luciferase expression in KB cells following treatment with different transfection materials. ** statistically significant difference compare to DNA, $P < 0.01$, one-way ANOVA with Bonferroni post test

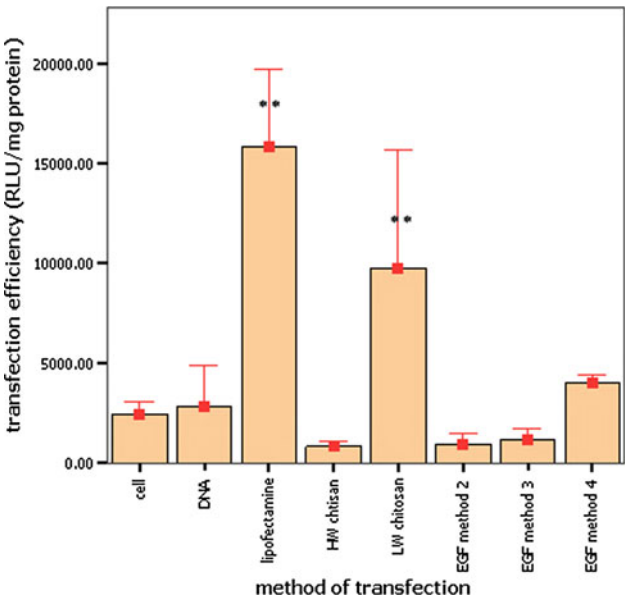

Fig. 2 Means and 95% confidence intervals of means of luciferase expression in fibroblasts following treatment with different transfection materials. ** statistically significant difference compared to DNA, $P < 0.01$, one-way ANOVA with Bonferroni post test

DNA by Lipofectamine™ 2000. The N/P ratio of HW chitosan/DNA used was 7:1 and N/P ratio of LW chitosan/DNA was 56:1. Figures 1 and 2 show the transfection efficiency into KB and fibroblast cells, respectively. Only transfection by Lipofectamine™ and the depolymerized chitosan (LW) gave significantly higher luciferase activity as compared to naked DNA transfection into both of KB and fibroblast cells ($P < 0.01$, ANOVA with Bonferroni post test).It was noted that transfection efficiencies of both Lipofectamine™ and LW chitosan were much higher in KB cells than in fibroblasts.

3.2 Molecular weight and degree of deacetylation

The determination of molecular weight by gel permeation chromatography revealed that the HW chitosan in this experiment had weight average (Mw) = 470 kDa and the average number molecular weight (Mn) = 56 kDa, which gave its polydispersity (Mw/Mn) = 8.39. LW chitosan, which is the depolymerization product of HW chitosan, had Mw about 16 kDa and Mn = 6.3 kDa and polydispersity = 2.54. The DDA performed by NMR showed that HW chitosan had about 80% and the LW chitosan had 54% DDA.

3.3 Agarose gel electrophoresis

Figure 3 revealed DNA binding ability of chitosans and chitosan derivatives, it was shown that HW (lane 2) and LW chitosan (lane 5) bound DNA completely as no band of DNA was detected. It was noted that DNA was bound without any free plasmid detection of both EGF conjugated to HW and LW chitosan using disulfide linkages in lane 3 and 5, respectively. However gel electrophoresis was not able to detect free plasmid as well as EGF conjugated chitosan–DNA polyplexes formulated by cross linking reagent in both HW and LW chitosan in lane 4 and 7, respectively.

3.4 Scanning electron microscopy

The morphology and average sizes of chitosan–DNA microparticles or chitosan–DNA polyplexes have been

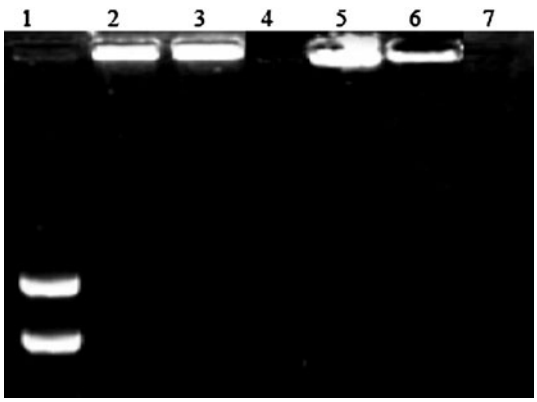

Fig. 3 Electrophoresis of different chitosan–DNA complexes on an agarose gel. Lane 1, DNA only; lane 2, HW chitosan–DNA; lane 3 EGF conjugated HW chitosan–DNA method 1; lane 4, EGF conjugated HW method 2; Lane 5, LW chitosan–DNA; lane 6, EGF conjugated LW chitosan method 3, lane 7, EGF conjugated LW chitosan method 4

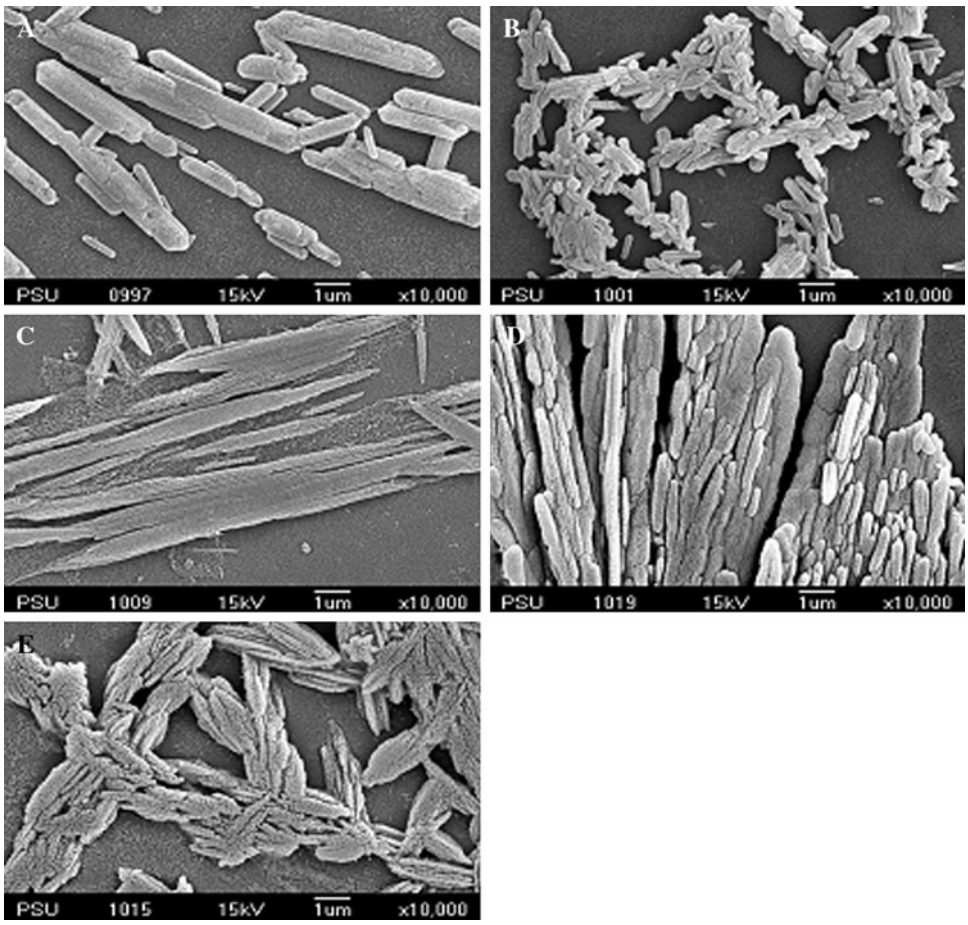

Fig. 4 Scanning electron micrograph of some different complexes. **a** HW chitosan–DNA complexes, the particles had different sizes from 0.5 μm (500 nm) to 10 μm. **b** LW chitosan–DNA, the particles size ranged from 0.5 to 1 μm complexes. **c** EGF conjugated to LW chitosan–DNA complexes using disulfide bond (method 3), the complexes had long needle shape and some were fused together. **d** EGF conjugated to LW chitosan–DNA complexes using NHS-PEO_4-Maleimide as a cross linker (method 4). **e** EGF conjugated to HW chitosan–DNA complexes using NHS-PEO_4-Maleimide as a cross linker (method 2)

shown in Fig. 4, where chitosan–DNA polyplexes formed by HW and LW chitosan had rod or capsule shapes. Chitosan–DNA microparticles formed by HW chitosan had sizes ranged from 500 nm to 10 μm, which are larger than chitosan–DNA microparticles formed by LW chitosan and which had sizes ranged from 500 nm to 1 μm and averaged sizes around 600 nm. It was noted that the sizes of LW chitosan–DNA microparticles did not vary as much when compared to HW chitosan–DNA microparticles, which had both small and large rod like particles. However, these two types of chitosan–DNA microparticles seemed to aggregate like polyplexes of particles. It was shown that EGF conjugated chitosan–DNA polyplexes rarely gave single particles as most of them are fused together. However, it can be noted that the surfaces of these polyplexes were covered with many small round globular particles.

4 Discussion

This study revealed that the HW chitosan gave low transfection efficiency, but still higher than the EGF conjugated chitosan–DNA polyplexes in all four methods used. This is contrast to the study of Blessing and colleagues [26], where they conjugated EGF with polyethylenimine (PEI)/DNA complexes and a covalent surface modification of EGF–PEI/DNA complexes with a poly (ethylene glycol (PEG) derivative and found the increased transfection efficiency in KB cells via a receptor-dependent fashion. There may be many factors that may related to the reduced in transfection efficiency. One possible explanation may involve the large particle size of the EGF–chitosan–DNA polyplexes. The size of EGF–PEI/DNA complexes from the study of Blessing et al. [26] varied from about 50–600 nm, while most of the particles of all EGF conjugated chitosan–DNA polyplexes in this study were fused together. This may have been caused by the polymerization process between the chitosan–DNA microparticles themselves, as the chitosan itself have amino groups that may react with the disulfide arms of the thiol group of the modified chitosan by SPDP or by the continuing reaction of the cross linking reagent. However, the aggregation of polyplexes may lead to increased sedimentation of the polyplexes on cells surface but the polyplexes may be too stable to released the DNA because the transfection efficiency of chitosan depends on the interaction strength between the chitosan vector and the DNA in the polyplexes [27]. Another reason could be that the EGF conjugated chitosan polyplexes may

lose plasmid DNA, especially from the conjugation process by using NHS-PEO_8-Maleimide as a cross linker in both HW and LW chitosan as shown from gel electrophoresis (Fig. 3). It was also noted from SEM that there were small round globular particles attached on the surfaces of the complexes in the groups of EGF conjugated chitosan–DNA polyplexes, which may be the EGF globular protein. However, further investigation of the expression of EGFR of both KB and primary fibroblast cells may provide more information.

Another possibility that may lead to the low transfection efficiency of EGF conjugated chitosan–DNA polyplexes may come from the bioactivity of EGF, which may increase cell proliferation and effect transfection efficiency due to the constant number of plasmid copies verses the increase in the number of cells. This may need further investigation. However, high dose of EGF (100 ng/ml) can inhibit keratinocyte growth due to negative feed back mechanism [28]. The EGF concentration used for these conjugation methods were high, but the EGF concentration conjugated in the polyplexes for each cells transfection experiment was not determined here, which could be investigated further. However, the number of KB cells in EGF conjugated chitosan–DNA polyplexes was not dramatically increased compared to KB cells transfected with other chitosan–DNA microparticles at the beginning of transfection or after 48 h of transfection.

The concentration of HW chitosan used was 0.05% while the LW chitosan was 0.02% with the fixed amount of 5 μg of plasmid DNA. This mixture ratio was derived from previous experiments (data not shown) by varying the amount of chitosan until a high and reliable transfection efficiency of each molecular weight of the chitosan was achieved. Many studies have reported the N/P ratio of the chitosan–DNA nanoparticles formation affected transfection efficiency [5, 6, 29]. The charge ratio (N/P) of chitosan/DNA was expressed as the ratio of moles of the amine groups of chitosan to those of the phosphate ones of DNA [30]. The N/P ratio of HW chitosan/DNA used in this study was 7:1 and N/P ratio of LW chitosan/DNA was 56:1. Both of them had stable DNA binding affinities as shown in gel electrophoresis.

Even though the transfection efficiency of EGF-conjugated chitosan DNA complexes and HW chitosan–DNA microparticles was much lower compared to lipofectamine, the transfection efficiency of the microparticles from LW chitosan, which is the depolymerized chitosan with Mw $\sim$ 16 kDa (Mn $\sim$ 6.3 kDa), gave high transfection efficiency in both KB and fibroblast cells. Results from SEM showed that the size of LW chitosan–DNA microparticles was the smallest compared to other complexes from SEM, which may be one factor that may promoted the transfection efficiency. It was noted that the chitosan–DNA microparticles of HW chitosan had high varieties of sizes, which may come from the high polydispersity that can affect the size of the particles [6]. However, using SEM for examination the size, as well as morphology of these polyplexes, gave limited information, because samples were dehydrated from freezed-dried in the preparation process, which may affect the particle dimension, morphology and aggregation.

MacLaughlin et al. [11] synthesized depolymerized chitosan oligomers Mw from 7 to 92, but the transfection efficiency was much lower than at higher Mw 102 and 230 kDa, respectively, and being about 1000 times lower in transfection efficiency compared to Lipofectamine™. Haung et al. [31] also found a decreased A549 cellular uptake with the decreasing Mw or DDA of chitosan and a N/P ratio of 6 was used in that study. But the present study revealed much higher transfection efficiency with the depolymerized chitosan at Mw $\sim$ 16 kDa (or Mn $\sim$ 6.3), this may be because of different chitosan/DNA ratio as their study used low N/P ratio, while our experiment used chitosan/plasmid at N/P ratio about 56:1, which meant than we used a much higher chitosan for the lower Mw. This corresponded with the findings of the study Romoren et al. [5], who found that low molecular weight chitosan was beneficial at higher charge ratio of the complexes. The study of Lavertu et al. [6] also found that the low molecular weight chitosan, which had number average molecular weight (Mn) about 10 and 80% DDA at N/P ratio 10:1, gave higher transfection efficiency at the same level as Lipofectamine™ at pH 6.5. However, the very low molecular weight (1.9–7.7 kDa) chitosans with high DDA were found to form aggregates easily, even at very low charge ratios [32] and this might lower the transfection. However, the depolymerized LW chitosan in this study had only 54% DDA, which may reduce the problem of particles aggregation and, after cells uptake the chitosan–DNA microparticles, the DNA may be released from the particles more easily as DNA binding efficacy was reduced as degree of DDA was decreased [33]. Hence, many factors may have to be considered for improving transfection efficiency of chitosan, not only the ligand binding, but also the method of binding or conjugation, the size and morphology of the particles, the aggregation of the complexes, and especially the chitosan itself, as molecular weight, DDA and charge ratio may have to be adjusted.

This study supported the premise that LW chitosan has potential as a non-viral DNA delivery reagent and it has no toxic and hemolytic effect. It can also be used as a parenteral drug or as DNA carriers. Further, these chitosan DNA complexes were more effective than poly (L-lysine) and protect against nuclease degradation [10].

It was noted that the transfection efficiency of fibroblasts was much lower than KB cells in both Lipofectamine™ and

LW chitosan, which may refer to the cell line dependency [7]. This may be related to differences in cell physiology affecting the internalization mechanism and subsequent internal trafficking of the vectors [34]. It has also been found that dividing cells had higher transfection ability compared to quiescent cells [35] and higher levels of gene expression have been observed just before or during mitosis [36], which may explain why KB cells, with their population doubling time being about 24 h [37], or higher mitotic activity, had higher transfection ability than the primary oral fibroblasts with their average population doubling time about 3.6 days [38].

5 Conclusion

This study found that EGF-conjugated chitosan–DNA complexes synthesized using either disulfide linkage or NHS-PEO_4-Maleimide as a cross linker in both HW and LW chitosan did not improve transfection efficiency. However, the LW chitosan, which was the depolymerization product of HW chitosan had significantly higher transfection efficiency approaching the same level as Lipofectamine™. LW chitosan showed the potential for further development as a safe and cost effectiveness non-viral gene delivery vehicle.

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References

1. Partridge KA, Oreffo RO. Gene delivery in bone tissue engineering: progress and prospects using viral and nonviral strategies. Tissue Eng. 2004;10:295–307.
2. Bowman K, Leong KW. Chitosan nanoparticles for oral drug and gene delivery. Int J Nanomedicine. 2006;1:117–28.
3. Dang JM, Leong KW. Natural polymers for gene delivery and tissue engineering. Adv Drug Deliv Rev. 2006;58:487–99.
4. Peng L, Cheng X, Zhuo R, Lan J, Wang Y, Shi B, et al. Novel gene-activated matrix with embedded chitosan/plasmid DNA nanoparticles encoding PDGF for periodontal tissue engineering. J Biomed Mater Res A. 2009;90:564–76.
5. Romoren K, Pedersen S, Smistad G, Evensen O, Thu BJ. The influence of formulation variables on in vitro transfection efficiency and physicochemical properties of chitosan-based polyplexes. Int J Pharm. 2003;261:115–27.
6. Lavertu M, Methot S, Tran-Khanh N, Buschmann MD. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. Biomaterials. 2006;27:4815–24.
7. Corsi K, Chellat F, Yahia L, Fernandes JC. Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan–DNA nanoparticles. Biomaterials. 2003;24:1255–64.
8. Dastan T, Turan K. In vitro characterization and delivery of chitosan–DNA microparticles into mammalian cells. J Pharm Pharm Sci. 2004;7:205–14.
9. Mao S, Shuai X, Unger F, Simon M, Bi D, Kissel T. The depolymerization of chitosan effects on physicochemical and biological properties. Int J Pharm. 2004;281:45–54.
10. Richardson SC, Kolbe HV, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system biocompatibility, body distribution and ability to complex and protect DNA. Int J Pharm. 1999;178:231–43.
11. MacLaughlin FC, Mumper RJ, Wang J, Tagliaferri JM, Gill I, Hinchcliffe M, et al. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J Control Release. 1998;56:259–72.
12. Huang M, Khor E, Lim LY. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharm Res. 2004;21:344–53.
13. Mansouri S, Lavigne P, Corsi K, Benderdour M, Beaumont E, Fernandes JC. Chitosan–DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. Eur J Pharm Biopharm. 2004;57:1–8.
14. Zhang T, et al. Synthesis and properties of a novel methoxy poly(ethylene glycol)-modified galactosylated chitosan derivative. J Mater Sci Mater Med. 2009;20:673–80.
15. Zhu D, et al. Hydrophilic/lipophilic *N*-methylene phosphonic chitosan as a promising non-viral vector for gene delivery. J Mater Sci Mater Med. 2010;21:223–9.
16. Mao HQ, et al. Chitosan–DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release. 2001;70:399–421.
17. Kim TH, Nah JW, Cho MH, Park TG, Cho CS. Receptor-mediated gene delivery into antigen presenting cells using mannosylated chitosan/DNA nanoparticles. J Nanosci Nanotechnol. 2006;6:2796–803.
18. Christensen ME. The EGF receptor system in head and neck carcinomas and normal tissues. Immunohistochemical and quantitative studies. Dan Med Bull. 1998;45:121–34.
19. Kloeckner J, Boeckle S, Persson D, Roedl W, Ogris M, Berg K, et al. DNA polyplexes based on degradable oligoethylenimine-derivatives: combination with EGF receptor targeting and endosomal release functions. J Control Release. 2006;116:115–22.
20. Lee H, Kim TH, Park TG. A receptor-mediated gene delivery system using streptavidin and biotin-derivatized, pegylated epidermal growth factor. J Control Release. 2002;83:109–19.
21. Kloeckner J, Prasmickaite L, Hogset A, Berg K, Wagner E. Photochemically enhanced gene delivery of EGF receptor-targeted DNA polyplexes. J Drug Target. 2004;12:205–13.
22. Tan ML, Choong PF, Dass CR. Cancer, chitosan nanoparticles and catalytic nucleic acids. J Pharm Pharmacol. 2009;61:3–12.
23. Saul JM, Annapragada AV, Bellamkonda RV. A dual-ligand approach for enhancing targeting selectivity of therapeutic nanocarriers. J Control Release. 2006;114:277–87.
24. Freshney RI. Induction of differentiation in neoplastic cells. Anticancer Res. 1985;5:111–30.
25. Sambrook J, Fritsch E. Molecular cloning: a laboratory manual. NY, Cold Spring Harbor: Laboratory Press; 1989.
26. Blessing T, Kursa M, Holzhauser R, Kircheis R, Wagner E. Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. Bioconjug Chem. 2001;12:529–37.
27. Strand SP, Lelu S, Reitan NK, de Lange Davies C, Artursson P, Varum KM. Molecular design of chitosan gene delivery systems with an optimized balance between polyplex stability and polyplex unpacking. Biomaterials. 2010;31:975–87.
28. Yamasaki K, et al. Keratinocyte growth inhibition by high-dose epidermal growth factor is mediated by transforming growth factor beta autoinduction a negative feedback mechanism for keratinocyte growth. J Invest Dermatol. 2003;120:1030–7.

29. Ishii T, Okahata Y, Sato T. Mechanism of cell transfection with plasmid/chitosan complexes. Biochim Biophys Acta. 2001;1514: 51–64.
30. Kim TH, Kim SI, Akaike T, Cho CS. Synergistic effect of poly(ethylenimine) on the transfection efficiency of galactosylated chitosan/DNA complexes. J Control Release. 2005;105: 354–66.
31. Huang M, Fong CW, Khor E, Lim LY. Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation. J Control Release. 2005;106:391–406.
32. Morris VB, Neethu S, Abraham TE, Pillai CK, Sharma CP. Studies on the condensation of depolymerized chitosans with DNA for preparing chitosan–DNA nanoparticles for gene delivery applications. J Biomed Mater Res B Appl Biomater. 2009;89B:282–92.
33. Kiang T, Wen J, Lim HW, Leong KW. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. Biomaterials. 2004;25:5293–301.
34. Douglas KL, Piccirillo CA, Tabrizian M. Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors. Eur J Pharm Biopharm. 2008;68:676–87.
35. Brunner S, Sauer T, Carotta S, Cotten M, Saltik M, Wagner E. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther. 2000;7:401–7.
36. Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 1999;6:403–11.
37. Weissfeld AS, Rouse H. Arginine deprivation in KB cells. I. Effect on cell cycle progress. J Cell Biol. 1977;75:881–8.
38. Ma RH, Tsai CC, Shieh TY. Increased lysyl oxidase activity in fibroblasts cultured from oral submucous fibrosis associated with betel nut chewing in Taiwan. J Oral Pathol Med. 1995;24: 407–12.