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Research Article

Surface Modification of Gelatin Nanoparticles with Polyethylenimine as Gene Vector

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A novel carrier on balancing the transfection efficiency and minimizing cytotoxicity was designed. Gelatin cross-linked with 1.8 kDa of PEI (GA-PEI 1.8 k) formed stable complex and resulted in high positive ζ potential (42.47 mV) and buffering effect. These nanoparticles with N/P ratio of 30 give high transfection efficiency 2.12×10^4 RLU/μg protein and cell viability (86.4%). These modified GA-PEI nanoparticles, with high transfection efficiency and low cell toxicity, can be a potential gene vector in gene therapy.

1. Introduction

Gene therapy has been considered as an attractive approach for correcting deficient genes and modulating gene expression [1]. It refers to transmiting the DNA that encodes a therapeutic gene into the targeted cells and consequently expressing the specific protein in tissue. Viral vectors constructed from adenovirus, retrovirus, adeno-associated virus, and herpes simplex virus-1 have been shown highly efficient to transfer genes into cells. However, the safety concerns, such as inflammatory, immunogenicity, oncogenicity, and other toxic side effect, are crucial issues to be solved [2, 3]. On the other hand, nonviral vectors composed of biodegradable polymers or lipids have been developed as potential alternatives. The advantage properties of nonviral vectors applied on gene delivery are low cost, noninfectivity, absence of immunogenicity, good compliance, well-defined characteristics, and possibility of repeated clinical administration [4]. Therefore, a safe and effective nonviral gene delivery system needs efforts to build up.

In recent years, nanoparticles have demonstrated unique physical and biological properties that can be applied to overcome the issues in gene and drug delivery systems. Cellular uptake of nanoparticles can often be influenced by the particle size, which plays an essential factor in tissue and organ distribution [5–8]. Comparing with larger size micron particles, the smaller nanoparticles are capable of penetrating through the submucosal layers [6, 9], and enhance the efficiency in gene transfection level. The cellular barriers to nonviral gene vectors are a series of steps, including nucleotides protection, initial cell membrane interactions, internalization through endosomal uptake, escape from the endosome/lysosome, and translocation through nuclear membrane to the nucleus [10]. A number of cationic polymers have been investigated as gene carriers, such as synthetic amino acid polymer, polylysine; cationic dendrimers, and pol-yethylenimine (PEI), and carbohydrate-based polymer such as chitosan.

Among these cationic polymers, PEI has high pH-buffering capacity as a "proton sponge" due to its specific feature. The property of endosomal and lysosomal buffering can protect the nucleotides from degradation and promote the nucleotides release from the acidic vesicles. It has been reported that lower molecular weight PEI has advantages for *in vivo* application as compared to higher molecular weight PEI [11–13] due to its lower cytotoxicity. As a gene carrier, PEI can display high transfection efficiency *in vitro* and *in vivo* [12, 14] thus has tremendous potential in gene therapy. Nevertheless, it has not yet advanced to clinical application since the cytotoxicity issue remains to be evaluated.

Gelatin is a natural biodegradable polymer, derived from the hydrolysis of collagen, and mainly composed of

hydroxyproline, proline, and glycine [15]. It is considered "generally regarded as safe (GRAS)" material by FDA and has been commonly used in pharmaceuticals, cosmetics, and food products manufacturing [16]. The amino group and carboxyl group of gelatin can be functionalized for crosslinking as well as gelatin modification. In addition, gelatin is an amphoteric protein with isotonic point between 5 and 9 depending on the method of production. Preparation of particles formed by gelatin has been reported in numerous investigations and in range of different sizes. In particular, a two-step desolvation has been demonstrated as an efficient process that can produce much smaller nanoparticles [17].

In this study, gelatin was formulated as nanoparticles and modified by different molecular weight of PEI on the particles to increase the surface charge, providing as a promising gene carrier candidate. Based on PEI with high pH-buffering capacity and high transfection efficiency, parts of amines on PEI can be conjugated with the carboxylate group of gelatin by EDC to enhance the ζ-potential of nanoparticles and buffering effect. The effects of particle size and ζ-potential on DNA/plasmid protection efficacy were evaluated. The cell cytotoxicity and transfection efficiency of GE-PEI nanoparticles were assayed *in vitro* and *in vivo*.

2. Experimental Details

2.1. Preparation of Gelatin and Gelatin-PEI Nanoparticles. Gelatin nanoparticles were prepared by desolvation process as described elsewhere [17, 18]. In brief, 0.5 g of gelatin (type A, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 10 mL distilled water, followed by addition of 10 mL acetone to precipitate high molecular weight gelatin. After discarding the supernatant, the precipitation was once redissolved in 10 mL of distilled water and stirred at 600 rpm under 40°C, and pH was adjusted to 2.5. Gelatin nanoparticles were formed by adding dropwise 40 mL acetone and then crosslinked with 0.1% glutaraldehyde overnight. The nanoparticles were separated by 12,000 rpm centrifugation for 10 minutes three times and redispersed in distilled water. Gelatin nanoparticles were stored at 2–8°C for further experiments. For gelatin-PEI nanoparticles, stock gelatin nanoparticles were further conjugated with various condition of PEI (Sigma-Aldrich) with addition of 0.5 g EDC (1-ethyl—3-(3-dimethylaminopropyl) carbodiimide) as crosslinker under pH of 4.0 for 18 hours to form amide bonds between the activated carboxyl group of gelatin with the amine group of PEI. The conjugated nanoparticles were then centrifugated at 12,000 rpm for 10 minutes followed by resuspension in distilled water three times to separate from unconjugated free PEI,and stored at 2–8°C until further use.

2.2. Characterization of Nanoparticles. The particle diameter and ζ potential of the GA and GA-PEI nanoparticles were determined by particle size analyzer (BIC 90-Plus, Brookhaven Instruments Corporation, Holtsville, NY, USA). Measurements were carried out at 25°C with a 35 mW He-Ne laser (wavelength of 633 nm) at a light-scattering angle of 90° and performed in triplicate.

2.3. Quantification of Free Amino Groups after Conjugating with PEI. Free amino groups were estimated by trinitrobenzene sulfonate (TNBS, Sigma, USA) according to Fields [19]. In principle, nanoparticles were rinsed and centrifuged four times at 100,000 ×g for 5 minutes and resuspended in distilled water. Samples were then mixed with 4% sodium tetraborate and TNBS and stirred for 2 hours at 40°C. Supernatant was separated by centrifugation at 100,000 g for 5 minutes and diluted with water. Samples were then assayed at 349 nm for unreacted TNBS using a UV/VIS spectrophotometer (Lambda 20 Perkin-Elmer, Norwalk, UK). The free amino group content of the nanoparticles was determined relative to a TNBS control.

2.4. GA-PEI Nanoparticles/DNA Complex Preparation. Gelatin-PEI nanoparticles/DNA (pCMV-Luc, a plasmid with firefly luciferase reporter gene) complexes were prepared at various charge ratios (N/P). The N/P ratios of PEI/DNA complexes were expressed as the molar ratios of amino group of PEI to phosphate group of DNA. Complexes were formed by incubating DNA with the GA-PEI nanoparticles in DMEM medium (Dulbecco's Modified Eagle Medium, T043-01, Biochrom AG, Germany) containing 10% Fetal Bovine Serum (FBS) for 30 min at room temperature.

2.5. Cell Viability and Transfection Efficiency. NIH 3T3 cells were seeded in 96 wells (10^3 cells/well) and maintained in DMEM supplemented with 10% FBS at 37°C in a humidified 5% carbon dioxide incubator until properly adhere. GA nanoparticles/DNA and various N/P ratios of GA-PEI nanoparticles/DNA were added to each well with a final amount of 1 μg DNA. Cells were then incubated for 4 hours and washed with PBS and further incubated in fresh complete medium for 24 hours for MTS assay.

MTS cell viability assay (Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Assay, Promega, Madison, WI, USA), an alternative method of the commonly used MTT assay, was used to define the viability of cells cultured with the GA-PEI/DNA complex after transfection. In brief, the reacted product with tetrazolium formed soluble formazan in the culture medium, and absorbance was measured at 490 nm in an ELISA 96-well plate reader. The measurements were represented by mean value and standard deviation parameter.

Transfection efficiency was evaluated by the luciferase activity expressed from the transfected cells (2×10^5 cells/well in the 6-well culture plate). Cells were harvested 48 hours after transfected with N/P ratios of 10, 20, 30, 40, and 50 GA-PEI nanoparticles/DNA complex then washed with PBS, and resuspended in 200 μL lysis buffer (Promega E1941). The cell lysate was centrifuged for 5 min at 10,000 ×g. The supernatant was diluted to proper concentration, and 20 μL specimen were reacted with 100 μL assay reagent (Promega) in a 96-well white plate. Luminescence intensity was measured by Luminescence Spectrometer (Infinite 200, Tecan,

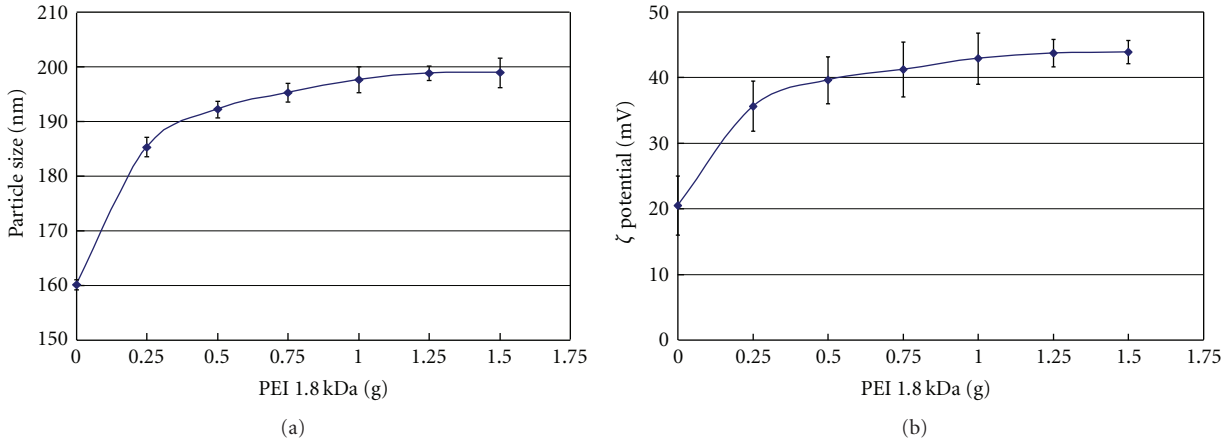

Figure 1: Characterization of particle size and ζ potential for GA-PEI (1.8 kDa) nanoparticles, ($n = 3$).

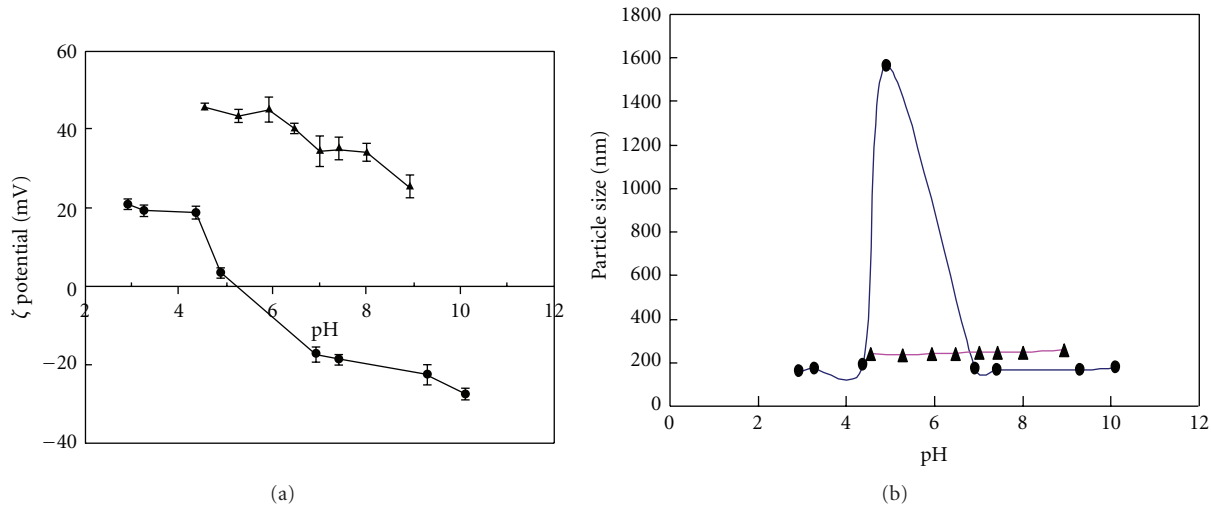

Figure 2: Influence of pH environment on the particle size and ζ potential. (a) shows the ζ potential change and (b) shows the particle size change for gelatin nanoparticles (•) and gelatin-PEI nanoparticles (▲), $n = 3$.

Männedorf, Switzerland), and the transfection efficiency was expressed as total light units per total cell lysate protein.

2.6. Statistical Analysis. All experiments were repeated three times with measurements collected in triplicate. Data are expressed as mean ± standard deviations. Statistical analysis was performed using Student's t-test. Differences were considered statistically significant with $P < .05$.

3. Results and Discussion

Polyethylenimine (PEI) has high positive charge provided by the nitrogen atoms and shown high affinity to DNA through the electrostatic interaction between the amino group of PEI and phosphate groups of the DNA [20]. We used low-molecular-weight PEI conjugated onto the surface of gelatin nanoparticles to provide a balance between enhancing transfection efficiency and minimizing cytotoxicity. Figure 1 shows the characterizations of particle size and ζ potential of 0.1 gram gelatin nanoparticles conjugated with different amount of 1.8 kDa PEI. In order to increase the surface charge of gelatin nanoparticles, excess of PEI was added. It was observed that particle size and ζ potential increased as more PEI was conjugated on to the nanoparticles. The particle size and ζ potential reached a plateau as the surface was saturated with PEI conjugation. Stable maximum size is close to 200 nm, and ζ potential of 42 mV were obtained when more than 1.0 gram of PEI were added for gelatin nanoparticles conjugation. As a result, an optimal weight ratio of gelatin to PEI to cross-linker agent (EDC) was determined to be 0.1 : 1.5 : 0.05 and was used for the remaining transfection efficiency and cell viability experiments.

PEI also exhibits a high positive charge density and buffering capacity [19]. Surface charges as well as particle sizes can be changed as a result of different pH microenvironments. Figure 2(a) demonstrates the influence of pH environment to the particle size and ζ potential for gelatin and gelatin-PEI nanoparticles. The gelatin nanoparticles

Table 1: Free amino group on nanoparticles.

Types of nanoparticles	Concentration of amino group (mM)
gelatin nanoparticles (unmodified)	0.14
gelatin-PEI 600 Da nanoparticles	1.56
gelatin-PEI 1.8 kDa nanoparticles	2.42
gelatin-PEI 10 kDa nanoparticles	4.18

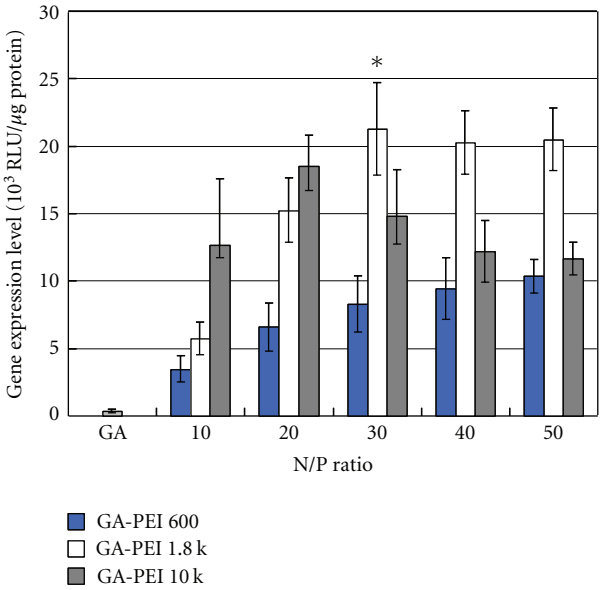

Figure 3: Transfection efficiency determined by luciferase activity of gelatin conjugated with 600, 1.8 k, and 10 kDa of PEI as function of various N/P ratio, ($n = 3$).

carried positive charge when formulated at an acidic environment of pH below 5, while carried negative charges when formulated at environment of pH above 5. However, gelatin-PEI nanoparticles exhibited high positive charge with slight decline throughout the ranges of pH 4 to pH 9. The results showed that the unmodified gelatin nanoparticles were not suitable as carrier for gene delivery at the physiological environment (pH = 7) due to its negative charge. Gelatin-PEI nanoparticles, under physiological pH of 7, can maintain its positive charge and the electrostatic interaction with the negative DNA to form stable complex. Figure 2(b) also demonstrates the influence of pH on the particle size of gelatin and gelatin-PEI nanoparticles. Gelatin-PEI particles showed stable particle size of 200 nm throughout the pH range of 4 to 9. In contrast, there is a dramatical change in the particle size of gelatin nanoparticles at pH = 5. This increase of particle size may be greatly due to the isoelectric point of gelatin at pH = 5, as demonstrated on Figure 2(a), where net balance of charges may contribute to the aggregation of particles hence particle size increase.

The ability of gelatin-PEI nanoparticles forming complex with DNA provides great potential as a carrier to deliver

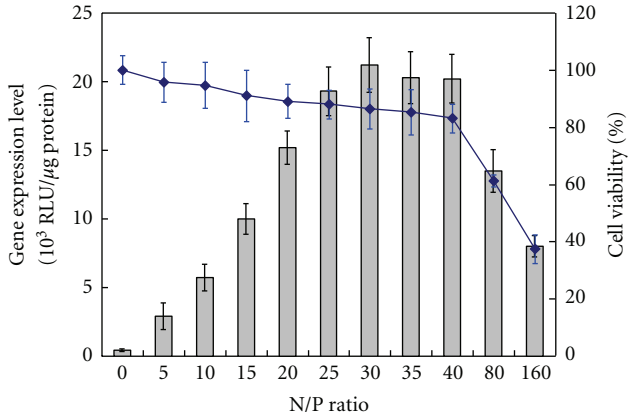

Figure 4: Transfection efficiency measured from luciferase activity (Bar chart) and cell viability (line) of GA-PEI 1.8 kDa as function of different N/P ratio, $n = 3$.

therapeutic gene. The free amino group on the gelatin nanoparticles and gelatin conjugated with different molecular weight of PEI were assayed by using TNBS and the results were shown in Table 1.

As a vector, the capacity of vectors carrying the gene is very important. The amount of amino groups on nanoparticles per phosphate group of DNA, represented as N/P ratio, was used as a parameter to determine the most optimal transfection condition. The complex containing 1.0 μg DNA of pCMV-Luc gene was prepared in various N/P ratios of 10, 20, 30, 40, 50 to evaluate the transfection efficiency by measuring the transfected luciferase activity per total protein amount. In addition, PEI molecular weights of 600 Da, 1.8 kDa, and 10 kDa were also investigated for the influence to transfection efficiency (as shown in Figure 3). The transfection efficiency was found to be enhanced as the N/P ratio of the GA-PEI nanoparticles increased. However, the gene expression activity of GA-PEI with PEI molecular weigh† of 10 kDa was found to be decreased for N/P ratio greater than 20. Furthermore, the GA-PEI conjugated with PEI molecular weight of 1.8 kDa reached to a plateau between N/P ratios of 30 to 50. The data suggested that the molecular weight played an important role in terms of cytotoxicity such that high cell viability was observed for low PEI molecular weight and hence more transfection was obtained. From the results, we found that the optimal condition of modification for the gelatin nanoparticles was using PEI 1.8 kDa at N/P ratio of 30.

Figure 4 shows the transfection efficiency of GA-PEI 1.8 k with various N/P ratios and the cell viability. Transfection increased linearly from 4.2×10^2 RLU/μg protein to 2.12×10^4 RLU/μg protein as the N/P ratio increased from 0 to 30. A stable plateau of transfection efficiency around 2.0×10^4 RLU/μg protein was reached for N/P ratios of 30 to 40 and then decreased to 8×10^3 RLU/μg of protein at N/P ratio of 160. Interestingly, cell viability dramatically decreased from 83.1% to 37.4% which was observed for N/P ratio beyond 40. This can be concluded that the GA-PEI 1.8 k with N/P ratio of 30 was the optimal transfection efficiency

of 2.1×10^4 RLU/μg of protein, while maintaining high cell viability of 86.4%.

4. Conclusion

The advantages of using nonviral vectors in gene delivery have attracted much attention for its low cost, noninfectivity, absence of immunogenicity, and good compliance. Combined with the advantages of high transfection efficiency of PEI and excellent biocompatibility of gelatin, we successfully developed the GA-PEI nanoparticles as gene delivery vectors. Gelatin nanoparticles conjugated with low molecular weight of PEI provide high transfection efficiency with minimizing cytotoxicity.

Experimental results had indicated that the particle size and ζ potential of GA-PEI can be altered by conjugating with increasing amount of PEI. Gelatin-PEI nanoparticles exhibited positive charges throughout all the ranges of pH and maintained stable particle size in both acidic and basic conditions. In order to form complex with DNA, gelatin-PEI nanoparticles were investigated with various N/P ratios. Transfection efficiency and cell viability were evaluated by the luciferase activity expression and MTS assay. It was concluded that GA-PEI 1.8 kDa with N/P ratio of 30 provided the optimal transfection efficiency and high cell viability. The results demonstrated GA-PEI 1.8 k nanoparticles as a potential vector for gene delivery.

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