

## Regular Article

## Methotrexate-Coated Complexes of Plasmid DNA and Polyethylenimine for Gene Delivery

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**Folate receptors are overexpressed on the surface cancer cells. We successfully constructed a new gene delivery vector of methotrexate (MTX)-coated plasmid DNA–polyethylenimine (pDNA–PEI) complexes (PEI complexes) by electrostatic binding. The stable anionic nanoparticle was optimized at MTX charge ratios of 120 or more. pDNA–PEI–MTX complexes (MTX complexes) demonstrated gene expression efficiency as high as cationic pDNA–PEI complexes in the mouse melanoma cell line, B16-F10. The MTX complexes were taken up by the cell-specific uptake mechanisms *via* the folate receptor. MTX-coated complexes are useful as endocytosis ligands. The MTX120 complexes exhibited no blood aggregation. The transgene efficiency of MTX120 complexes in the liver and spleen after their intravenous administration was higher than that of PEI complexes. Therefore, MTX complexes are expected as a new gene vector in the future.**

**Key words** methotrexate; gene delivery; ternary complex; DNA

Methotrexate (MTX) is an antagonist of folic acid (FA), which is required for DNA synthesis, and exerts anticancer effects on a multiple of cancer cells overexpressing folate receptors (FR) on their surfaces.<sup>1)</sup> MTX is major anticancer drugs which are used for several human malignancies such as breast cancer, malignant lymphoma, acute lymphoblastic leukemia, osteosarcoma, and head and neck cancer.<sup>2)</sup> On the other hand, FA is a water-soluble B vitamin constituted of a pteridine ring, glutamic acid, and paraminobenzoic acid. As FA has a high affinity for FR, FA-modified nanoparticles have been widely investigated as FR-mediated gene delivery systems.

Nucleic acid-based compounds are expected as the next generation therapeutic agents for cancer,<sup>3)</sup> but they have limited uptake into the cells due to anionic characteristics, high molecular weight, and instability in the body. Therefore, the success of nucleic acid-based compound therapy is entirely dependent on the delivery efficiency of the therapeutic gene. Non-viral vectors with cationic liposomes and polymers have been reported to date.<sup>4)</sup> Cationic vectors are known to exhibit high gene expression through specific mechanisms such as binding to the surface of cells, being taken up *via* the endocytotic pathway, and the plasmid DNA (pDNA) release from endosomes.<sup>5–9)</sup> However, cationic vectors caused undesirable gene expression and blood aggregation by their cationic charges.

We previously succeeded in the development of an anionic gene vector constructed by making electrostatically self-assembled pDNA, polyethylenimine (PEI), and FA.<sup>10)</sup> This ternary complex exhibited high gene transfer efficiency and pharmaceutical safety. Therefore, we hypothesized that MTX coating may be used as a ligand for the vector. There are many reports on the drug delivery system using MTX.<sup>11,12)</sup> However, MTX was used as an anticancer drug in these reports. There are no reports on the delivery using MTX as a ligand. The complexes may be taken up by cancer cells and be useful for gene delivery. Moreover, anionic nanoparticles are expected to reduce their hematological toxicity because the

cationic charges are neutralized.

In the present study, we constructed MTX-coated nanoparticles as a new gene vector with an increased affinity for cancer cells, and examined its usefulness.

## MATERIALS AND METHODS

**Chemicals** PEI (branched form, average molecular weight (MW) of 25000) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, U.S.A.). MTX was obtained from WAKO (Osaka, Japan). Fetal bovine albumin (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). RPMI 1640, OptiMEM I, and antibiotics (streptomycin 100 µg/mL and penicillin 100 U/mL) for the cell culture were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). 1-Methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) were obtained from Dojindo Laboratories (Kumamoto, Japan). Rhodamine-PEI (Rh-PEI) was prepared in our laboratory. Briefly, PEI and rhodamine B were dissolved in dimethyl sulfoxide (DMSO), and stirred at room temperature in the dark overnight. Rh-PEI was purified by gel filtration. Approximately 1.5% PEI nitrogen was labeled with rhodamine B. All other chemicals were of reagent grade.

**Preparation of pDNA** pCMV-Luc was constructed by subcloning the *HindIII/XbaI* firefly luciferase cDNA fragment from a pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). Enhanced green fluorescent protein (GFP)-encoding pDNA (pEGFP-C1) was obtained from Clontech (Palo Alto, CA, U.S.A.). The pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA dissolved in 5% dextrose solution was stored at –80°C prior to analysis. The concentration of pDNA was adjusted to 1 mg/mL by measuring the absorbance at 260 nm.

**Preparation of Complexes** pDNA solution were mixed with PEI solution (pH 7.4) by pipetting thoroughly and left for

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15 min at room temperature. Next, pDNA-PEI complexes and MTX were mixed by pipetting and left for another 15 min. We prepared each complex at a theoretical charge ratio: phosphate of pDNA:nitrogen of PEI:carboxylate of MTX=1:8:0 (PEI complexes), 1:8:15 (MTX15 complexes), 1:8:30 (MTX30 complexes), 1:8:60 (MTX60 complexes), 1:8:90 (MTX90 complexes), 1:8:120 (MTX120 complexes), and 1:8:150 (MTX150 complexes).

**Physicochemical Properties of Complexes** The particle size and  $\zeta$ -potential of complexes were measured by Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.). The particles diameter is shown as a number-fractioned mean value.

To verify the complex formation, complex solution containing 1  $\mu$ g of pDNA (20  $\mu$ L) were mixed with 4  $\mu$ L of loading buffer (0.2% bromophenol blue and 30% glycerol) and loaded into wells at one end of a 0.8% agarose gel. Electrophoresis (iMupid J; Cosmo Bio Co., Ltd., Tokyo, Japan) was carried out at 100 V in running buffer solution (40 mM acetic acid, 40 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)) for 60 min. pDNA retardation was visualized with ethidium bromide staining and was observed with a Gel Doc EZ System (BioRad Laboratories, Inc., Hercules, CA, U.S.A.).

**Transfection Experiments** B16-F10, mouse melanoma cell line, was purchased from American Type Culture Collection. B16-F10 cells were cultured in RPMI 1640 with 10% FBS and antibiotics (culture medium) in an incubator at 37°C with 5% CO<sub>2</sub>. B16-F10 cells were seeded in 24-well plates (Becton-Dickinson and Company, Franklin Lakes, NJ, U.S.A.) at a density of 1.0×10<sup>4</sup> cells/well and incubated in 500  $\mu$ L of culture medium. After 24-h pre-incubation, the medium was replaced with 500  $\mu$ L of Opti-MEM I medium, and each complex containing 1  $\mu$ g of pCMV-Luc was added to the cells and incubated for 2 h. The medium was replaced with culture medium after transfection and cells were incubated for a further 22-h in an incubator at 37°C with 5% CO<sub>2</sub>. After that, the cells were washed with phosphate buffered saline (PBS) and then were lysed with 100  $\mu$ L of lysis buffer (pH 7.8 and 0.1 M Tris-HCl buffer containing 2 mM EDTA and 0.05% Triton X-100). Each lysate sample (10  $\mu$ L) was mixed with 50  $\mu$ L of luciferase assay substrates (PicaGene; Toyo Ink Co., Ltd., Tokyo, Japan) and the produced light was instantly measured by a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, German). The protein content of the lysate was measured by the Bradford assay (Bio-Rad Laboratories, Inc.). Absorbance was measured by a microplate reader (Sunrise RC-R; Tecan Japan Co., Ltd., Kanagawa, Japan) at 595 nm. Luciferase activity was showed as relative light units (RLU) per mg of protein.

**Fluorescence Microscopy** B16-F10 cells were transfected with several complexes composed of pEGFP-C1, Rh-PEI, and MTX to visualize the cellular uptake of complexes and gene expression. After a 22-h incubation, Rh-PEI and GFP expression were observed by fluorescence microscopy (200× magnification; BZ-9000; KEYENCE, Osaka, Japan).

**Inhibition Study** For the inhibition study, the cells were transfected as described above with MTX120 complexes in transfection medium containing different concentrations of FA or MTX. The transfection medium was replaced with culture medium after transfection. The transfected cells were incubated for a further 22-h at 37°C with 5% CO<sub>2</sub>. After that, luciferase activities were measured by luminometer.

For confirmation of the endocytotic pathway, the cells were treated with 0.014 mM chlorpromazine (CPZ) (inhibitor of clathrin-mediated endocytosis), 0.2 mM genistein (inhibitor of caveolae-mediated endocytosis) or 1 mM amiloride (inhibitor of micropinocytosis) for 1 h after a 23-h pre-incubation. After that, the PEI complexes and MTX120 complexes were added to the transfection medium containing each inhibitor. The transfection medium was replaced with culture medium after 2 h transfection, cells were cultured for a further 22 h at 37°C with 5% CO<sub>2</sub>, and then the luciferase activities were measured by luminometer.

**Agglutination Study** Mice erythrocytes were washed with PBS three times by centrifugation at 5000 rpm (Kubota 3500; Kubota, Tokyo, Japan) at 4°C for 5 min and re-suspended in PBS. A 2% (v/v) stock suspension was prepared. Each complex was mixed with the erythrocytes (complexes-stock suspension=1:1). The suspensions were left to stand for 15 min at room temperature. The suspensions were placed on a glass plate and agglutination with erythrocytes was observed by microscopy (200×magnification).

**Animals** Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Male ddY mice (5–6 weeks old) were obtained from Japan SLC (Shizuoka, Japan). Mice were acclimatized to the breeding environment for at least one day before experiments.

**In Vivo Study** Each complex containing 40  $\mu$ g of pCMV-Luc (300  $\mu$ L) was intravenously administrated into mice to examine the transgene efficiency of complexes. The mice were sacrificed at 6 h after intravenous injection, and the liver, kidneys, spleen, heart, and lungs were surgically removed. These tissues were homogenized with lysis buffer. The homogenates were centrifuged at 15000 rpm (Kubota 3500; Kubota) at 4°C for 5 min and the luciferase activity of supernatants was

Table 1. Particle Size and  $\zeta$ -Potential of Complexes

Complexes	Size (nm)	(mV)
pDNA-PEI (PEI)	59.3±6.8	42.8±0.3
pDNA-PEI-MTX 15 (MTX 15)	n.d.	14.6±0.4
pDNA-PEI-MTX 30 (MTX 30)	318.1±23.5	4.2±0.1
pDNA-PEI-MTX 60 (MTX 60)	329.6±26.4	-2.7±0.1
pDNA-PEI-MTX 90 (MTX 90)	292.8±16.5	-7.7±0.1
pDNA-PEI-MTX 120 (MTX 120)	195.7±4.3	-10.9±0.1
pDNA-PEI-MTX 150 (MTX 150)	170.0±5.5	-13.8±0.2

n.d.: not detectable.

measured by luminometer. Luciferase activity was showed as RLU per gram of tissue.

**Statistical Analysis** Significant differences between two groups were evaluated by the Student's *t*-test. Multiple comparisons among groups were made by Tukey's test.

## RESULTS

**Physicochemical Properties of the Complexes** The  $\zeta$ -potential and particle size of the different complexes are shown in Table 1. The PEI complex particles had  $42.8 \pm 0.3$  mV  $\zeta$ -potentials and were  $59.3 \pm 6.8$  nm in size. The  $\zeta$ -potential of MTX complexes decreased with the increasing the addition of MTX concentration-dependently. The particle size of MTX15 complexes could not be measured by a Zetasizer Nano ZS because of complex aggregation; however, stable nanoscale complexes with anionic charge were observed at a charge ratio greater than 1:8:120.

Gel retardation assays were conducted to confirm the complex formations (Fig. 1). The band of naked pDNA was detected on the agarose gel. However, PEI complexes and MTX complexes showed no band.

**In Vitro Transfection Efficiency of the Complexes** FR-positive B16-F10 cells were transfected with different complexes to examine the transgene efficiencies of the complexes (Fig. 2). The PEI complexes showed high luciferase activities ( $2.24 \times 10^{10}$  RLU/mg protein), whereas the MTX15 complexes showed a significantly lower luciferase activities than PEI and other MTX complexes (except for MTX150 complexes) ( $p < 0.01$ ). As MTX increased, however, the transgene efficiency of MTX complexes increased, and MTX30, 60, 90, 120, and 150 complexes had a transgene efficiency as high as PEI complexes.

Based on the physicochemical properties and *in vitro* transfection efficiency, we performed further studies using the MTX120 complexes.

**Fluorescence Microscopy** To visualize the uptake of the complexes and gene expression, the cells were transfected with PEI complexes, and MTX120 complexes containing Rh-PEI and pEGFP-C1 (Fig. 3). Red dots of Rh-PEI and bright green fluorescence of GFP was observed to the same degree for both complexes.

**Inhibition Study** Inhibition studies were performed with several inhibitory agents. Figure 4A shows the gene expression of the MTX120 complexes in medium containing

different concentrations of FA or MTX. Both FA and MTX inhibited the transgene effect of the MTX120 complexes concentration-dependently.

We also examined the effects of endocytotic inhibitors on the transgene effects of the MTX120 complexes (Fig. 4B). Genistein significantly decreased the luciferase activity of the MTX120 complexes ( $p < 0.01$ ) to lower than 20%. On the other hand, CPZ and amiloride showed no inhibitory effect on the transfection efficiency of the MTX120 complexes.

**Agglutination Study** We examined the blood agglutination activity of PEI complexes and MTX120 complexes (Fig. 5). The PEI complexes demonstrated strong agglutination. On the other hand, the MTX120 complexes showed no agglutination.

**In Vivo Study** The PEI complexes and MTX120 complexes were intravenously administered to mice (Fig. 6). The PEI complexes showed high gene expression in the liver, spleen, and lungs. The gene expression in the liver and spleen of MTX120 complexes was significantly higher than that of PEI

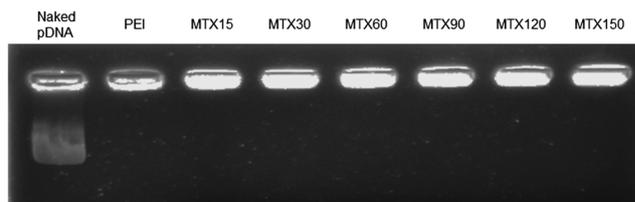


Fig. 1. Electrophoretic Analysis of Complexes

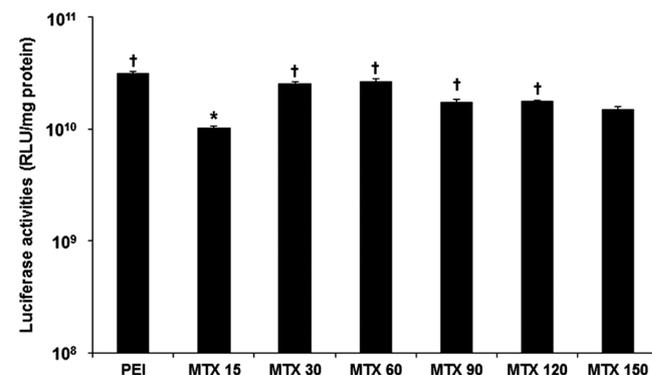


Fig. 2. *In Vitro* Transgene Efficiencies of Several Complexes

Each bar is the mean  $\pm$  S.E. of at least three experiments. \* $p < 0.01$  vs. PEI complexes, †:  $p < 0.01$  vs. MTX15 complexes.

### The uptake of Rh-PEI (red) and the expression of GFP (green)

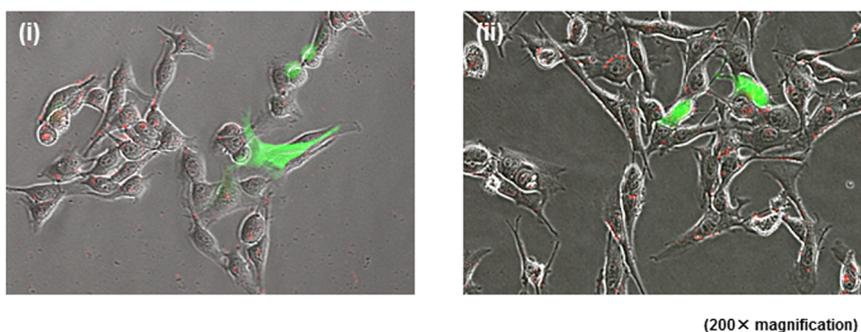


Fig. 3. Fluorescence Microscopy Image of B16-F10 Cells Transfected with Complexes

The uptake of Rh-PEI (red) and expression of GFP (green) were observed. (i) PEI complexes; (ii) MTX120 complexes. (Color figure can be accessed in the online version.)

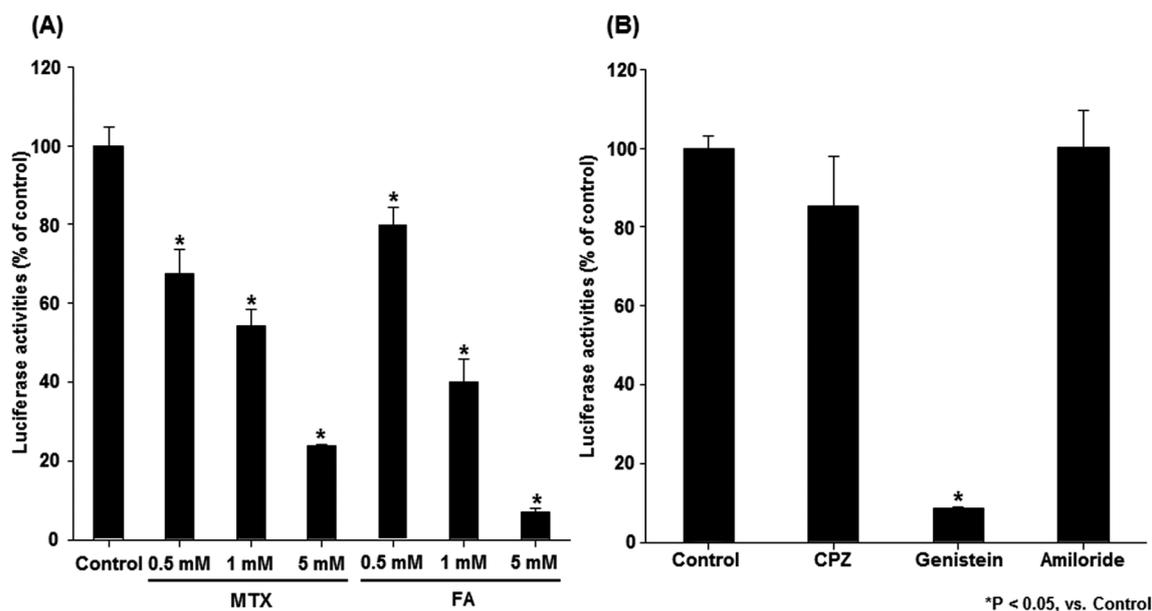


Fig. 4. Influence of MTX and FA (A), and Endocytotic Inhibitors (B) on Transgene Expression Efficiency of MTX120 Complexes

Each bar is the mean  $\pm$  S.E. of at least four experiments. \* $p < 0.05$  vs. Control.

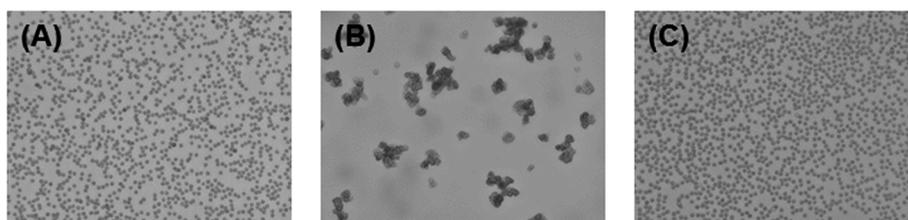


Fig. 5. Agglutination of Complexes with Erythrocytes (400 $\times$  Magnification)

(A) PBS; (B) PEI complexes; (C) MTX120 complexes.

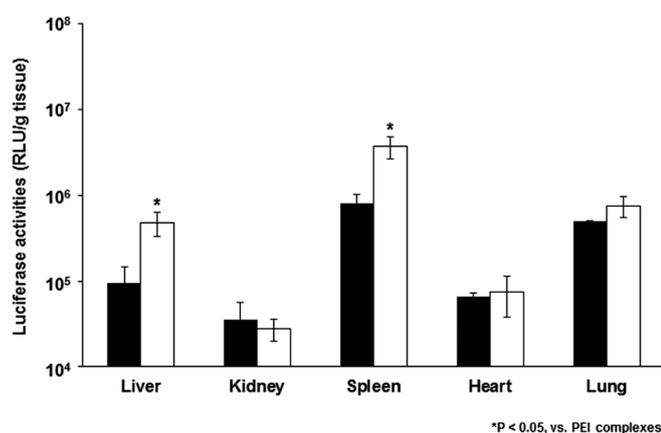


Fig. 6. *In Vivo* Transgene Efficiencies of the Complexes

PEI complexes (■), MTX120 (□) Each bar is the mean  $\pm$  S.E. of at least four experiments. \* $p < 0.05$  vs. PEI complexes.

complexes ( $p < 0.05$ ).

## DISCUSSION

FR is known as a glycosphosphatidylinositol-linked cell surface receptor and is a high affinity membrane folate-binding glycoprotein. FR is overexpressed in a variety of cancer cells, which are auxotrophic. Therefore, FA is an expected com-

ponent of the vector.<sup>13–15</sup> The anticancer drug MTX has a similar chemical structure with folic acid. MTX inhibits the synthesis of tetrahydrofolate (active folate) by dihydrofolate reductase inhibitory effects, stopping the synthesis of DNA. This mechanism of action is exerted through FR. We formed a hypothesis which MTX can electrostatically coat the PEI complexes because it has an anionic character. Furthermore, MTX-coated complexes may be taken up by cells through the FR-mediated pathway because their chemical structure is similar with FA.<sup>13–15</sup>

The MTX decreased the  $\zeta$ -potential of PEI complexes concentration-dependently. MTX complexes that formed became anionic nanoscale particles at a charge ratio of more than 120 (Table 1). Also the  $\zeta$ -potential of MTX complexes did not reach the plateau. Most of MTX may cover the PEI complexes and there are little the free MTX. Furthermore, MTX did not push pDNA from the PEI complexes (Fig. 1). These results suggest that MTX was coated without disrupting the conformation of PEI complexes, and complexes formed tightly stable anionic nanoparticles enclosing pDNA.

Next, we examined the gene expression of each complex with different charge ratios in B16-F10 that constitutively expresses FR. In general, high gene expression of cationic gene delivery vectors is thought to be due to electrostatic interaction of vectors with a cell membrane.<sup>6,16,17</sup> PEI complexes exhibited high gene expression levels (Fig. 2). On the other hand, anionic complexes generally repulse the cellular membrane

and are not able to be taken up spontaneously by cells. However, anionic MTX complexes had high gene expression levels at charge ratios of more than 30, comparable with cationic PEI complexes. Moreover, high uptake and gene expression of both complexes were also confirmed by the observation of Rh-PEI and GFP (Fig. 3). On the other hand, PEI complexes and MTX complexes showed equivalent cytotoxicity in the preliminary experiment. Regardless of the results, the MTX complexes showed different results from the PEI complexes in blood aggregation (Fig. 5) and *in vivo* experiments (Fig. 6). However, further study is necessary because their cytotoxicity might affect the results.

To clarify the mechanism, we evaluated the effects of competitive antagonists on the cellular uptake of MTX-coated particles. The additive concentrations of FA or MTX were sufficiently higher than MTX concentrations in MTX120 complexes. Gene expression of MTX120 complexes was found to decrease in a concentration-related fashion in the presence of FA or MTX. This result suggested that MTX complexes were taken up *via* the FR-mediated pathway and were showed to a high gene expression (Fig. 4A).

FA and FA-mediated drug delivery systems were reported to be taken up by the pathway of caveolae-mediated endocytosis with the interposition of FR.<sup>18–20</sup> We also conducted inhibition experiments by adding several endocytosis inhibitors. The gene expression of MTX120 complexes was significantly decreased in the presence of genistein (Fig. 4B). These results suggested that MTX120 complexes were also taken up *via* the caveolae-mediated endocytotic pathway with the interposition of FR in common with previously reported FA-mediated drug delivery systems.

Cationic gene delivery vectors often agglutinated the erythrocytes and produced a variety of adverse effects such as embolism and inflammatory reactions.<sup>21,22</sup> The PEI complexes exhibited markedly strong agglutination activities. On the other hand, the MTX120 complexes did not exhibit any agglutination (Fig. 5). These results cause to maintaining the anionic surface charge of the MTX complexes. Thus, MTX complex, which is effective and safe gene delivery vector, has suitability for *in vivo* use.

Therefore, gene expression of each complex was assessed in order to examine the basic kinetics *in vivo*. We administered the complexes intravenously into mice, and the gene expression in the liver, kidneys, spleen, heart, and lungs was measured (Fig. 6). The PEI complexes showed high luciferase activities in the liver, spleen, and lungs. On the other hand, MTX120 complexes exhibited significantly higher luciferase activities in the liver and spleen than PEI complexes. Nanoparticles are generally taken up in the reticuloendothelial tissue such as liver and spleen. Furthermore, MTX120 complexes may have been also taken up by these organs through the FR-mediated pathway because these organs except for liver were FR-positive.<sup>23</sup>

In this study, we successfully developed MTX complexes with electrostatic interactions. The MTX complexes were taken up by cells through the pathway of caveolae-mediated endocytosis with the interposition of FR and showed high transgene efficiency. The MTX complexes also exhibited high gene expression in the liver and spleen, which are FR-positive organs, through the FR-mediated pathway after intravenous administration. These results suggested MTX complexes to

be useful for effective gene therapy. MTX-coated gene vectors are expected to be used in combination with nucleic acid-based compounds, such as pDNA and small interfering RNA, and have anti-cancer effects in the future, but further studies are necessary.

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**Conflict of Interest** The authors declare no conflict of interest.

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