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The role of adenosine receptor and caveolae-mediated endocytosis in oligonucleotide-mediated gene transfer

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ABSTRACT

We previously reported the preparation and characterization of ternary nanoparticles with the negative surface charge, which comprises histidine-conjugated polyallylamine (PAA-HIS)/DNA core complex and a single-stranded oligonucleotide outer layer, to transfect various cell lines. As a continued effort, here the investigations on the endocytotic mechanisms involved in the uptake of the oligonucleotide-coated PAA-HIS/DNA complexes are reported. Interestingly, these complexes showed enhanced transfection efficiency only when deoxyadenosine-containing oligonucleotides were deposited on the PAA-HIS/DNA complex surface. The addition of uncomplexed oligonucleotide, free adenosine and adenosine receptor antagonist significantly inhibited the transfection efficiency of oligonucleotide-coated PAA-HIS/DNA complexes. These results indicated that the oligonucleotide-coated PAA-HIS/DNA complexes could specifically recognize adenosine receptors on the cell surface and were taken up by adenosine receptor-mediated process. Uptake and transfection experiments with various endocytic inhibitors suggested that, after receptor/ligand binding, oligonucleotide-coated PAA-HIS/DNA/complexes were mainly internalized via caveolae-mediated pathway to result in effective intracellular processing for gene expression. In conclusion, both adenosine receptor and caveolae-mediated endocytosis play important roles in oligonucleotide-mediated gene transfer.

1. Introduction

Due to recent progress in the area of non-viral vector-mediated gene delivery, a variety of polycations have been shown to compete in DNA delivery such as polyethylenimine (PEI), poly(L-lysine) (PLL) or polyallylamine (PAA) [1–6]. These polycations can condense DNA and bind to cells through electrostatic interaction between the net positive surface charges of complexes and negatively charges of cellular membrane. Therefore, when an additional layer of polyanion is deposited on the surface of the polycation/DNA complex, the ternary anionic complex should show minimal cellular uptake and transfection if specific targeting ligands are not exposed on the complex surface [7–10]. Previously, we prepared ternary anionic complexes comprising histidine-conjugated PAA (PAA-HIS)/DNA core and anionic outer layer, such as deoxyadenosine-rich ($C_{10}A_{20}$) and deoxythymidine-rich ($C_{10}T_{20}$) oligonucleotides [11]. Accidentally, we discovered that $C_{10}A_{20}$ could, but $C_{10}T_{20}$ could not mediate PAA-HIS/DNA complexes to exhibit high gene expression even they did not conjugate any specific targeting molecules. Although not all oligonucleotides could be well utilized for designing the special transfection agent, in this study, we further demonstrated both *in vitro* and *in vivo* gene expression of various polycation/DNA complexes could be improved by the technique of assembling appropriate oligonucleotides with positively charged core complexes.

The successful design of a non-viral gene delivery system requires a comprehensive understanding of the mechanisms involved in the interaction of the systems with the target cells. There are a number of studies that have revealed various cellular processes that are involved in the vector-mediated transfection of cells [12–16]. They include cellular binding and subsequent internalization pathway for gene transcription. Clearly, the gene expression of oligonucleotide-coated polycation/DNA complexes was strongly dependent on the difference of chemical structure between thymidine and adenosine. Therefore, the role of adenosine in oligonucleotide-mediated gene transfer was first analyzed for efficient cellular binding. Subsequently, the intracellular mechanism of oligonucleotide-coated polycation/DNA complexes was elucidated. Consequently, the purpose of this work was to study how the technology of coating oligonucleotides on polycation/DNA complexes could pass through these cellular processes efficiently for efficient gene delivery.

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2. Materials and methods

2.1. Preparation of binary complexes

PAA-HIS/DNA and PEI/DNA complexes were formulated by mixing 20 μg/mL DNA solution (enhanced green fluorescent protein (EGFP) plasmid (4.7 kbp; Clontech Laboratories, Palo Alto, CA) or luciferase plasmid (4.1 kbp; Clontech Laboratories, Palo Alto, CA)) with 100 μg/mL PAA-HIS and 1.45 μg/mL PEI solution in MilliQ water, respectively.

2.2. Preparation of ternary oligonucleotide-coated polycation/DNA complexes

Single-stranded oligonucleotides (Bio Basic Inc., Canada) dissolved in MilliQ water at 100 μM was added to PAA-HIS/DNA at oligonucleotide/PAA-HIS molar ratio of 3. PEI/DNA/oligonucleotides complexes were prepared by mixing binary complexes with oligonucleotides at 9 of oligonucleotide/PEI molar ratio. All oligonucleotides mentioned in this study are $C_{10}A_{20}$, unless indicated otherwise. The particle size and surface charge of oligonucleotide-coated polycation/DNA complexes were determined by using a Zetasizer (ZS-90, Malvern, UK).

2.3. Preparation of quaternary oligonucleotide-coated PAA-HIS/DNA complexes

The complementary single-stranded oligonucleotide ($C_{10}T_{20}$, Bio Basic Inc., Canada) dissolved in MilliQ water at 100 μM was mixed with ternary PAA-HIS/DNA/oligonucleotide complexes at $C_{10}A_{20}/C_{10}T_{20}$ M ratio of 1. In addition, alternative quaternary DNA complexes (PAA-HIS/DNA/$C_{10}T_{20}$/$C_{10}A_{20}$) were prepared with a similar process.

2.4. Cell line

The model cell line used in this study was a human cervix carcinoma cell line, HeLa cell (BCRC number: 60,005). The used medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel).

2.5. Oligonucleotide-coated polycation/DNA complexes for in vitro and in vivo gene delivery systems

HeLa cells were cultured in 12-well plates at a density of 10^5 cells/well and then were transfected with PAA-HIS/DNA/$C_{10}A_{20}$ as mentioned above [11]. In addition, HeLa cells in 12-well plates (10^5 cells/well) were treated with binary PEI/DNA and ternary PEI/DNA/$C_{10}A_{20}$ complexes, respectively.

For preliminary *in vivo* transfection assay, PEI/DNA/oligonucleotide complexes at 9 of oligonucleotide/PEI molar ratio and subsequently were delivered to the shaved abdominal region of ICR mice (three per group) using a helium-driven gene gun (BioWare Technologies, Taipei, Taiwan). Each vaccination consisted of four shots around the shaved abdominal region, resulting in a total of around 2 μg of EGFP plasmid carried with binary or ternary complexes being introduced into skin. After 24 h, mice were observed the EGFP expression by fluorescent microscope (AZ100, Nikon, Japan).

2.6. Transfection studies of oligonucleotide

HeLa cells were cultured in 12-well plates at a density of 10^5 cells/well. After incubation for 24 h, the DMEM medium was replaced with OPTI-MEM medium containing PAA-HIS/DNA/oligonucleotide complexes and incubated for another 24 h. For comparison, C_{30}, A_{30}, and T_{30} were used to be as oligonucleotides in constitution effect study. In sequence effect assay, $(C_5A_{10})_2$, and $(C_2A_5C_3A_5)_2$ were used to form ternary complexes. In addition, $C_{10}A_{20}$, $C_{10}A_{40}$, and $C_{10}A_{50}$ were used to analyze the effect of molecular weight for transfection efficiency. For concentration-dependent experiment, HeLa cells were pretreated with oligonucleotides for 60 min at the concentration of oligonucleotides was 0.3–1.8 μM. For pretreated time-dependent experiment, the cells were incubated with 1.8 μM of oligonucleotide for 0, 15, 30, and 60 min. For shielding effect of complementary oligonucleotides, the cells were incubated with quaternary complexes which were prepared by mixing complementary oligonucleotides with ternary complexes at $C_{10}T_{20}/C_{10}A_{20}$ M ratio of 1. In competition effect of nucleotides, the cells were pretreated with adenosine at the concentration was 10–1000 μM for 60 min. In inhibition experiments, the cells were pretreated with 8-(p-Sulfophenyl) theophylline hydrate (8-SPT) at 1 mM for 60 min. After incubation for another 24 h, the luciferase activity assay was performed according to the manufacturer's protocol. Briefly, cells were lysed by lysis reagent (Promega, USA) for 20 min and then centrifuged at 12,000 g at 4 °C for 30 min. The relative luminescence and total protein of supernatant were determined using a chemiluminometer (Monolight Luminometer 2010, Becton–Dickinson, San Jose, CA) and Bio-Rad protein assay (USA), respectively. Luciferase activity was indicated as relative light units per mg protein (RLU/mg protein).

2.7. Temperature-dependent oligonucleotide-coated PAA-HIS/DNA complexes gene expression

In order to determine energy-dependency of nanoparticle uptake, cells were transfected with DNA nanoparticles at either 37 or 4 °C [14]. HeLa cells were cultured in 12-well plates at a density of 10^5 cells/well. After incubation for 24 h, the DMEM medium was replaced with 1 mL phosphate buffer saline (PBS) and the cells were incubated for 30 min, either 37 or 4 °C. After that, the cells were incubated with PAA-HIS/DNA/oligonucleotide complexes containing 2 μg plasmid DNA in OPTI-MEM medium at either 37 or 4 °C for an additional 3 h. Finally, the cells were extensively washed three times with PBS at the corresponding temperatures before OPTI-MEM medium at 37 °C was added. After 48 h of incubation at 37 °C for both conditions, the cells were analyzed for luciferase activity as mentioned above.

2.8. Cellular uptake studies with endocytosis inhibitors

To analyze the uptake of PAA-HIS/DNA/oligonucleotide complexes in the presence of inhibitors, HeLa cells cultured in 6-well tissue culture polystyrene plates (2.5×10^5 cells/well) were incubated with inhibitors (chlorpromazine (10 μM, Aldrich, St. Louis, MO), genistein (200 μM, Aldrich, St. Louis, MO), or wortmannin (10 nM, Aldrich, St. Louis, MO)) 1 h prior to incubated with PAA-HIS/DNA/oligonucleotide complexes containing YOYO-1-labeled DNA. After incubation for another 2 h, the cells were rinsed twice with PBS before they were detached with 500 μL of trypsin-EDTA (0.25%, GibcoBRL). Subsequently, the cells were washed twice with PBS before they were transferred into tube (REF 352054, Falcon) for flow cytometry analysis (Becton–Dickinson).

2.9. Cytotoxic studies with endocytosis inhibitors

Cytotoxicity of oligonucleotide-coated ternary complexes was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide; Sigma) colorimetric assay. HeLa cells were cultured in 24-well tissue culture polystyrene plates (Costar, USA) at a density of 2×10^4 cells/well. After incubation for 24 h, the DMEM medium was removed and the cells were incubated with OPTI-MEM medium containing chlorpromazine (10 μM), genistein (200 μM), or wortmannin (10 nM). After incubation for another 3 h, the cells were extensively washed three times with PBS and incubated with 200 μL of MTT solution (5 mg/mL) for 3 h at 37 °C. After incubation, the MTT solution was aspirated and the formazan reaction products were dissolved in DMSO and shaken for 20 min. The optical density of the formazan solution was read on an ELISA plate reader (SpectraMax M2e, Molecular Probe) at 570 nm. The results are shown as a percentage of untreated cells with 100% viability.

2.10. Transfection studies with endocytosis inhibitors

For gene expression study with endocytosis inhibitors, HeLa cells were cultured in 24-well plates at a density of 2×10^4 cells/well for 24 h, and then pretreated with chlorpromazine (10 μM), genistein (200 μM), or wortmannin (10 nM) for 1 h prior to addition of ternary complexes to the cells, respectively. Afterward, HeLa cells were incubated at 37 °C with binary or ternary complexes for 2 h in the presence and absence of the inhibitors. Subsequently, the medium was replaced with fresh OPTI-MEM medium. The cells incubated with the polyplexes were maintained 24 h after transfection, and then the gene expression was determined by luciferase activity assay as mentioned above.

3. Results

3.1. Oligonucleotide-coated polycation/DNA complexes for in vitro or in vivo gene delivery system

In our previous study [11], $C_{10}A_{20}$ oligonucleotide-coated polycation/DNA complexes have been demonstrated to possess high gene expression *in vitro* (Fig. 1(a)). The polycation and DNA used were PAA-HIS and EGFP plasmid, respectively. In this study, we further demonstrated that PEI/DNA complexes coated with $C_{10}A_{20}$ oligonucleotides still exhibited higher gene expression *in vitro* than binary PEI/DNA complexes (Fig. 1(b)), indicating the technique of coating an oligonucleotide layer on complex surface could be applied to different combinations of polycation and DNA. Following *in vitro* transfection studies, we administered $C_{10}A_{20}$ oligonucleotide-coated PEI/DNA complexes to the shaved abdominal skin of mice using the gene gun. Apparently, the ternary PEI/DNA/$C_{10}A_{20}$ complexes showed higher EGFP expression than the binary PEI/DNA complexes under fluorescent microscope (Fig. 1(c)). Based on these studies, both *in vitro* and *in vivo* gene expression of polycation/DNA

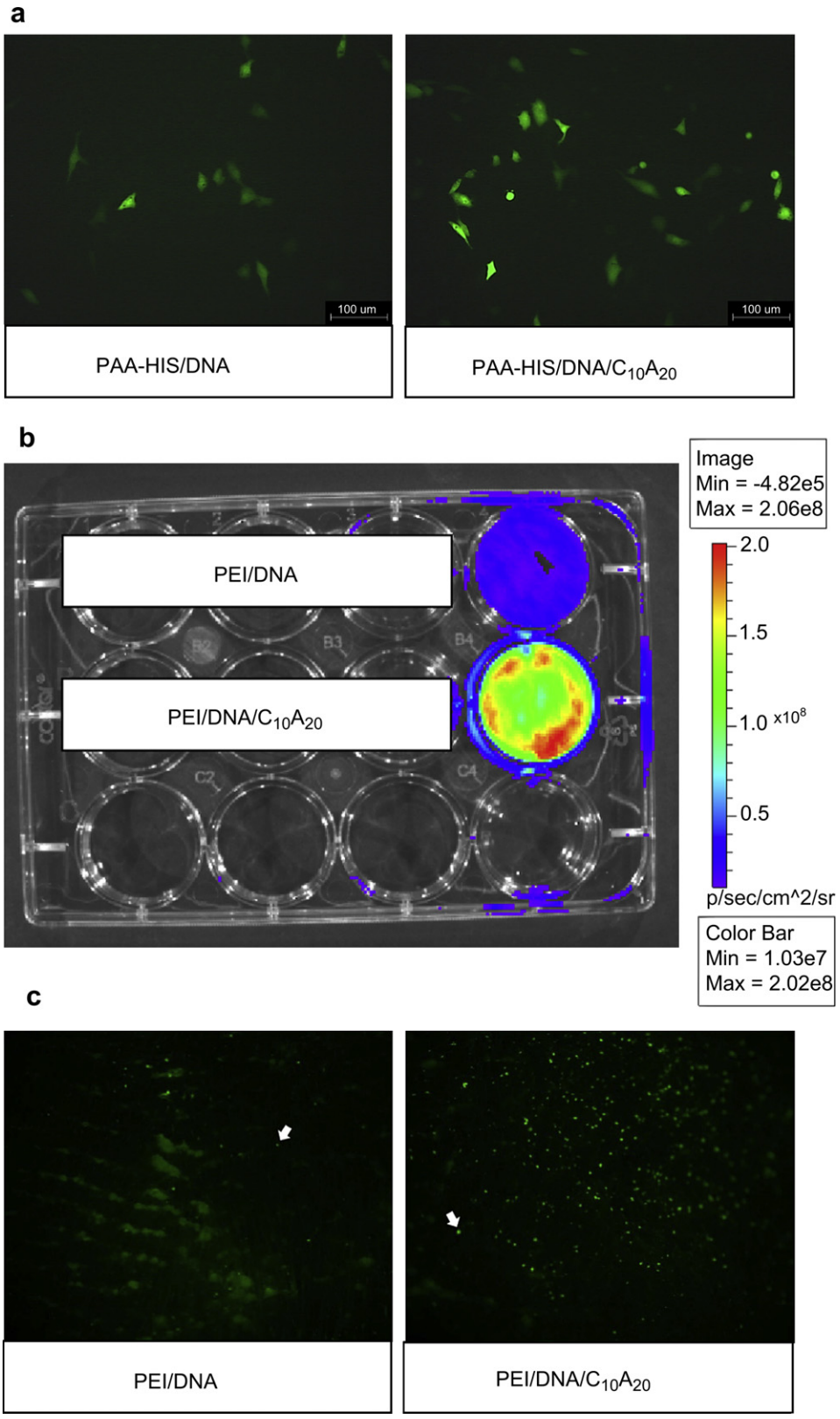

Fig. 1. Transfection efficiency assay. HeLa cells were treated with (a) PAA-HIS/EGFP plasmid and PAA-HIS/EGFP plasmid/$C_{10}A_{20}$ complexes at 3 of oligonucleotide/PAA-HIS molar ratio for 24 h. (b) PEI/luciferase plasmid and PEI/luciferase plasmid/$C_{10}A_{20}$ complexes at 9 of oligonucleotide/PEI molar ratio for 24 h. (c) Fluorescent microscope images of transfection EGFP plasmid into the shaved abdominal skin of mice using the gene gun with PEI/DNA and PEI/DNA/$C_{10}A_{20}$ complexes at 9 of oligonucleotide/PEI molar ratio for 24 h.

complexes could be improved by the technique of assembling oligonucleotides with binary polycation/DNA complexes.

3.2. The effect of constitution, sequence, and molecular weight of oligonucleotides on the physicochemical properties of oligonucleotide-coated PAA-HIS/DNA complexes

Table 1 shows the physicochemical properties of oligonucleotide-coated PAA-HIS/DNA complexes by using different oligonucleotides, A_{30}, T_{30}, and C_{30}. Compared to binary PAA-HIS/DNA complexes, ternary PAA-HIS/DNA/A_{30} and PAA-HIS/DNA/T_{30} complexes possessed negative surface charge but did not significantly increase their size, similar to our previous report [11]. However, PAA-HIS/DNA/C_{30} complexes showed an extreme aggregation degree and still presented positive surface charge, indicating the deoxycytosine oligonucleotide (C_{30}) could not be applied to prepare ternary complexes. Thus, only deoxyadenosine oligonucleotide (A_{30}) and deoxythymidine oligonucleotide (T_{30}) were further applied to gene delivery in the following investigation.

Compared with luciferase expression of T_{30} oligonucleotide-coated PAA-HIS/DNA complexes ($1.4 \pm 0.4 \times 10^6$ RLU/mg protein), A_{30} oligonucleotide-coated PAA-HIS/DNA/oligonucleotide complexes ($5.4 \pm 0.7 \times 10^7$ RLU/mg protein) showed about 40-fold higher transfection efficiency in HeLa cells, similar to our previous study that $C_{10}A_{20}$ exhibited higher level of luciferase expression than $C_{10}T_{20}$ did [11]. Since these two ternary complexes showed similar anionic charges (~ -20 mV), the higher level gene expression of PAA-HIS/DNA/A_{30} complexes was attributed to the difference of chemical structure between thymidine and adenosine. Furthermore, Fig. 2 shows no significant difference in gene expression and this was observed when the sequence ($C_{10}A_{20}$, $(C_5A_{10})_2$, $(C_2A_5C_3A_5)_2$) and molecular weight (($C_{10}A_{20}$, $C_{10}A_{40}$, $C_{10}A_{50}$)) of oligonucleotides were varied. These results indicate transfection efficiency of oligonucleotide-coated PAA-HIS/DNA complexes is only sensitive to the constituent of oligonucleotides.

3.3. Inhibition effect of complementary oligonucleotides

Since oligonucleotide coated on the outer layer of binary complex possesses high binding affinity toward its complement by nucleic acid base-paired interaction, the shielding effect of complementary oligonucleotides on the inhibition of gene expression of oligonucleotide-coated PAA-HIS/DNA complexes was investigated. As shown in Fig. 3, various ternary and quaternary complexes, such as PAA-HIS/DNA/$C_{10}A_{20}$, PAA-HIS/DNA/$C_{10}A_{20}$/$C_{10}T_{20}$, PAA-HIS/DNA/$C_{10}T_{20}$, and PAA-HIS/DNA/$C_{10}T_{20}$/$C_{10}A_{20}$, were compared in this study. Fig. 4 shows the luciferase activity of ternary PAA-HIS/DNA/$C_{10}A_{20}$ complexes was significantly inhibited by an approximately 1000-fold drop ($p < 0.05$) while the complementary oligonucleotide $C_{10}T_{20}$ was administered, indicating the interaction between adenosine and cell was interrupted by the presence of $C_{10}T_{20}$ between them. However, the gene expression of PAA-HIS/DNA/$C_{10}T_{20}$ complexes was either not increased due to the hybridization of $C_{10}A_{20}$ with $C_{10}T_{20}$. PAA-HIS/DNA/$C_{10}T_{20}$, was not increased due to the hybridization of $C_{10}T_{20}$ with $C_{10}A_{20}$. Therefore, $C_{10}A_{20}$ would lose its characteristics to enhance the gene expression of nanoparticle when it binds with $C_{10}T_{20}$, regardless of whether it is located at the topmost or inner layer of complex surface.

Table 1
Particle sizes and surface charges of PAA-HIS/DNA/oligonucleotide complexes with different constitution of oligonucleotides at 3 of oligonucleotide/PAA-HIS molar ratio.

Polymer complex	Particle size (nm)	Surface charge (mV)
PAA-HIS/DNA/C_{30}	1496.6 ± 489.7	27.3 ± 0.9
PAA-HIS/DNA/A_{30}	82.4 ± 27.3	−21.4 ± 0.2
PAA-HIS/DNA/T_{30}	98.07 ± 42.6	−20.9 ± 7.66

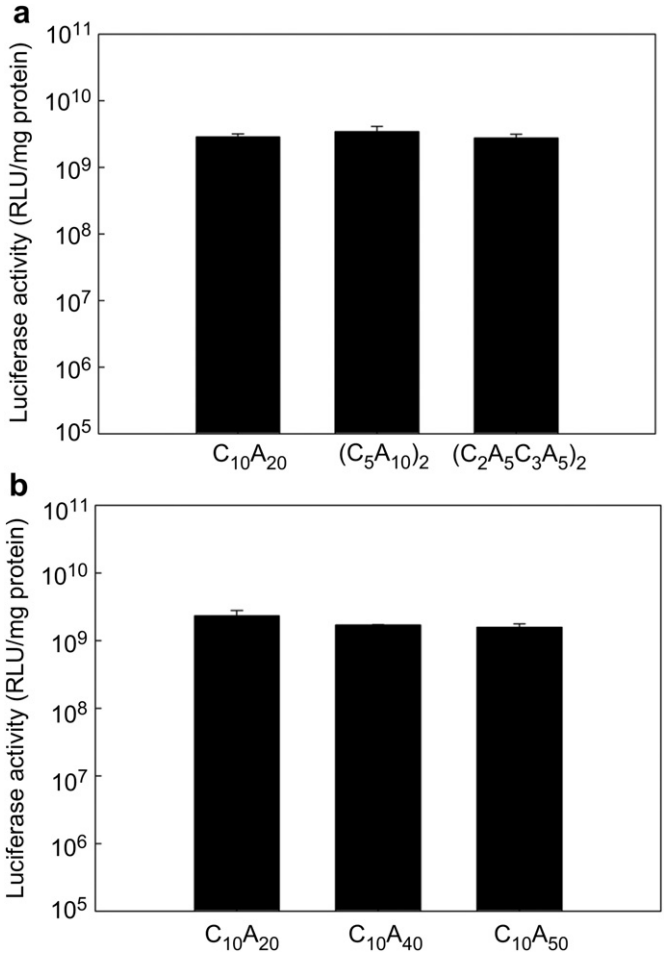

Fig. 2. Effect of (a) sequence and (b) molecular weight of oligonucleotides. Transfection into HeLa cells incubated with various oligonucleotide-coated PAA-HIS/DNA complexes at 3 of oligonucleotide/PAA-HIS molar ratio for 24 h. The cells were analyzed for luciferase activity. Asterisk (*) denotes significant differences ($p < 0.05$) compared with PAA-HIS/DNA/$C_{10}A_{20}$ complexes.

3.4. Inhibition effect of uncomplexed oligonucleotides

To investigate the inhibition effect of uncomplexed oligonucleotides, HeLa cells were pre-incubated with free $C_{10}A_{20}$ oligonucleotides prior to the addition of $C_{10}A_{20}$ oligonucleotide-coated PAA-HIS/DNA complexes to the cells. Fig. 5(a) shows free $C_{10}A_{20}$ oligonucleotides inhibiting the transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes in a dose dependent manner and its effect was significant when the concentration of $C_{10}A_{20}$ greater than 0.6 μM ($p < 0.05$). Furthermore, Fig. 5(b) shows that the transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was either decreased with increase in the pretreated time ($p < 0.05$). These results imply the interaction between uncomplexed $C_{10}A_{20}$ oligonucleotides and HeLa cells would obstruct the introduction of plasmid DNA of PAA-HIS/DNA/$C_{10}A_{20}$ complexes into cells and further transfection efficiency.

3.5. Inhibition effect of nucleotides and 8-SPT

Adenosine receptors, a G-protein coupled receptor on cellular membrane, have been demonstrated to interact with different agonists and antagonist ligands [17,18]. Thus, it is reasonable to

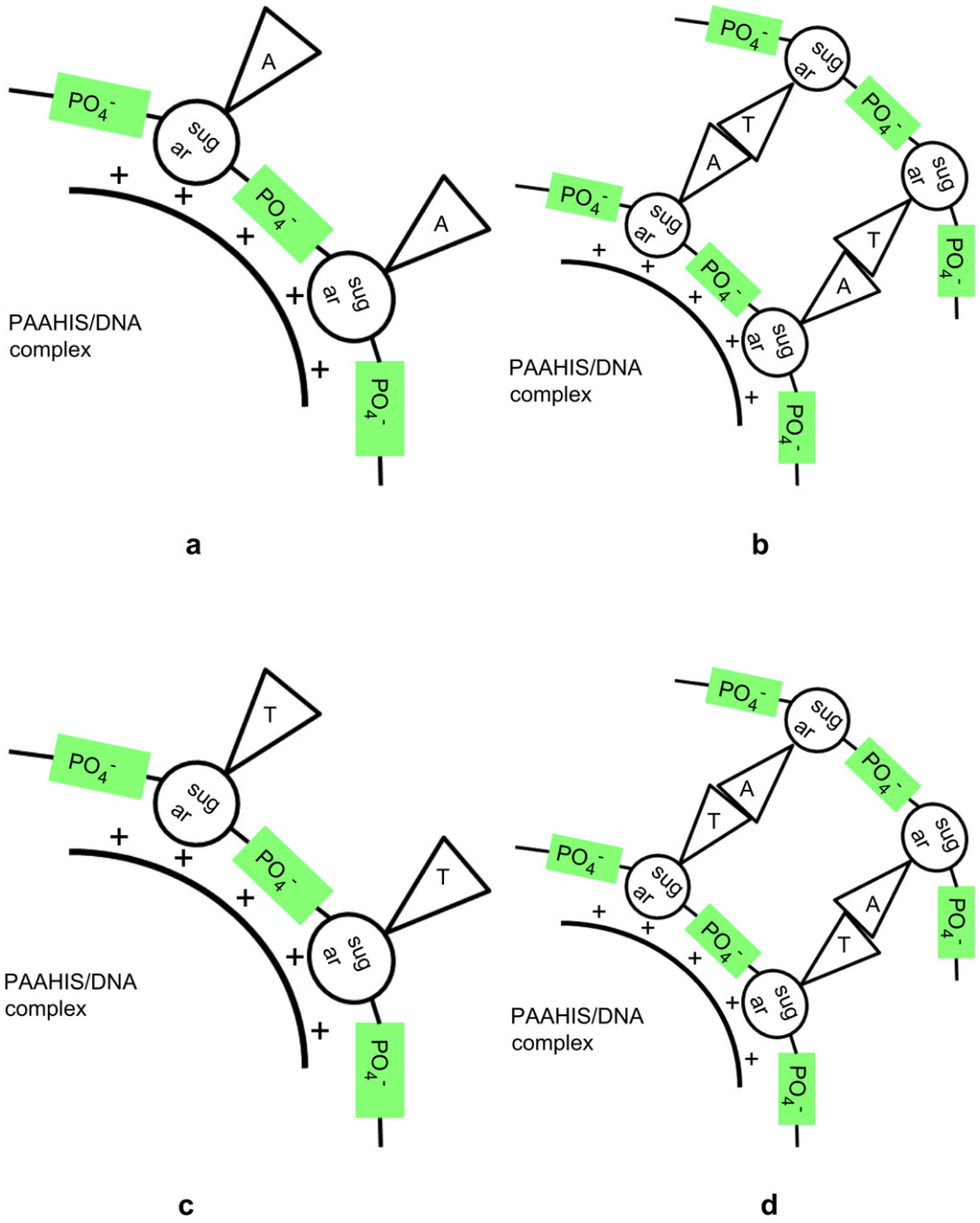

Fig. 3. Schematic representation of the structure of ternary and quaternary complexes. (a) PAA-HIS/DNA/$C_{10}A_{20}$, (b) PAA-HIS/DNA/$C_{10}A_{20}$/$C_{10}T_{20}$, (c) PAA-HIS/DNA/$C_{10}T_{20}$, and (d) PAA-HIS/DNA/$C_{10}T_{20}$/$C_{10}A_{20}$ complexes.

assume the transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes could be reduced when the receptor was blocked. Pretreatment of HeLa cells with adenosine and 8-SPT was performed for the inhibition study. Fig. 6(a) shows adenosine reducing the luciferase activity of the PAA-HIS/DNA/$C_{10}A_{20}$ complexes in a concentration-dependent manner. The transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes at 1000 μM of adenosine was greatly reduced to 7 ± 0.2% relative to its initial level in the absence of adenosine. Also, pretreatment of HeLa cells with 8-SPT, an adenosine receptor antagonist, significantly reduced the transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes by 80% (Fig. 6(b)). Since no significant toxicity was observed (data not shown), the decrease of transfection efficiency was attributed to the inhibition effect of 8-SPT. Thus, the introduction of PAA-HIS/DNA/$C_{10}A_{20}$ complexes into HeLa cells could be effectively inhibited by blocking adenosine receptors.

3.6. Inhibition effect of hypothermia

Generally, positively charged complexes reduce their gene expression at lower temperature. Similarly, it was found that the gene expression of negatively charged PAA-HIS/DNA/$C_{10}A_{20}$ complexes at 4 °C was only 36 ± 7% as compared to transfection efficiency at 37 °C. This indicates the effect of hypothermia inhibition and oligonucleotide-coated PAA-HIS/DNA complexes mediated by energy-dependent uptake processes.

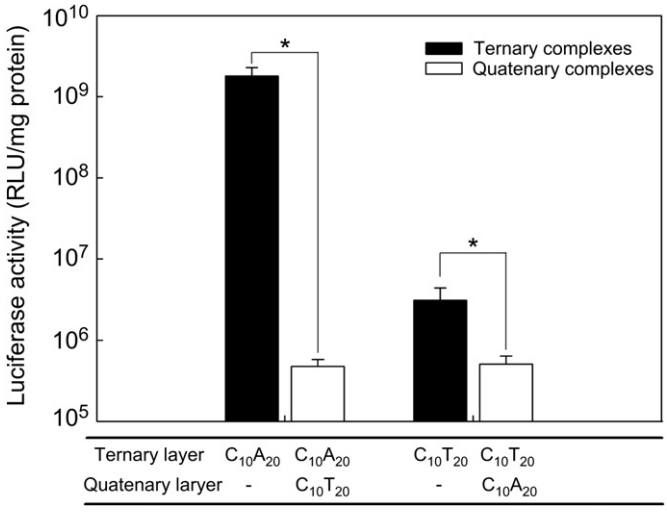

Fig. 4. Shielding effect of complementary oligonucleotides. Transfection into HeLa cells incubated with ternary and quaternary complexes (see Fig. 4) at 3 of oligonucleotide/PAA-HIS molar ratio for 24 h. The cells were analyzed for luciferase activity. Asterisk (*) denotes significant differences ($p < 0.05$) between ternary and quaternary complexes.

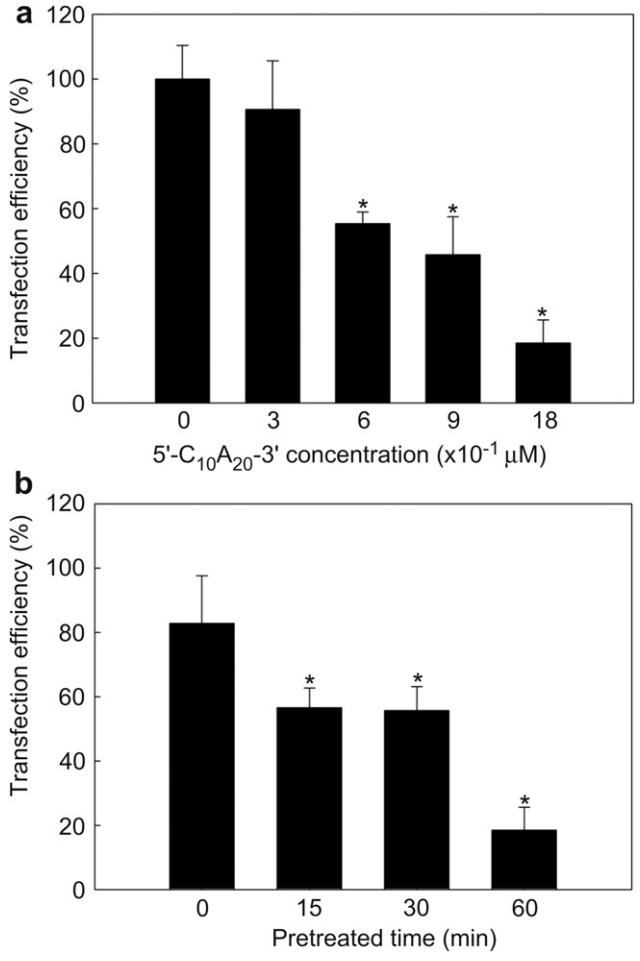

Fig. 5. Inhibition effect of uncomplexed oligonucleotides. (a) Transfection of PAA-HIS/DNA/$C_{10}A_{20}$ complexes into HeLa cells pre-incubated with free $C_{10}A_{20}$ at different concentrations for 60 min. (b) Transfection of PAA-HIS/DNA/$C_{10}A_{20}$ into HeLa cells pre-incubated with 1.8 μM of $C_{10}A_{20}$ at different treating times. The cells were analyzed for luciferase activity. Asterisk (*) denotes significant differences ($p < 0.05$) compared with the cells without pretreatment.

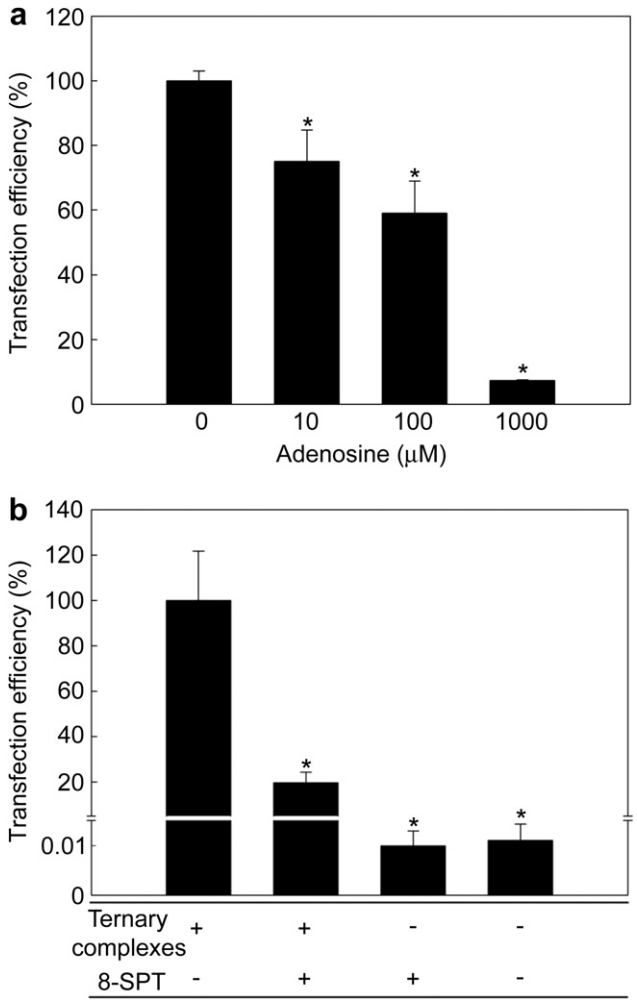

Fig. 6. Inhibition effect of adenosine and 8-SPT. Transfection of PAA-HIS/DNA/$C_{10}A_{20}$ complexes into HeLa cells pre-incubated with (a) different adenosine concentrations and (b) 1 mM of 8-SPT for 60 min. The cells were analyzed for luciferase activity. Asterisk (*) denotes significant differences ($p < 0.05$) compared with the cells without pretreatment.

3.7. Effect of endocytosis inhibitors on uptake of oligonucleotide-coated PAA-HIS/DNA complexes into HeLa cells

To visualize the uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes in the absence and presence of endocytosis inhibitors, HeLa cells were incubated with the prepared complexes containing YOYO-1-labeled pDNA, followed by washing and analysis by flow cytometry (Fig. 7). Simultaneously cell viability assay was measured to confirm not toxic inhibitor concentrations. As shown in Fig. 8(a), chlorpromazine, genistein and wortmannin used at concentrations were not cytotoxic in HeLa cells, in which all cell viability were more than 80% compared to untreated cells. Fig. 7 shows uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was only inhibited to a greater extent by caveolar inhibitor (genistein), which decreased the uptake to 49%. Conversely, inhibition of clathrin-mediated uptake by chlorpromazine almost did not reduce the uptake of oligonucleotide-coated PAA-HIS/DNA complexes. For inhibition of macropinocytosis by wortmannin, uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was reduced by 8%. These results indicate ternary oligonucleotide-coated PAA-HIS/DNA complexes were predominantly internalized by caveolae-mediated endocytosis.

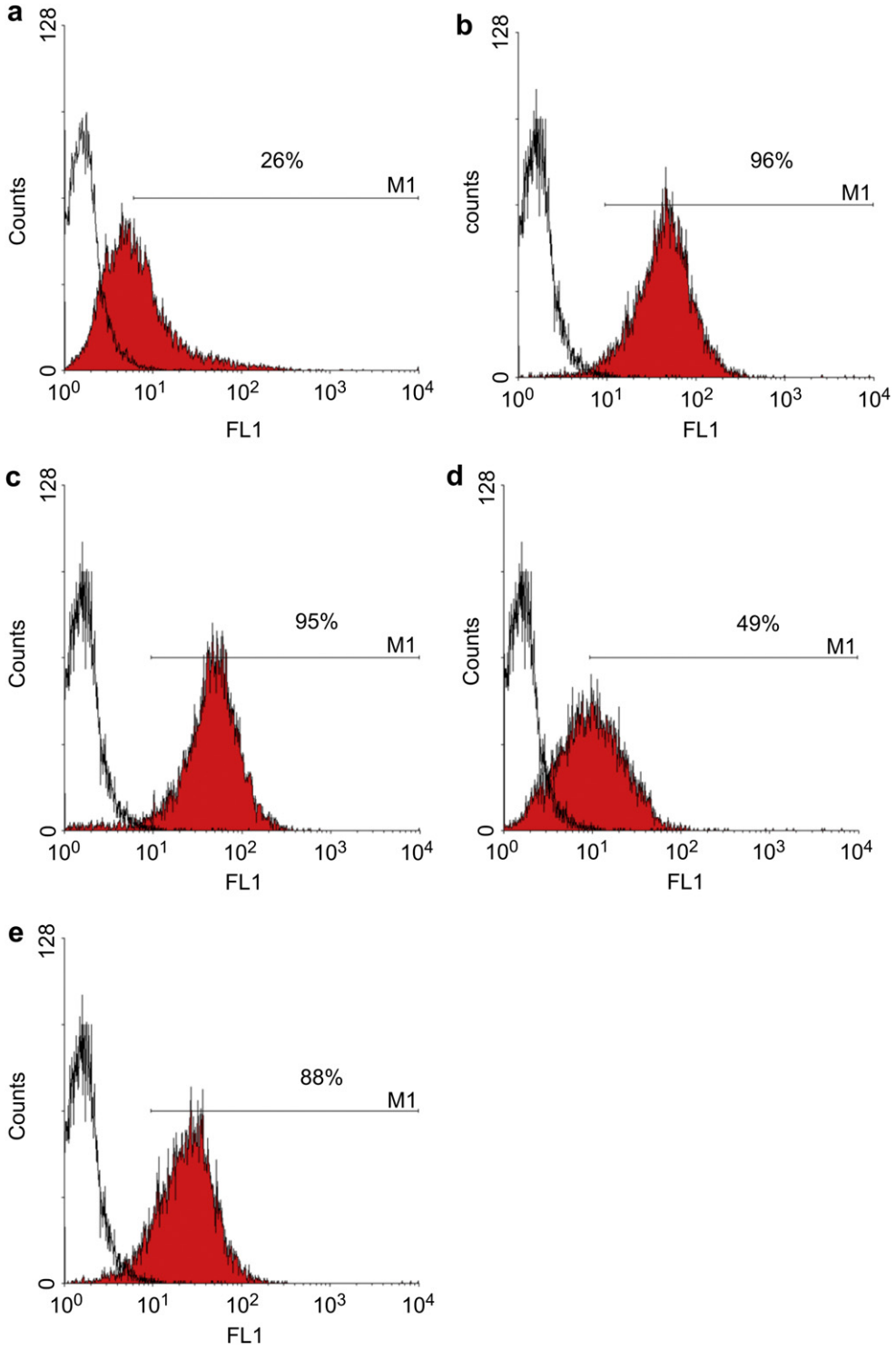

Fig. 7. Effect of endocytosis inhibitors on cellular uptake. HeLa cells were pre-incubated with various inhibitors for 1 h and then co-incubated with the prepared complexes containing YOYO-1-labeled pDNA, followed by washing and analysis by flow cytometry. Uptake of (a) PAA-HIS/DNA complexes without endocytosis inhibitor, (b) PAA-HIS/DNA/$C_{10}A_{20}$ complexes without endocytosis inhibitor, (c) PAA-HIS/DNA/$C_{10}A_{20}$ complexes with 10 μM of chlorpromazine, (d) with 200 μM of genistein, and (e) with 10 nM of wortmannin.

3.8. Effect of endocytosis inhibitors on transfection efficiency of oligonucleotide-coated PAA-HIS/DNA complexes

Finally, transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes in HeLa cells with the addition of endocytosis inhibitors was investigated. In the presence of genistein and wortmannin, transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was significantly reduced by 96 ± 2% and 66 ± 10% relative to inhibitor-free conditions ($p < 0.05$), respectively, whereas in the presence of chlorpromazine, transfection efficiency was not reduced significantly (Fig. 9(a)). Furthermore, transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was analyzed in the presence of double

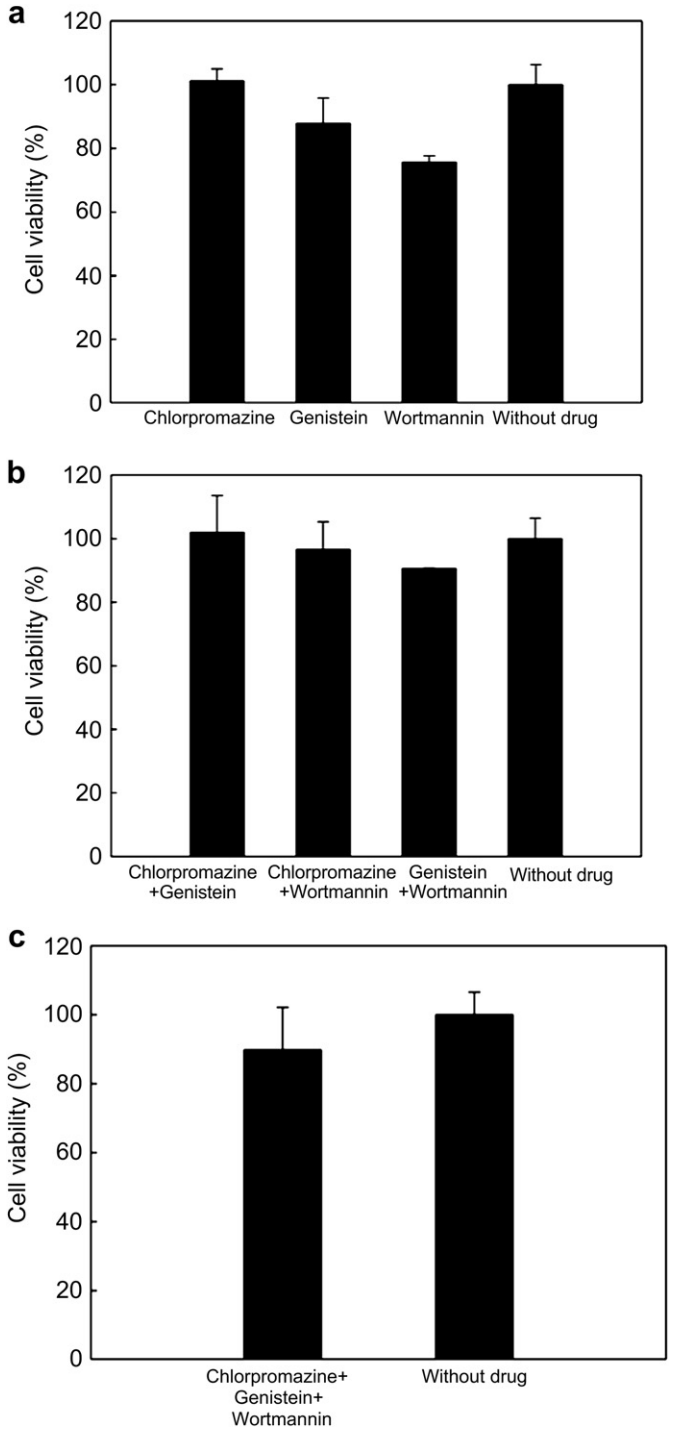

Fig. 8. Cytotoxicity assay of endocytosis inhibitors. HeLa cells were incubated with (a) single, (b) double, and (c) triple endocytosis inhibitors for 3 h. The cell viability was determined by MTT assay. The results are expressed as percentage of cell viability relative to untreated cells. Asterisk (*) denotes significant differences ($p < 0.05$) compared with the cells without inhibitor treatment.

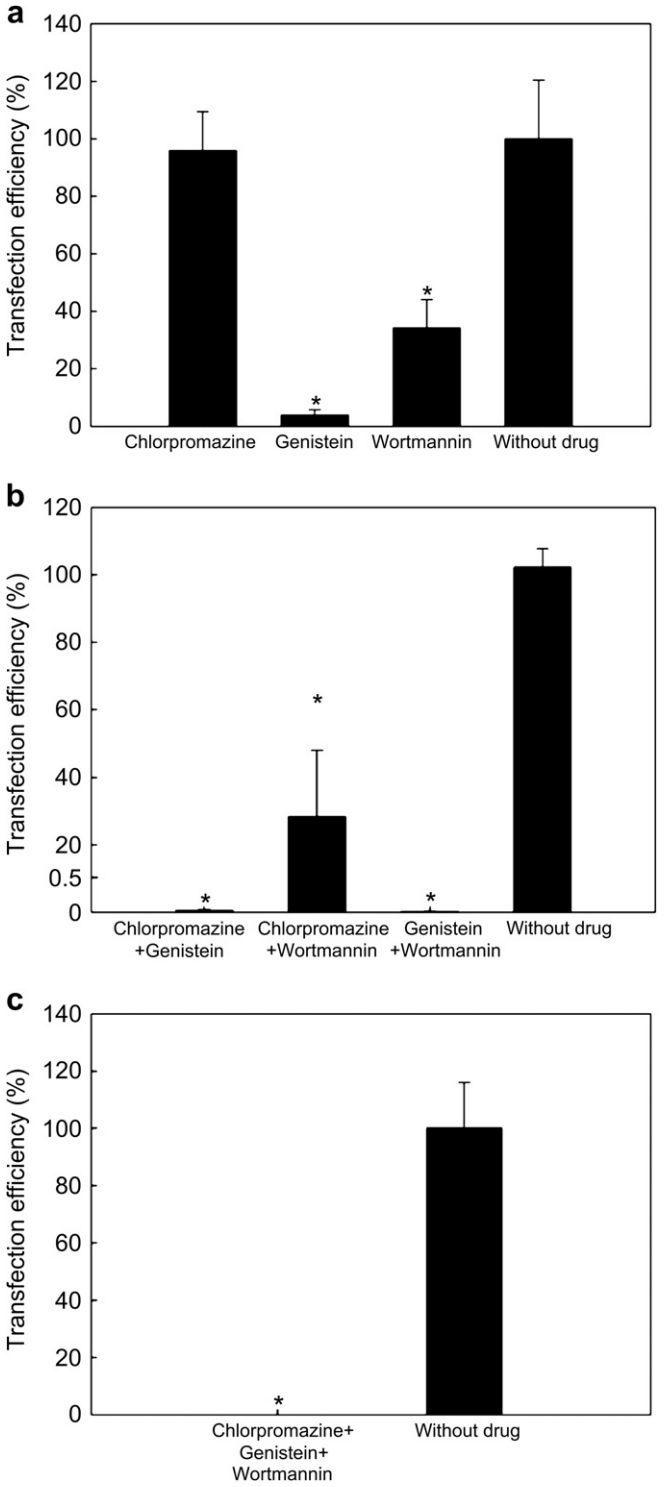

Fig. 9. Effect of endocytosis inhibitors on transfection efficiency. HeLa cells were pre-incubated with (a) single, (b) double, and (c) triple endocytosis inhibitors for 1 h and then co-incubated with PAA-HIS/DNA/$C_{10}A_{20}$ complexes. The cells were analyzed for luciferase activity. Asterisk (*) denotes significant differences ($p < 0.05$) compared with the cells without endocytosis inhibitor.

and triple inhibitors (Fig. 9(b) and (c)). At this time, cell viability in the presence of double and triple inhibitors was still above 80% (Fig. 8(b) and (c)). For double inhibitors with chlorpromazine and wortmannin, transfection efficiency of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was reduced to about 28% in comparison to that of control cells, which is similar to the effect of only wortmannin present in the medium. Therefore, macropinocytotic uptake mechanism could contribute the gene expression of oligonucleotide-coated PAA-HIS/DNA complexes in HeLa cells. However, regardless of double or triple inhibitors, gene expression of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was found to be less 1% when genistein was added into the medium. Since genistein almost completely block gene expression, caveolae-mediated endocytosis could be assigned as primary uptake

pathway for gene expression of oligonucleotide-coated PAA-HIS/DNA complexes.

Fig. 10 shows the gene expression of HeLa cells treated with PAA-HIS/DNA and PEI/DNA complexes with or without coating a $C_{10}A_{20}$ oligonucleotide layer in the presence of endocytosis inhibitors. Compared with uncoated binary PAA-HIS/DNA complexes, the additional $C_{10}A_{20}$ oligonucleotide layer completely did not modify the effect of endocytosis inhibitors on gene expression of HeLa cells. In terms of PEI/DNA/$C_{10}A_{20}$ complexes, inhibitor of caveolae-mediated endocytosis still drastically reduced gene expression of HeLa cells relative to inhibitor-free conditions, while it had no such deleterious effect on transfection efficiency of binary PEI/DNA complexes. Conversely, wortmannin reduced the gene delivery efficiency of uncoated PEI/DNA complexes to about 17 ± 3.3% in comparison to that of control cells, indicating oligonucleotide coated on the PEI/DNA complexes could change gene delivery pathway in HeLa cells.

4. Discussion

Gene therapy offers a potential method to treat diseases by transferring exogenous nucleic acids into cells to alter protein expression profiles. Several biological barriers met in the process of transfection must be overcome to achieve efficient non-viral gene delivery, which includes binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, transport into nucleus and enabling gene expression [4]. Therefore, the successful design of a non-viral gene delivery system requires a comprehensive understanding of the mechanisms involved in the interaction of the systems with the target cells. Generally, anionic complexes cannot be taken up by cells due to the electrostatic repulsion. A variety of polyamines such as PEI, PAA and PLL have been widely investigated to develop non-viral gene delivery vector for DNA delivery application [19,20]. However, it was found that the negatively charged oligonucleotide-coated PAA-HIS/DNA complexes could be entirely internalized by the living HeLa cells to exhibit high gene expression in our previous study [11]. As shown in Fig. 1, $C_{10}A_{20}$ oligonucleotide-coated PAA-HIS/DNA and PEI/DNA complexes exhibited higher gene expression *in vitro* and *in vivo* than their binary complexes. Therefore, the purpose of the study is to understand how oligonucleotide-coated PAA-HIS/DNA complexes bind and transfect cells.

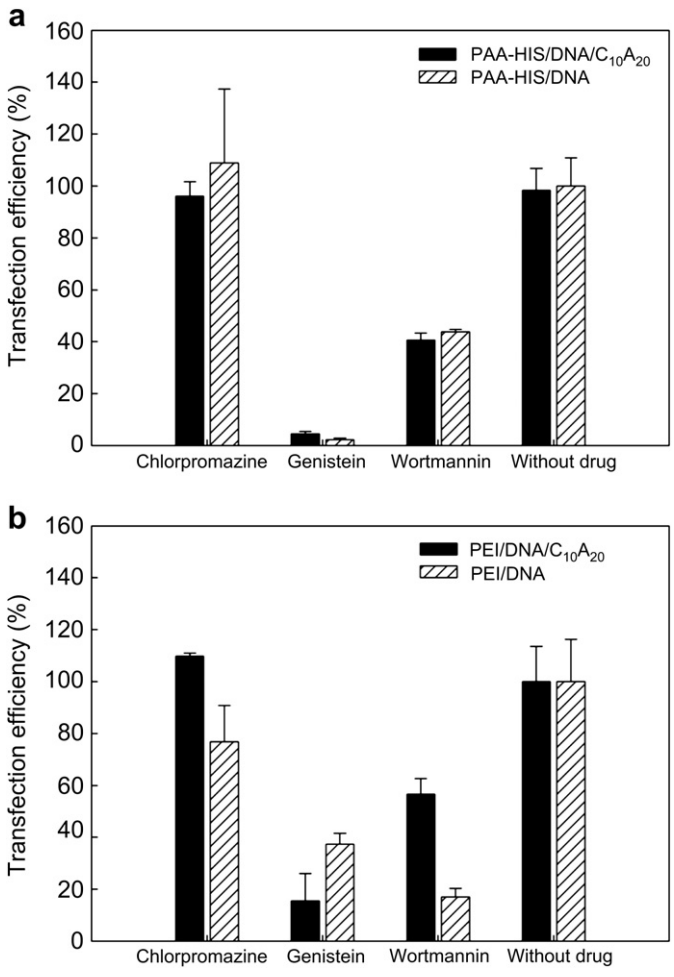

Fig. 10. Effect of endocytosis inhibitors on transfection efficiency. HeLa cells were pre-incubated with various inhibitors for 1 h and then co-incubated with (a) PAA-HIS/DNA/$C_{10}A_{20}$ or PAA-HIS/DNA complexes, and (b) PEI/DNA/$C_{10}A_{20}$ or PEI/DNA complexes. The cells were analyzed for luciferase activity.

At first, we investigated why $C_{10}A_{20}$, but not $C_{10}T_{20}$ oligonucleotide could be used to modify polycation/DNA complexes to enhance transfection efficiency [11]. Table 1 shows oligonucleotides with the simplest constituent were designed to prepare ternary complexes, including A_{30}, T_{30}, and C_{30}. It was found only A_{30} and T_{30} could be successfully coated on PAA-HIS/DNA complexes with nanosize and negative surface charge. Furthermore, deoxythymidine T_{30} oligonucleotide-coated PAA-HIS/DNA complexes only exhibited limited transfection efficiency, indicating deoxyadenosine constituent plays an important role in $C_{10}A_{20}$ oligonucleotide-coated PAA-HIS/DNA complexes with high transfection efficiency. In addition, Fig. 2 shows no significant difference in gene expression of HeLa cells was observed when the sequence ($C_{10}A_{20}$, $(C_5A_{10})_2$, $(C_2A_5C_3A_5)_2$) and molecular weight (($C_{10}A_{20}$, $C_{10}A_{40}$, $C_{10}A_{50}$)) of oligonucleotides were varied. These results confirm transfection efficiency of polycation/DNA complexes could be enhanced by the technique of assembling deoxyadenosine-containing oligonucleotides with binary polycation/DNA complexes.

Fig. 3 shows a layer of complementary oligonucleotide that was deposited on the ternary complexes (PAA-HIS/DNA/$C_{10}A_{20}$ and PAA-HIS/DNA/$C_{10}T_{20}$) to form quaternary complexes (PAA-HIS/DNA/$C_{10}A_{20}$/$C_{10}T_{20}$ and PAA-HIS/DNA/$C_{10}T_{20}$/$C_{10}A_{20}$). However, regardless of the site of $C_{10}A_{20}$, quaternary complexes could not achieve enhanced transfection efficiency (Fig. 4). This could be ascribed to the fact that nitrogenous bases of the oligonucleotide on both quaternary complex surfaces could not turn outside to contact cell surface directly when two complementary oligonucleotides were combined (Fig. 3). Previous studies have been reported that effective anionic complexes were transported into cells principally by ligand–receptor interaction [9,10]. Since the transfection efficiency of our ternary complexes were regulated by negatively-charged oligonucleotides, it is reasonable to assume oligonucleotide-coated PAA-HIS/DNA complexes ware also transferred into cells by ligand–receptor interaction. Therefore, during uptake of oligonucleotide-coated PAA-HIS/DNA complexes, adenosine cannot be shielded by its complementary thymidine to prevent the binding between adenosine and its receptor on the cell surface.

Subsequently, the inhibition effect on PAA-HIS/DNA/$C_{10}A_{20}$ complexes was observed when HeLa cells were pretreated with uncomplexed $C_{10}A_{20}$ oligonucleotide, free adenosine and adenosine receptor antagonist 8-SPT (Figs. 5 and 6). These results imply PAA-HIS/DNA/$C_{10}A_{20}$ complexes was in competition with uncomplexed $C_{10}A_{20}$ oligonucleotides or free adenosine to bind cells, and the introduction of PAA-HIS/DNA/$C_{10}A_{20}$ complexes into HeLa cells could be effectively inhibited by blocking adenosine receptor. Thus, the ligand ($C_{10}A_{20}$)-receptor interactions were present on HeLa cells. In addition, it is known receptor-mediated uptake is usually categorized to energy-dependent endocytosis [12]. PAA-HIS/DNA/$C_{10}A_{20}$ complexes were shown to enter HeLa cells by energy-dependent

mechanism as hypothermia reduced transfection efficiency by 64%. These experiments indicate that the plasmid DNA of oligonucleotide-coated PAA-HIS/DNA complexes was transferred into cells for further gene expression by adenosine receptor-mediated and energy-dependent process.

Several morphologically distinct endocytosis by energy-dependent pathway have been characterized, such as clathrin-mediated, caveolae-mediated endocytosis, and macropinocytosis [13–16]. To classify the intracellular fate of oligonucleotide-coated PAA-HIS/DNA complexes, inhibition of uptake and gene expression experiments were performed in the presence of optimized inhibitor concentrations of chlorpromazine (clathrin-mediated pathway), genistein (caveolae-mediated pathway), and wortmannin (macropinocytosis). Inhibition of either clathrin-mediated endocytosis or macropinocytosis did not affect the level of uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes, analyzed by flow cytometry (Fig. 7). Only genistein was able to significantly inhibit uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes into HeLa cells by about 50%. Similarly, transfection efficiency was unaffected when clathrin-mediated endocytosis was inhibited (Fig. 9). Thus, clathrin-mediated endocytosis of PAA-HIS/DNA/$C_{10}A_{20}$ complexes did not contribute to transfection. Surprisingly, although uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was hardly affected by macropinocytosis, gene expression of luciferase was greatly decreased by 66%. This suggests that wortmannin cannot inhibit cellular uptake, but cellular processing via macropinocytosis results in a reduction of gene expression.

On the other hand, the effect of genistein on blocking caveolae-mediated endocytosis resulted in an almost complete loss of oligonucleotide-mediated gene expression in HeLa cells, indicating that caveolar processing is the efficiency means of oligonucleotide-coated PAA-HIS/DNA complex trafficking in HeLa cells. Therefore, when uptake of PAA-HIS/DNA/$C_{10}A_{20}$ complexes was in the presence of genistein, regardless of double or triple inhibitors, gene expression of luciferase was decreased almost completely (Fig. 9). This further shows the importance of caveolae-mediated uptake for the rational design of oligonucleotide-coated gene delivery system. Thus, gene expression of binary cationic complexes and ternary oligonucleotide-coated PEI/DNA complexes might exhibit different dependency of inhibitors due to different combination of uptake and intracellular pathways (Fig. 10).

5. Conclusion

In summary, the “receptor” on the cell surface for oligonucleotide-mediated gene delivery is an adenosine receptor. Upon receptor/ligand binding, oligonucleotide-coated PAA-HIS/DNA complexes are internalized via caveolae-mediated pathway to result in effective intracellular processing for gene expression, showing a feasible approach to further develop new anionic gene delivery systems.

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