DNA/PEI/Alginate Polyplex as an Efficient *In Vivo* Gene Delivery System

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Abstract A novel non-viral gene delivery system comprised of a DNA/PEI/Alginate (DPA) polyplex was prepared and assessed *in vitro* and *in vivo*. Coating the positively charged DNA/PEI (DP) complex with a polyanion resulted in a high level of *in vitro* reporter gene transfection in the presence of 50 vol% serum due to the minimized cytotoxicity of PEI and the reduced non-specific interactions with serum components. Among the tested anionic polymers, which included sodium alginate, poly(methacrylic acid) and poly(acrylic acid), the sodium alginate showed the highest gene transfection efficiency. The DPA polyplex also showed a reduced level of erythrocyte aggregation in target cells when compared with the DP complex. According to *in vivo* studies in which reporter genes encoding green fluorescent protein (GFP) and luciferase were used, injection of the DPA polyplex into tumor cells in six week old female C57/BL6 mice resulted in a much higher level of GFP expression and approximately 7 fold higher luciferase expression than treatment with the DP complex. Taken together, these results demonstrated that the anionic alginate coating of the DP complex contributed to efficient gene delivery *in vitro* and *in vivo*. © KSBB

Keywords: alginate, polyethylenimine, reporter gene, polyplex, gene delivery

INTRODUCTION

Development of a novel intracellular method to deliver genes to target cells is one of the most important issues in gene therapy [1]. There have been extensive studies conducted in an effort to develop non-viral vectors, such as cationic liposomes [2] and cationic polymers [3-5], and polyethylenimine (PEI) has emerged as an efficient gene delivery vehicle because it is very stable and easy to handle and manipulate, and also because it is inexpensive when compared to cationic liposomes and other commercially available transfection reagents [1]. In addition to being able to condense DNA, PEI also exhibits endosomolytic activity at acidic endosomal pHs, therefore it is considered as one of the most efficient non-viral vectors [1]. However, it has been reported that PEI-mediated *in vivo* gene delivery has a low transfection efficiency in the presence of serum, and that this may be caused by the non-specific interaction of the DNA/PEI (DP) complex with blood components, such as serum proteins [4,5]. Accordingly, several studies have been conducted in an attempt to inhibit the interaction of serum proteins with DNA-carrier complexes, and methods such as the self-assembly of DNA with block copolymers containing the cationic segment of PEI attached to poly(*N*-2-hydroxypropyl methacrylamide) [PHPMA], poly(acrylic acid) [PAA] [6,7], and poly(ethylene glycol) [PEG] [9,8] have been developed. The resulting complexes have shown improved biophysical properties with decreased surface charge, lower toxicity, and reduced non-specific binding to cells and serum proteins [6,7]. In addition, electrostatic neutralization using synthetic polyanions also appears to increase the efficiency of PEI-mediated gene transfection in the presence of serum. However, the non-ionic blocks are known to interfere with DNA condensation at a high serum protein concentration, which results in a potential negative effect as a result of repeated injection of synthetic polymers [6].

In order to develop a non-viral gene delivery system with high serum stability and low toxicity while maintaining a high efficiency of gene delivery, a negatively charged biodegradable polymer, sodium alginate, was used to coat the positively charged DP complex [10,11]. Sodium alginate is a polysaccharide comprised of β-(1-4)-D-manuronic acid and α-(1-4)-L-gluronic acid that is currently used in the food and pharmaceutical industries, therefore it has the potential for

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biomedical applications. Additionally, polysaccharides may be useful for targeted delivery because they have specific receptors on certain cells or tissues and they display well-documented biodegradability and biocompatibility [11,12]. It has been reported that the DNA encapsulated alginate delivery system can mitigate the immune response and enhance trans-gene expression [11], and it was also reported that the blending of alginate with PEI decreased its charge-associated cytotoxicity and improved its gene transfection efficiency *in vitro* [10]. However, there are few reports on the alginate based non-viral gene delivery system for *in vivo* applications. In this study, a novel non-viral system for the delivery of a DNA/PEI/Alginate (DPA) polyplex was prepared and assessed *in vitro* and *in vivo*. Reporter genes encoding green fluorescent protein (GFP) and luciferase were used as models for the gene delivery system. Among the tested anionic polymers of sodium alginate, poly(methacrylic acid) [PMA] and poly(acrylic acid) [PAA], the DP complex coated with sodium alginate showed the highest *in vitro* gene delivery efficiency in the presence of serum. Therefore, this novel non-viral gene system for the delivery of DPA polyplex was assessed *in vivo* using an erythrocyte aggregation test in target cells.

MATERIALS AND METHODS

Materials

Sodium alginate (three grades with a low viscosity of 20~40 cP, a medium viscosity of 250 cP, and a high viscosity of 3,500 cP), poly(methacrylic acid) [PMA], poly(acrylic acid) [PAA], boric acid, tris(hydroxymethyl)aminomethane, ethylenediaminetetraacetic acid (EDTA), and ethidium bromide were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) and trypsin-EDTA (0.25%) were purchased from Hyclone (Logan, UT, USA). Iscove's modified Dulbecco's medium (IMDM) and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Grand Island, NY, USA) and polyethylenimine (branched, M_r = 25,000) was obtained from Aldrich (Milwaukee, WI, USA). Luciferase assay reagent was obtained from Promega (Madison, WI, USA). The reporter plasmids of pCMV-Luc and pEGFP-N1 were obtained from Clontech (Mountain View, CA, USA). All reagents were used without further purification.

C3 Tumor Cell Culture

The C3 tumor cell line, which was derived from murine fibroblasts of C57BL/6 origin by HPV infection [13], was cultured in IMDM supplemented with 8% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 55 mM 2-mercaptoethanol at 37°C in a 5% humidified CO_2 incubator.

Preparation of DNA/PEI Complex

In order to prepare the DNA/PEI (DP) complex, 1 μg of plasmid DNA was mixed with 1.5 μL of 20 mM PEI (N/P ratio of 10) and then incubated for 10 min in a 1.5 mL eppendorf tube at room temperature. Next, distilled water and 3 μL of NaCl (5 M) were added to the above solution to give a final volume of 100 μL.

Preparation of DNA/PEI/Polyanion Polyplex

The DNA/PEI/Alginate (DPA) polyplex was prepared by coating the prepared DP complex with alginate. To accomplish this, various amounts of alginate were added to the pre-incubated DP complex, which was then incubated for 10 min at room temperature. The weight ratio of alginate to DNA varied from 0.015 to 1.5. For the polyanionic coating of the DP complex, PMA or PAA with a weight ratio of polyanion to DNA of 0.15 was added to the pre-incubated DP complex, which was then incubated for 10 min at room temperature.

Characterization of the DNA/PEI/Alginate Polyplex

The particle size and surface charge of the DP complex and the DPA polyplex were measured using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka Electronics, Osaka, Japan). The cytotoxicity of the DP complex and the DPA polyplex was analyzed using an MTS assay as described below. Briefly, C3 cells were seeded in 96-well plates at a density of 3×10^4 cells/well and then incubated for 24 h. Next, the medium was replaced with fresh, serum-free IMDM that contained DP complex or DPA polyplex. The C3 cells were then incubated with DP complex or DPA polyplex for 24 h, with untreated C3 cells being used as a control. Finally, the cytotoxicity of the DP complex and the DPA polyplex was analyzed using a Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

In Vitro Transfection Test

In vitro gene transfection tests were carried out using C3 cells. Briefly, C3 cells were seeded at a density of 3×10^4 cells/well in a 24-well plate containing 0.4 mL of IMDM. After 24 h of incubation, the medium was exchanged for 0.4 mL of fresh medium containing various concentrations of FBS, up to 50 vol%. Next, each well received 100 μL of freshly prepared DP complex or DPA polyplex containing 1 μg of DNA (pCMV-Luc gene). The C3 cells were then incubated in a 5% CO_2 incubator at 37°C for 48 h. Next, the cells were washed twice with PBS and re-suspended in 100 μL of cell lysis reagent. The cell debris were then pelleted by centrifugation at 12,000 rpm and 4°C for 20 min. Next, the supernatant (5 μL) was mixed with 25 μL of luciferase assay solution, and the luciferase activity was then measured with a luminomate (Biolumat LB 9500, Berthold, Germany).

Erythrocyte Aggregation Assay

Fresh blood was collected from C57/BL6 mice and immediately mixed with sodium citrate (25 mM). The erythrocytes were washed three times with cold Ringer solution on

Table 1. Characterization of the DNA/PEI/Alginate polyplexes that contained various Alginate/DNA (w/w) ratios. The N/P ratio of PEI to DNA was fixed at ten

Alginate/DNA (w/w)	0	0.015	0.075	0.15	0.75
Particle size (nm)	282 ± 7.67	240 ± 5.76	275 ± 6.72	261 ± 3.42	360 ± 9.72
Zeta potential (mV)	26 ± 2.34	21 ± 1.56	17 ± 0.723	15 ± 1.23	12 ± 0.57
Cytotoxicity (%)[a]	60 ± 8.7	95 ± 3.75	95 ± 2.75	93 ± 1.75	97 ± 3.67

[a]Cytotoxicity (%) data represent relative cell viability compared to the control (no treatment).

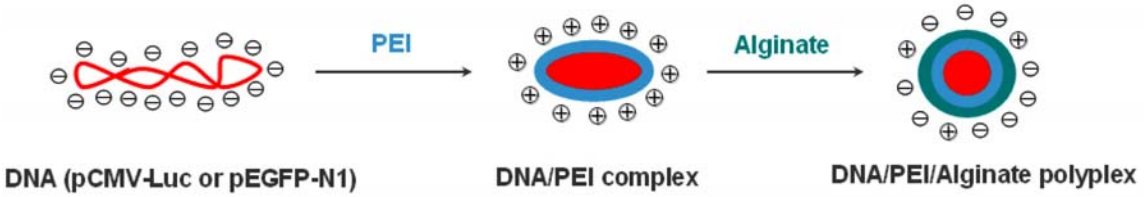

Fig. 1. Schematic representations of the preparation of the DNA/PEI complex and the following DNA/PEI/Alginate polyplex.

ice, and the washed erythrocyte suspension (200 μL) was then mixed with DP complex or DPA polyplex in a 24-well plate (Nunc, Rochester, NY, USA) and incubated at 37°C for 1 h. The erythrocyte aggregation was then observed with a microscope (NiKon TS100, Tokyo, Japan).

In Vivo Transfection Test

C3 cells (1×10^6 cells) were re-suspended in 100 μL of PBS and then injected subcutaneously into six week old female C57/BL6 mice (n = 3, KRIBB, Daejeon, Korea). Within two weeks, tumors between 6 and 7 mm in diameter had developed. DP complex (N/P = 10) or DPA polyplex (DNA:Alginate = 1:0.15 in weight ratio) containing 10 μg of DNA (pEGFP-N1 or pCMV-Luc) was then diluted with 100 μL of 5% (w/v) glucose solution and directly injected into the C3 tumors in the mice. The animals were then sacrificed 7 days later and the tumors and the marginal surrounding tissues were surgically removed and immediately embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA), and then sectioned in a cryostat-microtome at −20°C. The fluorescence from green fluorescent protein was then examined using an Olympus BX60 fluorescence microscope (Melville, NY, USA) to observe the gene expression. Tumors injected with pCMV-Luc reporter gene were homogenized in 500 μL of culture lysis reagent, and the cell debris was then pelleted by centrifugation at 12,000 rpm and 4°C for 20 min. Next, the supernatant (50 μL) was mixed with 100 μL of luciferase assay solution and the luciferase activity was measured using a photoluminometer for 30 sec.

RESULTS AND DISCUSSION

Although non-viral gene delivery systems have been shown to have significant transfection efficiency *in vitro*, their *in vivo* applications have been hampered by decreased transfection efficiency, primarily due to non-specific interactions with serum components [1]. We designed a novel ternary polyplex comprised of DNA/PEI/Alginate (DPA) that had a decreased positive surface charge and compared its gene delivery efficiency with that of a DNA/PEI (DP) complex. Fig. 1 shows schematic representations for the preparation of the DP complex and the DPA polyplex. The negatively charged alginate can form a charge-mediated polyplex with the positively charged DP complex. In addition, the poly-L-gluronate sequence of alginate has been reported to associate with the amines of PEI [14], therefore, the alginate coating of the DP complex was expected to reduce the positive charge on the surface of the DP complex and prevent its binding with blood components, thereby leading to increased *in vivo* transfection efficiency [10,15].

Table 1 summarizes the particle size, zeta potential and cytotoxicity of the DPA polyplex used in this study. As the alginate content increased, the polyplex size decreased slightly and then increased, and the zeta potential decreased due to the negative charge of the alginate. In addition, the cytotoxicity of the complex was drastically reduced by the coating of alginate. Fig. 2 shows the luciferase gene transfection efficiency of the DPA polyplex in C3 cells with an increasing ratio of alginate to DNA. Based on the results shown in Fig. 2, the N/P ratio of the DNA/PEI complex was fixed at a value of 10 for the remainder of the experiments conducted in this study. The DPA polyplex showed a 10~30 fold higher gene transfection efficiency than the DP complex in the presence of 50 vol% serum. The high luciferase gene transfection efficiency of the DPA polyplex might be ascribed to the ability of the alginate to provide an electrostatic stabilization to the polyplex, as has been demonstrated with other hydrophilic polymers such as poly(acrylic acid) [PAA], poly(propyl acrylic acid) [PPAA], and poly(*N*-2-hydroxypropyl methacrylamide) [PHPMA] [6,7,10]. In addition, the effect of the molecular weight of alginate on the luciferase gene transfection was investigated using alginates with three different grades of viscosity, low (20~40 cP), medium (250

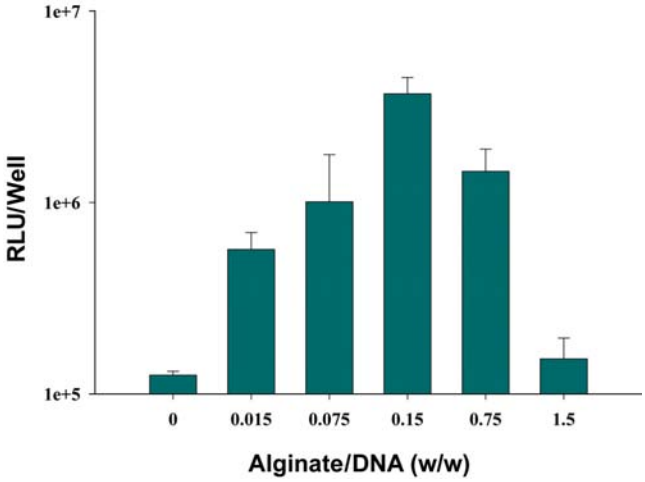

Fig. 2. Effect of coating the DNA/PEI complex with alginate on the luciferase gene transfection efficiency in C3 cells in the presence of 50 vol% serum (N/P = 10, mean ± SD, *n* = 3).

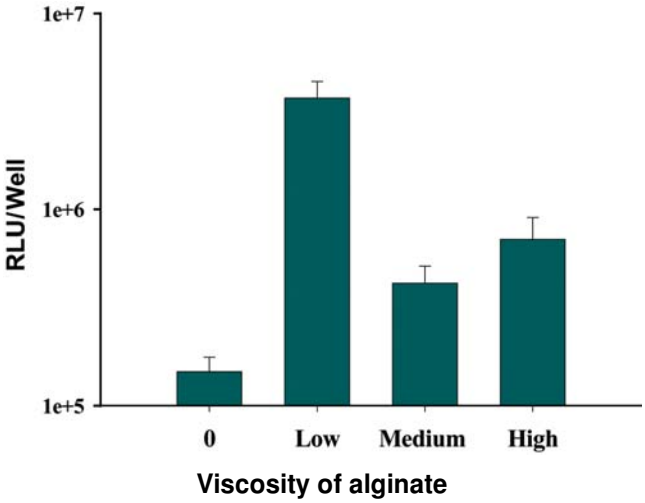

Fig. 3. Effect of the viscosity of alginate used to coat the DNA/PEI complex on the luciferase gene transfection efficiency in C3 cells in the presence of 50 vol% serum (N/P = 10, mean ± SD, *n* = 3): Low, alginate with a viscosity of 20~40 cP; Medium, alginate with a viscosity of 250 cP; and High, alginate with a viscosity of 3,500 cP.

cP), and high (3,500 cP). As shown in Fig. 3, the luciferase gene transfection efficiency of the DPA polyplex in the presence of 50 vol% serum was greater than that of the DP complex regardless of the alginate viscosity. In addition, the DP complex that was coated with a low viscosity alginate showed the highest luciferase gene expression. It was believed that the relatively low gene transfection of the high viscosity alginate was caused by aggregation of the DPA polyplex, which resulted in poor absorption to the target cells. As seen

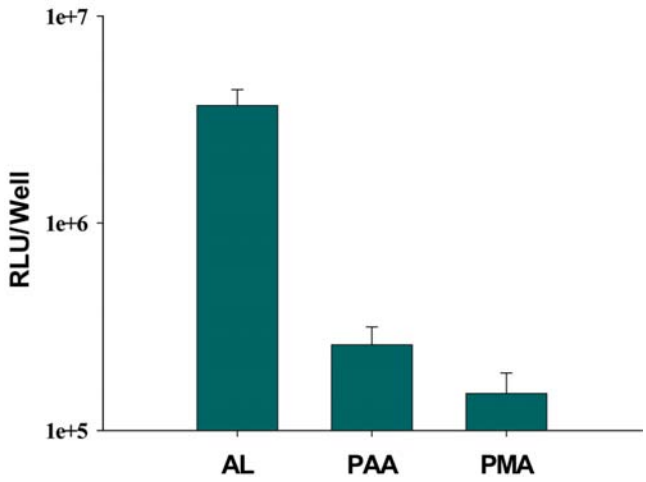

Fig. 4. Effect of coating the DNA/PEI complex with a polyanion on the luciferase gene transfection efficiency in C3 cells in the presence of serum (N/P = 10, mean ± SD, *n* = 3): AL, alginate, PAA, poly(acrylic acid); and PMA, poly (methacrylic acid).

in Figs. 2 and 3, the DP complex that was coated with a low viscoscity alginate and had an N/P ratio of 10 and an alginate to DNA ratio of 0.15 resulted in the highest rate of gene transfection.

In Fig. 4, the amount of luciferase gene transfection that occurred using the alginate coated DP complex is compared with the amount of transfection that occurred after treatment with PAA or PMA coated DP complexes. Alginate coating resulted in the highest transfection efficiency, possibly due to its additional biocompatibility and reduced toxicity [10]. Although the electrostatic neutralization that occurred as a result of coating the complex with synthetic polyanions increased the efficiency of PEI-mediated luciferase gene transfection in the presence of serum, the repeated use of large amounts of synthetic polymers may cause undesirable side effects in the body. Because alginate is biocompatible in its hydrophilic state, it may form a non-fouling surface that would in turn inhibit the serum protein adsorption and the concomitant complex disruption [11,16]. The alginate coating of the DP complex did not appear to disturb the PEI-mediated endosomal escape, which in turn increased the transfection efficiency in the presence of serum.

In order to investigate the interaction of the DPA polyplex with erythrocytes, an erythrocyte aggregation assay was performed as described elsewhere [17]. Fresh mouse erythrocytes were incubated with either the DP complex, the DPA polyplex, or alone as a control. As shown in Fig. 5A, there was no aggregation between the C3 cells in the control samples. However, the addition of DP complex caused severe aggregation of the erythrocytes due to their electrostatic interactions, as indicated by the arrows in Fig. 5B. Furthermore, when cells were incubated in the presence of a DPA polyplex with an alginate to DNA weight ratio of 0.15, the aggregation level was drastically reduced (Fig. 5C). The

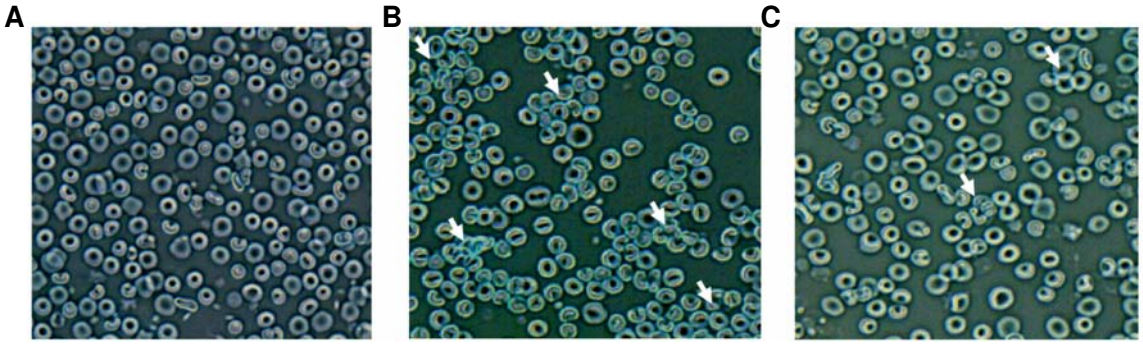

Fig. 5. Effect of the alginate (A) coating of the DNA/PEI (DP) complex on its binding with erythrocytes prepared from C57/BL6 mice: (a) untreated erythrocytes, (b) erythrocytes treated with the DP complex, and (c) erythrocytes treated with the DPA polyplex. The erythrocytes were observed using a microscope at a magnification of 100.

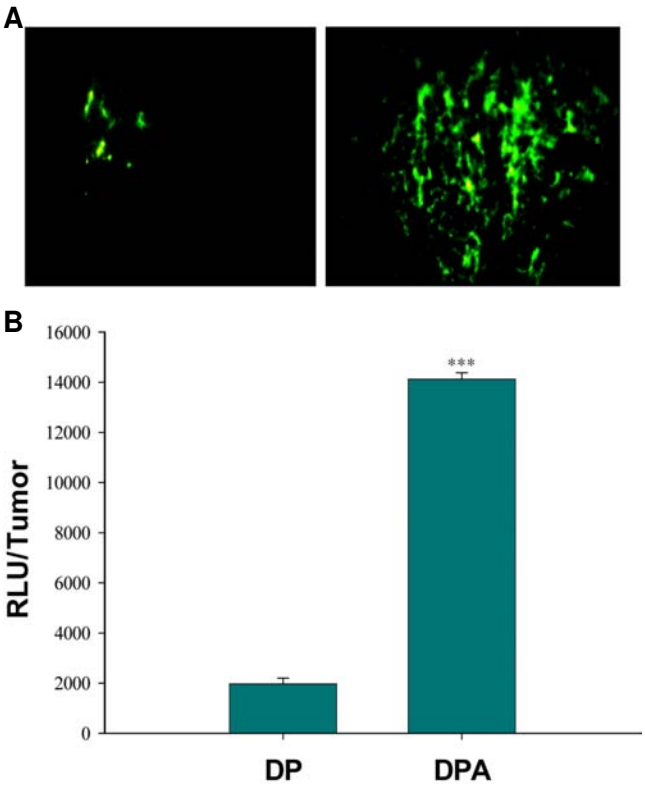

Fig. 6. Transfection efficiency of the DNA/PEI (DP) complex or the DNA/PEI/Alginate (DPA) polyplex in C57/BL6 mouse tumor models (n = 3) prepared by the subcutaneous injection of C3 cells: (A) fluorescent microscopy of green fluorescent protein gene (pEGFP-N1) expressing tumor tissues treated with (left) DP complex and (right) DPA polyplex at a magnification of 100, and (B) luciferase gene (pCMV-Luc) expression level in C3 tumors treated with DP and DPA polyplex (mean ± SD, n = 3); *** significantly different from DP by t-test ($p < 0.001$).

anionic and hydrophilic nature of alginate appeared to inhibit the non-specific interaction of the DP complex with erythrocytes, resulting in reduced erythrocyte aggregation, which may eventually contribute to the high transfection efficiency that occurs in the presence of serum.

Based on the *in vitro* studies described above, the DPA polyplex appeared to be a novel gene delivery system with a high luciferase gene transfection efficiency that resulted in a minimal amount of erythrocyte aggregation, therefore, the *in vivo* transfection efficiency of the DPA polyplex was assessed in six week old female C57/BL6 mice. Subcutaneous injection of C3 cells into C57/BL6 mice produced tumors that were between 6 and 7 mm in diameter in two weeks. After the tumors formed, either DP complex or DPA polyplex with a weight ratio of alginate to DNA of 0.15 was injected into the C3 cell-induced model tumors, and the GFP and luciferase reporter gene expressions within the tumors were then investigated. Fig. 6A shows the fluorescent microscopic images of the GFP gene (pEGFP-N1) expression in tumor tissues treated with DP complex (left) and DPA polyplex (right). Tumors treated with DPA polyplex showed significantly higher GFP gene expression than those that were treated with the DP complex. In addition, when pCMV-Luc was used as a reporter gene, tumors treated with DPA polyplex exhibited approximately 7 fold higher luciferase gene expression than those treated with DP complex (Fig. 6B). Based on the results of a t-test ($p < 0.001$), it was obvious that the luciferase level in the C3 tumors treated with DPA polyplex was significantly higher than that of tumors that had been treated with the DP complex. Taken together, these results indicate that the alginate coating of the DP complex alleviated its interaction with proteins in the interstitial fluid and blood around tumor tissues, and/or enhanced the stability of the DP complex, which resulted in a high gene transfection efficiency *in vivo*. Polyplexes are generally more resistant to inactivation by serum [18-20], therefore this novel DPA polyplex will be further investigated for the development of a clinical gene therapy protocol.

CONCLUSION

Coating a DP complex with alginate resulted in a higher

gene transfection efficiency *in vitro* in the presence of serum than was observed when an uncoated DP complex was used, which may have occurred due to the enhanced biocompatibility and reduced non-specific binding of the coated DP complex with serum components. Furthermore, erythrocyte aggregation in target cells that were treated with the DPA polyplex decreased due to electrostatic neutralization of the complex by the coating. In addition, an *in vivo* study in which a reporter gene encoding GFP or luciferase was transfected indicated that tumor cells treated with the DPA polyplex showed a much higher level of GFP expression and approximately 7 fold higher luciferase gene expression than the DP complex. The novel DPA polyplex will be further investigated for the development of a clinical gene therapy protocol.

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