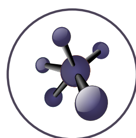
Ito et al., J Nanomater Mol Nanotechnol 2016, 5:6
DOI: 10.4172/2324-8777.1000202



Journal of Nanomaterials & Molecular Nanotechnology

Research Article

A SCITECHNOL JOURNAL

Effect of pDNA Complex Particle Size on Gene Expression: Difference between *In Vitro* and *In Vivo* Experiments

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Abstract

We have developed a novel gene-transfection system comprising very small (70-150 nm) plasmid/polyethyleneimine/hyaluronan (HA) ternary complexes having negative surface charge, and reduced nonspecific interactions with bio-components. They showed high *in vivo* gene expression efficiency. However, in *in vitro* transfection experiments, such small ternary complexes often demonstrated rather much lower gene expression than the conventional DNA/polycation complex. In this study, we investigated the influence of the complex particle size on gene expression efficiency, focusing on the difference between *in vitro* and *in vivo* transfection.

Size of the DNA complex particles strongly depended on the concentration of the mixing solutions, and the higher concentration resulted in the larger particles. As for the *in vitro* transfection, DNA/polyethyleneimine binary complexes could readily be bound to the cells through the electrostatic attractions, regardless of their size. Smaller binary complexes particles showed higher gene expression than the larger ones, likely due to their high internalization efficiency. On the other hand, small DNA/polyethyleneimine/HA ternary complexes having negative surface charge kept dispersing in the medium away from the cells, and demonstrated lower *in vitro* gene transfection efficiency than larger ones. In regards to *in vivo* transfection after intravenous injection, only small ternary complexes showed high gene expression, because reduced nonspecific interactions and small size of the complex particles are required to be distributed in the body, and accumulated in tumor. The superiority and inferiority of the vectors for *in vivo* transfection cannot be always correctly predicted by the results of *in vitro* transfection.

Keywords

DNA delivery; Ternary complex; Hyaluronic acid; Gene expression; Particle size

Introduction

Although certain polycations and cationic lipids which electrostatically bind to DNA achieved fairly high gene transfer efficiency in cultured cells, *in vivo* gene expression levels by those transfection reagents is still very low [1,2]. One of the major reasons for this low *in vivo* transfection efficiency is non-specific interactions of the DNA complex with bio-components such as proteins, cells, and extracellular matrices [1-4]. We reported that certain several acidic mucopolysaccharide containing hyaluronic acid (HA) or chondroitin sulfate (CS) could deposit onto DNA/polycation (or cationic lipid) complexes to recharge the surface to negative, and DNA/polycation/polyanion ternary complex was formed [5,6]. The negative charge of the ternary complexes effectively diminished adverse interactions with bio-components [7-10]. The polyanion could play a role as not only protective-coating, but also as a ligand to target cells, and a transcriptional enhancer [7].

Most serious problem remained unsolved which causes the low transfection efficiency of the plasmid complex in living body was the difficulty in achieving and maintaining the small size of the complex particles. A diameter less than 100 nm is required for particles to be distributed in the body and accumulate in tumor tissue by enhanced permeability and retention (EPR) effect [11]. Size and its distribution of the DNA particles strongly depend on the preparation conditions. When prepared at very low concentration, small particles are formed [12], and the higher concentration of the mixing solution resulted in the larger aggregate particles. Plasmid DNA complex particles with less than 100 nm can only be obtained at very low concentrations ([DNA]<20 μg/ml). However, for *in vivo* transfection, highly concentrated DNA complex suspension ([DNA]>150 μg/ml) is required to administer an adequate dose of DNA for high gene expression. Mixing of DNA with polycation under such highly concentrated conditions usually gives much larger particles (>200 nm) [8]. Thus, for sufficient *in vivo* transfection, it is required to make small DNA particles at very low concentration followed by condensation to more than ten times higher. However, condensation of DNA/polycation (or cationic lipids) always results in inactivation. Centrifugation, ultra-filtration, or evaporation always causes the particle aggregation. Freeze-drying of the DNA/polycation complex is also known to induce strong aggregation, and a significant loss of transfection efficiency [13,14].

Recently, we found that the HA-coated DNA/polycation ternary complexes could be freeze-dried without loss of gene transfection activity, owing to the protective effect of HA against the aggregation of the DNA complex particles [15]. Moreover, it enabled the preparation of small DNA complex particles (<70 nm) suspensions with high concentration (200 μg/ml) by complex formation at very low concentration followed by condensation through lyophilization-and-rehydration procedure. Those concentrated finely dispersed DNA/polycation complexes realized very high *in vivo* gene transfection efficiency in tumor tissues [6,15].

On the other hand, in *in vitro* transfection experiments, such small ternary DNA complexes often demonstrated rather much lower gene expression efficiency than the conventional large DNA/polycation complex particles. The superiority or inferiority of the preparation conditions in terms of gene expression efficiency was often contrary between *in vitro* and *in vivo* transfection, thus, making it difficult to design the ideal vectors. In this study, we examined the influence of particle size of DNA/PEI/HA ternary complexes on gene expression efficiency, focusing on the difference between *in vitro* and *in vivo* transfection.

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Received: September 09, 2016 **Accepted:** September 20, 2016 **Published:** September 25, 2016

SciTechnol International Publisher of Science, Technology and Medicine

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doi: 10.4172/2324-8777.1000202

Materials and Methods

Materials

Plasmid DNA with luciferase gene was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from the pGL3-control vector (Promega, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, CA, USA). B16 cells were obtained from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan). Cell culture lysis reagent and luciferase assay substrate were purchased from Promega Corp (WI, USA). Protein assay kit was obtained from Bio-Rad Laboratories (CA, USA). Linear polyethylenimines (PEIs) (Polyethylenimine Linear MW 25,000 in a free base form, and Polyethylenimine "Max" in a hydrochloride salt form comparable to MW 25,000 in a free base form were obtained from Polyscience, Inc (PA, USA).

Preparation of DNA complex

Typically; An aqueous solution of plasmid DNA (12.5 µl, 800 µg/ml; [P] = 2.424 mM) was diluted by 0, 100, 400, or 1600 µl of water, and then mixed with solutions of hyaluronic acid sodium salt (from Microorganism) (Nacalai Tesque, Inc, Kyoto, Japan) (25 µl; 5.82 mg/ml; [COOH]=14.5 mM), and PEI (12.5 µl; [NH]=29.1 mM), in this order. The mixing ratio of DNA:PEI:HA was 1 : 12 : 12 in charge. All the solutions were prepared in pure water. After standing for 30 min, they were freeze-dried, and the resulting white spongy complexes were rehydrate with pure water or 5% glucose to be [DNA]=200 µg/ml. Various concentrations of fresh DNA complex without freeze drying process was also similarly prepared.

ζ-Potential and size measurement

The freeze-dried complex containing 1.2 µg of DNA was rehydrated with water (6 µl). After 30 min, it was diluted with 800 µl of water, and ζ-potential and size were measured by a particle analyzer (MALVERN Zetasizer Nano ZS, Worcestershire, UK). Freshly prepared complexes were also analyzed similarly.

In vitro transfection

B16 cells, a mouse melanoma cell line, were seeded to 24-well plates at 1.2×10^5 cells per well, and cultured for 2 days in Eagle's Minimum Essential Medium (EMEM) media supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 unit/ml), and streptomycin sulfate (0.1 mg/ml). The primary growth medium was then replaced with 500 µl of fresh EMEM with FBS and the antibiotics. Lyophilized DNA complex was rehydrated by pure water to be [DNA]=200 µg/ml, and diluted with the same volume of double-concentrated PBS. After 30 min, they were added to the cells (1.25 µg of plasmid per well), and incubated for 4 hours at 37°C in a 5% CO_2 incubator. Fresh medium (500 µl) was then added to the wells. After additional 20-h incubation at 37°C, the cells were lysed, and transgene expression and protein content in the lysate were assessed with the corresponding assay kits. Transfection with fresh DNA complex suspensions without freeze drying process was also performed, as follows. The complexes were prepared at [DNA]=200 µg/ml (in 6.25 µl water), or [DNA]=22 µg/ml (in 56.8 µl water), and prior to addition to the cells, they were diluted with the same volume of double-concentrated PBS, or one-tenth volume of 11 times-concentrated PBS, respectively.

Cellular uptake *in vitro*

Amount of the DNA complex taken up by the cells, and that remained in the supernatant medium after the transfection procedure was evaluated by the complexes of fluorescently labeled plasmid DNA. Plasmid DNA was labeled in water with YOYO-1 iodide (YOYO) (Molecular Probes, Inc., OR, USA) (YOYO/DNA=1/15 in mole on the basis of nucleotide). It was diluted by a given volume of pure water, and then mixed with HA and PEI. One-tenth volume of 11 times-concentrated PBS was added to the YOYO-DNA complex suspension, and the mixture was diluted with PBS to be [DNA]=5.5 µg/ml. It was incubated with the cells similarly to the *in vitro* transfection experiments. After incubation for 24 h at 37°C in 5% CO_2, the supernatant was collected and centrifuged at 1000 rpm for 5 min. DNA complex remained in the supernatant and the cellular uptake efficiency were evaluated by measuring the fluorescence intensity of the supernatant and the cells, respectively, by a microplate reader, XFluor4GENiosPro (TECAN, Zürich, Switzerland) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. To determine the proportion of the DNA complex present in the supernatant or taken-up into the cells, the DNA complex suspension of each concentration was prepared and incubated in the same culture dish without cells. They were then properly diluted and assayed for fluorescence intensity to make a calibration curve.

In vivo transfection

Animal experiments and maintenance were performed under conditions approved by the animal research committee of Otsuma Women's University. B16 cells were subcutaneously inoculated into male 5-week-old ddY mice (8.3×10^6 cells per mouse). When the size of the tumor reached 8-10 mm in diameter, fresh or lyophilized-and-rehydrated DNA complex suspension containing 50 µg of plasmid (in 250 µl of 5% glucose) was intravenously injected. After 24 h, the mice were sacrificed, and the tumor was excised. It was homogenized in 1 ml of lysis-buffer, and centrifuged. The supernatant was assayed for luciferase activity and protein content.

Results

Characterization of DNA complexes

DNA complexes were prepared by mixing with PEI in the presence or absence of HA at concentrations of [DNA]=6, 22, 67, or 200 µg/ml in water. After lyophilized-and-rehydrated to [DNA]=200 µg/ml, they were diluted with water, and assessed for ζ-potential and size. Freshly prepared complexes before freeze drying were also similarly prepared and measured. Mixing ratio of the DNA/PEI binary- or DNA/PEI/HA ternary-complexes were 1:12, or 1:12:12 (in charge), respectively, since the ternary complex at this ratio demonstrated the best *in vivo* gene transfection efficiency [8]. ζ-Potential of DNA/PEI binary complexes prepared at the mixing ratio of 1:12 (in charge) was around 40 mV both before and after freeze-drying, regardless of the preparation concentration of the DNA complexes. On the other hand, the ternary complexes (at 1:12:12 charge ratio) showed highly negative surface charge (about -50 mV), also regardless of the preparation conditions (Figure 1).

Size of the DNA complexes highly depended on the concentration of the mixing solutions. The proportion of the DNA complexes in each size range is illustrated in Figure 2. When the DNA complexes were prepared at relatively high concentration (200 µg/ml), both binary- and ternary-complexes are mostly comprised of large aggregates, even before freeze-drying, and only a small portion could be detected in small particle range (<100 nm). Fresh binary- and ternary-complexes prepared at low concentration (before lyophilization) included high proportion of the particles less than 100 nm in diameter. Freeze-drying of the binary complexes caused remarkable aggregation, and small particles (<100 nm) could hardly be observed in the lyophilyzed-

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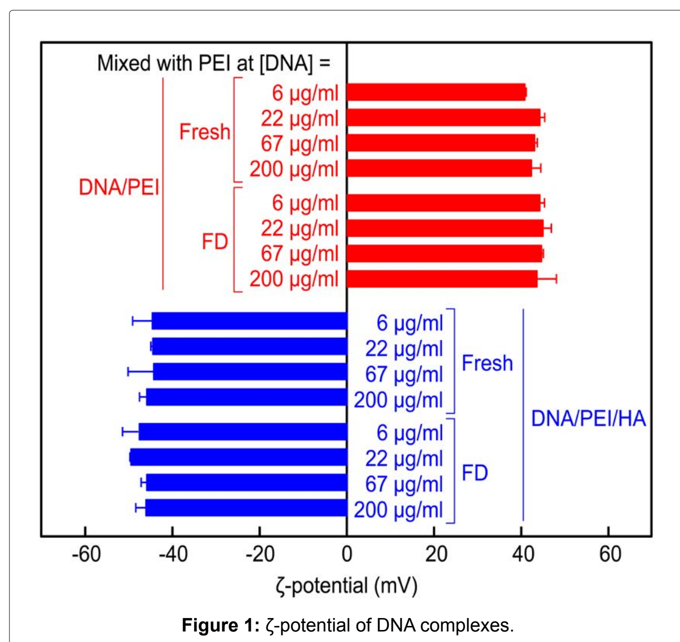

Figure 1: ζ-potential of DNA complexes.

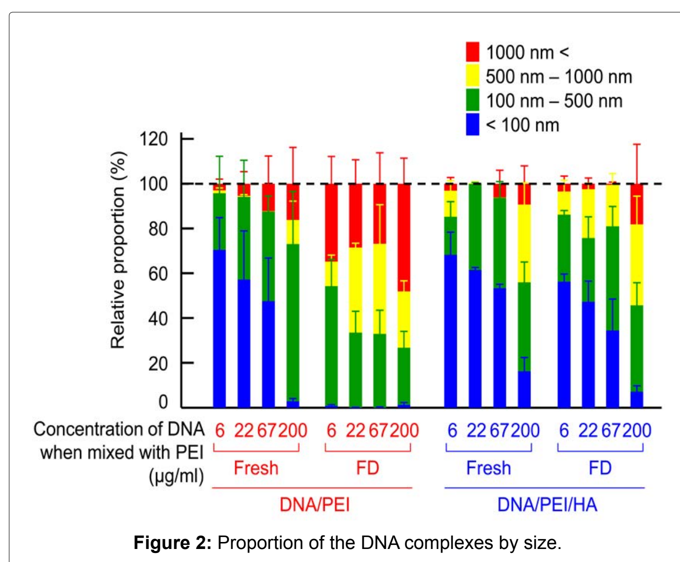

Figure 2: Proportion of the DNA complexes by size.

and-rehydrated binary complexes regardless of the preparation concentration. On the other hand Size distribution of the DNA/PEI/HA ternary complex scarcely changed by freeze-drying, and those prepared at [DNA]=6 μg/ml included large portion (55% volume) of fine particles smaller than 100 nm even after freeze-drying and rehydration. DNA/polycation binary complexes easily adhere to each other to form a large aggregates, while DNA/PEI/HA ternary complexes are stably suspended, and did not aggregated through the lyophilizing-and-rehydrating procedure. As was reported in our previous studies, this high dispersion stability of the DNA/PEI/HA ternary complex allowed the condensation of the small complex particles remaining their small sizes. Small DNA/PEI/HA ternary complex at a high concentration sufficient for injection of the required amount of DNA could be obtained by condensation through the lyophilizing-and-rehydrating procedure. Small complexes containing 100-200 μg of plasmid DNA suspended in 0.1 mL of aqueous media were then prepared, and high *in vivo* gene expression in tumour tissue was achieved [6].

Cellular uptake of the DNA complexes

In order to evaluate the cellular uptake efficiency of the DNA complexes during the *in vitro* transfection, they were prepared with fluorescently labelled plasmid under various conditions. Fresh DNA complexes prepared without freeze-drying were incubated with the cells, and fluorescence intensity of the supernatant medium or the cells were measured. The proportions of the DNA complexes present in the supernatant medium or in the cells are illustrated in Figure 3. In the case with naked plasmid DNA, fluorescence intensity associated with the cells was very low (Figure 3b), and more than 90% of the DNA still remained in the medium after 24 h incubation (Figure 3a). It is known that naked DNA is not readily taken up by the cells because of it's too large size and electrostatic repulsion. On the other hand, positively charged DNA/PEI binary complexes would easily stick to the cell surfaces through the electrostatic interaction, and the cells incubated with the binary complexes showed high fluorescent intensities irrespective of the preparation conditions or the particle size (Figure 3b). On the other hand, cellular uptake efficiency of the DNA/PEI/HA ternary complexes much depended on their preparation conditions. The DNA/PEI/HA ternary complexes prepared at low concentration ([DNA]=6 μg/ml), which comprise high proportion of small particles less than 100 nm, largely (more than 60%) continued to be suspended in the medium without precipitation. The higher were the concentration of the complex preparation solutions, the lower proportion of the complex remained in the supernatant (Figure 3a). It is in good accordance with the proportion of the small particles (<100 nm) in the complex suspension (shown in Figure 2). Relatively small ternary complex particles should have high dispersion stability, and kept suspended in the medium, causing lower cellular uptake efficiency.

In vitro and *in vivo* gene expression efficiency

Comparison of *in vitro* and *in vivo* gene expression efficiency of the binary- or ternary-complexes before or after lyophilization-and-rehydration was then examined. Freeze-drying of the DNA/polycation binary complex induced not only the aggregation, but a significant loss of transfection efficiency, as was shown in our previous study [15]. So the transfection by freeze-dried DNA/PEI binary complex was not

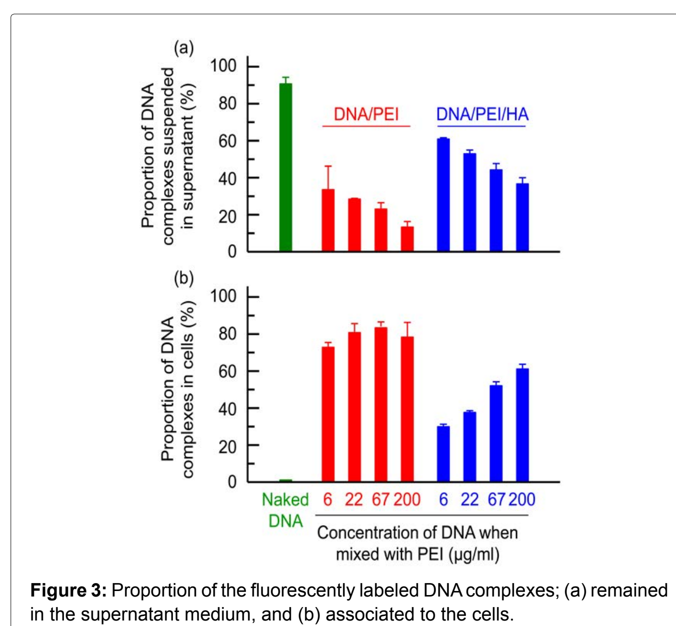

Figure 3: Proportion of the fluorescently labeled DNA complexes; (a) remained in the supernatant medium, and (b) associated to the cells.

doi: 10.4172/2324-8777.1000202

carried out in this study. *In vivo* transfection of the fresh DNA complex prepared at low concentration (22 μg/mL) could not be performed because, at this concentration, the administration volume became too large to be injected.

When the cells were transfected *in vitro* by the fresh DNA/PEI binary complexes, those prepared at low concentration (22 μg/mL) demonstrated higher gene expression than those prepared at higher concentration (200 μg/mL) (Figure 4a). The DNA/PEI complex particles have positive surface charge, and easily stick to the cells. Even small complexes obtained at 22 μg/mL could also readily bind to the cells in high efficiency (Figure 3). On the other hand, DNA/polycation binary complexes are very easy to aggregate, and most DNA/PEI complexes at high concentration existed in large aggregates (Figure 2), which are too large to be captured into the cells. Small binary complexes prepared at lower concentration would, thus, induce higher gene expression. As for ternary DNA complexes coated by HA, *in vitro* transfection efficiency was in good accordance with the cellular uptake efficiency. Those prepared at lower concentration, having low cellular uptake efficiency as shown in Figure 3, demonstrated lower gene expression in the cultured cells than those obtained at higher concentration having higher uptake efficiency (Figure 4a).

In *in vivo* transfection study, the ternary complex showed opposite results to the *in vitro* transfection. The smaller DNA complex, being prepared at lower concentration, induced the higher extra gene expression in tumor tissue (Figure 4b). High *in vivo* gene expression in tumor tissue could only be obtained by the DNA/PEI/HA ternary-complex prepared at very low concentration, followed by condensation through lyophilization-and-rehydration procedure.

Discussion

The superiority or inferiority of the plasmid DNA complexes in terms of gene expression efficiency much depended on the preparation conditions, especially on the concentration of the mixing solution, which is a determinant of the particle size (Table 1). When DNA was mixed with polycation at low concentration ([DNA]=6-22 μg/ml), suspensions of both binary- and ternary-complexes comprised high proportion of the small particles (<100 nm). The higher concentration of the mixing solutions invited the formation of larger particles for both binary- and ternary-complexes.

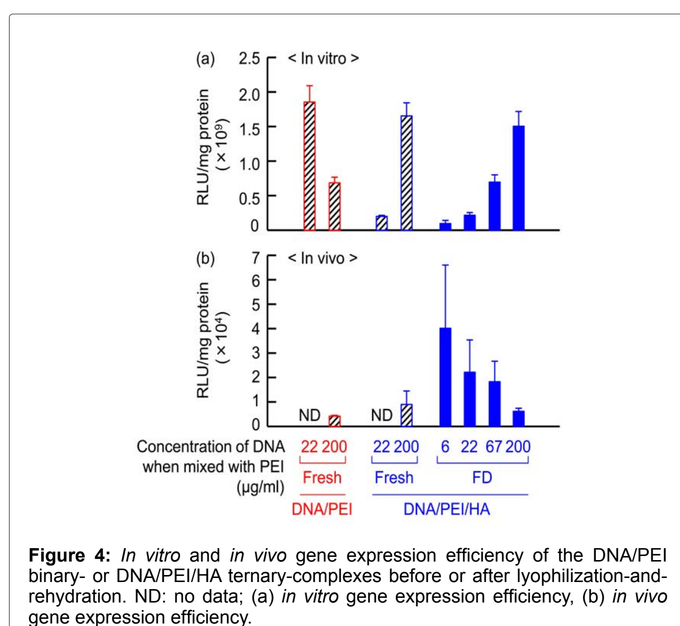

Figure 4: *In vitro* and *in vivo* gene expression efficiency of the DNA/PEI binary- or DNA/PEI/HA ternary-complexes before or after lyophilization-and-rehydration. ND: no data; (a) *in vitro* gene expression efficiency, (b) *in vivo* gene expression efficiency.

In *in vitro* transfection experiments, DNA binary complexes could be associated to the cells by electrostatic interaction regardless of the preparation conditions. Gene expression efficiency depended not to association- but to the internalization-efficiency of the particles. Smaller particles could be taken-up into cells and transported to nucleus in high efficiency, and would introduce higher reporter gene expression. On the other hand, behavior of the DNA ternary complexes was much affected by the preparation conditions. Small ternary complex particles prepared at low concentration had high dispersion stability, but are not electrostatically attracted by the cells. So they remained in the medium not precipitating or attaching to the cells for long time. Preparation of the ternary complexes at higher concentration afforded relatively larger particles, which would precipitate onto the cells to be taken-up. After internalization, the DNA/PEI/HA ternary complexes would be able to mediate high gene expression because of their high transcription efficiency [7]. Thus, for efficient *in vitro* transfection by the DNA/PEI/HA ternary complexes, large particle size and high tendency to precipitate onto the cells are required. Precipitation rate would be gene expression efficiency-determining factor for such stably dispersed ternary complex particles.

In regards to *in vivo* transfection after intravenous injection, body distribution efficiency is the bottleneck for gene expression. As was discussed in our previous reports [8,15], reduction of the adverse side effects of the DNA complexes, which mainly caused by their cationic surface charge, and sufficiently small size of the complex particles are essential to the successful delivery. HA-coated ternary complex has a negative surface charge regardless of the preparation concentration, and have diminished unexpected interaction with bio-components such as blood cells, endothelial cells, or extracellular matrices. Size of the complex particles is also very important factors to determine the gene expression efficiency in target tumor tissues. Particles with diameter less than 100 nm are known to distribute in the body and accumulate in tumor by enhanced permeability and retention (EPR) effect [11]. Small size of the particles is, thus, strongly required for *in vivo* tumor transfection. As shown in Figure 4b, *in vivo* gene expression in tumor tissue was highly dependent on the concentration of the preparation solution. The lower was the concentration of the complexing solution, that is to say, the smaller was the DNA complex particle, the higher extra gene activity was detected in tumor.

Although high dispersion stability and small particle size are very important factors for the delivery efficiency to the cells in *in vivo* study, they are not necessarily required for *in vitro* transfection, and sometimes invited rather low gene expression because of their tendency to keep floating in the supernatant. This favorability difference should be carefully considered when the transfection system is evaluated.

Conclusion

For *in vitro* transfection by DNA/PEI binary complex, smaller particles were favored because of their higher internalization efficiency into cells than the larger binary complexes, whereas small DNA/PEI/HA ternary complex particles kept dispersing in the medium away from the cells resulting lower gene expression than the larger ternary complexes. On the other hand, in *in vivo* transfection, smaller DNA ternary complex particles showed much higher extra gene activity in tumor than larger ternary complexes. High diffusivity along with stable

doi: 10.4172/2324-8777.1000202

Table 1: Summary of influence of the concentration of the mixing solutions on the particle size, cellular uptake-, and gene expression-efficiencies.

		Concentration of the mixing solutions	Binary complex	Ternary complex
Particle size before FD		Low	Small	Small
		High	Large	Large
Particle size after FD		Low	Large	Small
		High	Large	Large
Cell uptake		Low	High	Low
		High	High	High
Gene expression	***In vitro***	Low	High	Low
		High	Medium	High
	In vivo	Low	Low	High
		High	Low	Low

dispersibility in the body is required to accumulate in tumor tissues. Thus, for the development of the vector having high therapeutic effect by plasmid complexes, transfection efficiency should be evaluated in light of this remarkable difference between *in vitro*- and *in vivo*-experiments. The superiority and inferiority of the vectors for *in vivo* transfection cannot be always correctly predicted by the results of *in vitro* transfection.

Acknowledgment

We acknowledge Prof. T. Sato (Keio University) and Prof. Y. Tokudome (Josai University) for their help in measuring particle sizes and ζ-potentials. We wish to thank Prof. S. Fumoto (Nagasaki University) for offering linear PEI. This work was partly supported by JSPS KAKENHI Grant Number 26350533.

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