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# Effect of endocytosis inhibitors on gene transfection efficiency with Chitosan/Poly-L-arginine/DNA Complex in HeLa cells

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**Keywords:** polyarginine; gene transfer; chitosan; endocytosis; inhibitor

**Objectives:** To investigate the effect of endocytosis inhibitors on gene transfection efficiency of poly-L-arginie/DNA/chitosan (PLA/DNA/CS) complexes in human cervical carcinoma cells (HeLa cells).
**Methods:** Chitosan salt (CS; chitosan HCl, MW of 45 kDa and 87% degree of deacetylation) combined with poly-L-arginine (PLA, MW of > 70 kDa) was formulated. The ternary complexes (PLA, DNA and CS) at various weight ratios were formulated and characterized by gel electrophoresis. Their particle sizes and charges were measured using Zetasizer Nano ZS. The cell viability was evaluated by MTT assay. The effect of endocytosis inhibitors (ammonium chloride, filipin III, chlorpromazine, nocodazole, genistein and wortmannin) on enhanced green fluorescent protein (EGFP) gene delivery efficiency in human cervical carcinoma cells (HeLa cells) was then analysed. Polyethylenimine (PEI, 25 kDa) complexed with DNA (PEI/DNA) at the weight ratio of 1 were used as a positive control.
**Results:** The ternary complexes (PLA/DNA/CS) were completely formed at the weight ratio above 0.5. The mean size and zeta potential of these complexes were in the range of 200-260 nm and 30-35 mV, respectively. For cytotoxicity studies with MTT assay, the ternary complexes at various weight ratios showed an average cell viability of over 80%. However, the transfection efficiency of the ternary complexes was lower than that of PEI/DNA. When pre-incubated cells with each inhibitor for 30 min prior PLA/DNA/CS complexes treatment, both ammonium chloride and filipin III significantly increased gene transfection efficiency. On the contrary, other inhibitors caused the decrease in transfection efficiency. The results demonstrated the possibility of using an association of ammonium chloride or filipin III with PLA/CS as an attractive improved DNA delivery potential *in vitro*.
**Conclusion:** Ammonium chloride and filipin III might be preferable for the enhancement of gene transfer, while chlorpromazine, nocodazole, genistein and wortmannin may reduce the gene transfer with their endocytosis inhibitory properties.

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## Introduction

Chitosan (CS) is a polymer of N-acetyl-glucosamine and glucosamine, produced by alkaline deacetylation of chitin. CS is a nontoxic biodegradable polycationic polymer; therefore, it can be complexed with either negatively charged DNA[1]. CS/DNA complexes have been used to transfect various cell types. Moreover, poly-L-arginine is recently reported as a good gene carrier [2)]. The barriers for transgene trafficking to the nucleus are the cell membrane and cytopasmic compartment. Endocytosis is an active cellular uptake mechanism to internalize small molecules, macromolecules and particles[3-5]. Various forms of endocytosis have been demonstrated to be involved in the internalization of polymeric nanoparticles: clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis. Different route of entry strongly affected transfection efficiency of gene carriers. In order to further improve the transfection efficiency of cationic polymers, poly-L-arginine (PLA) mixed with CS polyplexes were investigated. We examined to find if chemical endocytosis inhibitors could affect the transfection efficiency. Understanding the physicochemical properties of the gene carriers in association with the uptake pathways can lead to rational design of nanoparticles that target pathways and improve the intracellular fate of the particles.

## Methods

***Ternary complexes formation*:** CS and pEGFP-C2 DNA were fixed at 4 and 1 µg, respectively, with various weight ratio of PLA MW > 70 KDa. The order of mixing was as follows: adding DNA solution to PLA solution and then added CS solution. The mixture was gently mixed by autopipette for a few seconds after that left for 30 min at room temperature.
***Agarose gel electrophoresis*:** The ternary complexe formation was analyzed by agarose gel electrophoresis (50 min at 100 V). Agarose gel was prepared with 1% agarose solution in TAE buffer with 0.5 µg/ml ethidium bromide.
***Particle size and zeta potential determination*:** The particle size and zeta potential of the complexes were measured using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature.

***In vitro transfection on HeLa cells:*** The day before transfection, HeLa cells were seeded into a 24-well plates at a density of 2 x $10^4$ cells/well in 1 ml of MEM medium containing : 1% L-Glutamine, 1% non-essential amino acids, 10% FBS. The cells were incubated overnight in an incubator at 37°C and 5% $CO_2$. On the day of transfection experiments, after media was removed and rinsed once with PBS, ternary complexes were added in 1 ml of serum free media and the cells were incubated with the complexes for 24 h at 37°C and 5% $CO_2$. Then, 1 ml of fresh complete medium was replaced, and the incubation was continued for further 24-48 h to allow for GFP expression. Non-treated cells and cells transfected with naked DNA and PEI/DNA were used as controls. All transfection experiments were performed in triplicate.

***Cell viability assay:*** Cytotoxicity evaluation was performed using MTT assay. The cells were seeded in a 96-well plate at a density of 8 x $10^3$ cells/well in 100 µl of growth medium and grown overnight, prior to incubation with PLA/DNA/CS growth medium was removed. 100 ml of ternary complexes at the same weight ratio as the transfection efficiency and PEI were applied onto the cell in culture medium without serum at concentrations and cell viability was compared to cells treated with culture medium without serum only, then incubated for 24 h. After 24 h, 100 ml of MTT (1 mg/ml in culture medium without serum) was added to each well and incubated for 4 h under normal growing conditions. Next, all media was removed and 100 ml DMSO was added. The formazen crystals were dissolved and then the absorbance was measured at 550 nm using a plate reader (A Packard BioScience Company). Viability of non-treated control cells was arbitrarily defined as 100%.

***Effect of various endocytosis inhibitors on in vitro transfection:*** Non-toxic concentration of endocytosis inhibitors was selected previously with MTT assay. Those were 5 µM chlorpromazine, 20 mM ammonium chloride, 5 µg/ml filipinIII, 20 nM wortmannin, 10 µM nocodazole and 50 µM genistein. Prior transfection on HeLa cells was carried out, cells were treated with each inhibitor for 30 min. Then transfection was performed as described above.

**Results**

***Characterization of ternary complexes of PLA/DNA/CS***: To determine the complete complexation of ternary complexes formed with fixed weight of DNA and CS at 1 and 0.01 µg, respectively, and varied weight of PLA ranging from 0 to 2 µg, 1% agarose gel electrophoresis were done. As shown in figure 1, the complete complexes were retained at the weight ratio above 0.5 (Lane 6).

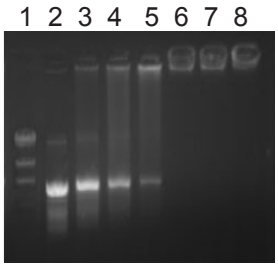


Figure 1. Gel retarding analysis of PLA/DNA/CS complexes with different weight ratio.
Lane 1 DNA marker; lane 2 pEGFP-C2 plasmid;
lanes 3-8 weight ratios 0.01, 0.05, 0.1, 0.5, 1 and 2, respectively.

Next, the ternary complexes were formulated using DNA and CS fixed at the weight of 1 and 4 µg, respectively, and varied weight PLA of PLA ranging from 0 to 4 µg. Then, the physical properties of those complexes including particle size and zeta potential that influence the gene transfer into cells were measured. As illustrated in figure 2, particle size (a) and zeta potential were represented against weight ratio of PLA to CS and DNA. The mean particle size of the complexes was in nano-range of 200-260 nm, and increased with increasing weight ratio from 1 to 4. Zeta potential value was positive and in the range of 30-35 mV.

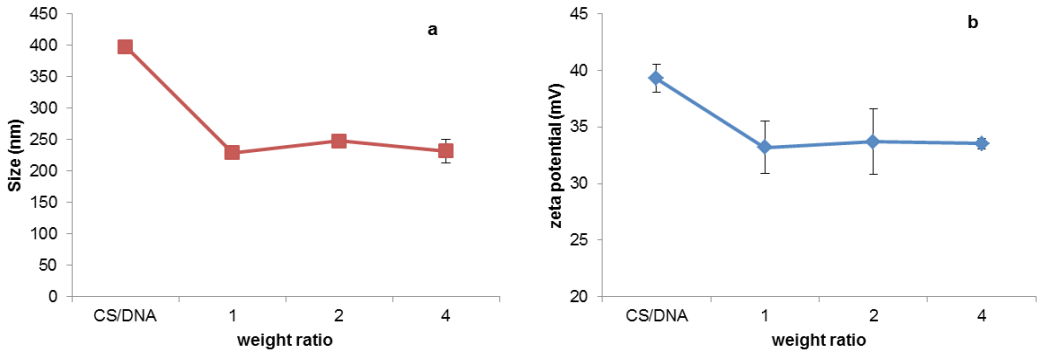


Figure 2. The particle sizes (a) and zeta-potential (b) of the ternary complexes with different weight ratio.

***Cytotoxicity and in vitro gene transfection of PLA/DNA/CS***: To evaluate the cytotoxicity of ternary complexes, HeLa cells were treated with complexes from PLA (at various weight of 1-4 µg)/ DNA (1 µg)/CS (4 µg) for 24 h. The viability of cells without treatment was used as a control. The mitochondrial activity of living cells was measured by the MTT assay.

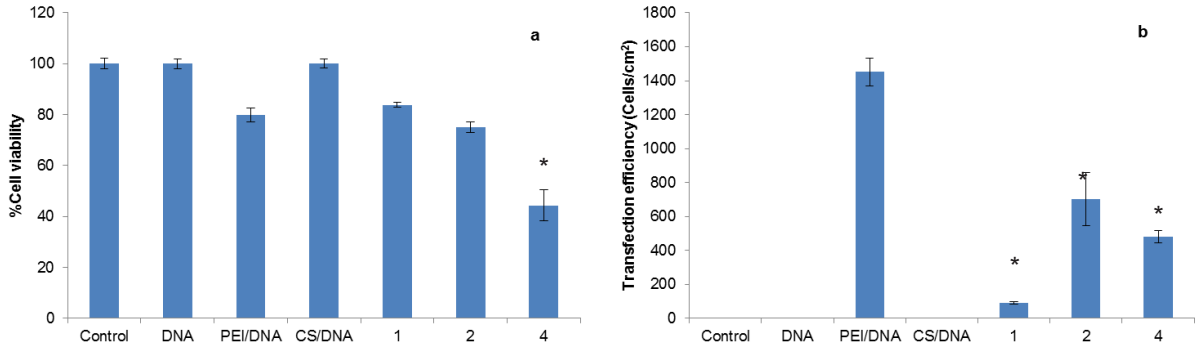


Figure 3. Effect of weight ratios on
(a) cell viability and
(b) transfection efficiency of PLA/DNA/CS complexes with various weight ratios of 1–4 in HeLa cells.
Values shown are the means±SD of triplicate experiments (* indicates $p \leq 0.05$). *, $p<0.05$ vs control

As shown in figure. 3a, there was a significant difference in mitochondrial activity observed between cells treated with PLA/DNA/CS at weight ratio of 4 and the negative control or positive control ($p<0.05$). However, no significant difference in cell viability was noticed between cells treated either with PLA/DNA/CS at weight ratio of 1 or 2 and the positive control ($p<0.05$). *In vitro* gene transfection was evaluated by transfected-cell counting using images obtained by fluorescence microscope and the transfected-cell was calculated. Polyethylenimine (PEI, 25 kDa) was used as a positive control. The results of gene transfection efficiency for PLA/DNA/CS are shown in Fig 3b. The difference of the transfection efficiency depended on the weight ratio, maximum transfection efficiency was found with PLA/DNA/CS at weight ratio of 2.

***The effect of endocytosis inhibitors on in vitro gene transfer mediated by PLA/DNA/CS***: After having determined cytotoxicity of different endocytosis inhibitors (data not shown), we chose non-toxic concentration of each inhibitor to elucidate their effect on transfection efficiency in HeLa cells. Transfection of HeLa cells by PLA/DNA/CS complexes was affected by clathrin-mediated endocytosis inhibitor chlorpromazine, caveolae inhibitor genistein and PI-3 kinase inhibitor wortmanin. Gene transfer efficacy was almost completely blocked by microtubule-disrupting agent nocodazole. By contrast, endosome acidification inhibitor ammonium chloride and cholesterol-dependent endocytosis inhibitor filipin III rather augmented the gene transfer (figure 4). Both ammonium chloride and filipin III significantly increased transfection efficiency about 3.0 and 2.0-fold ($p < 0.05$), respectively.

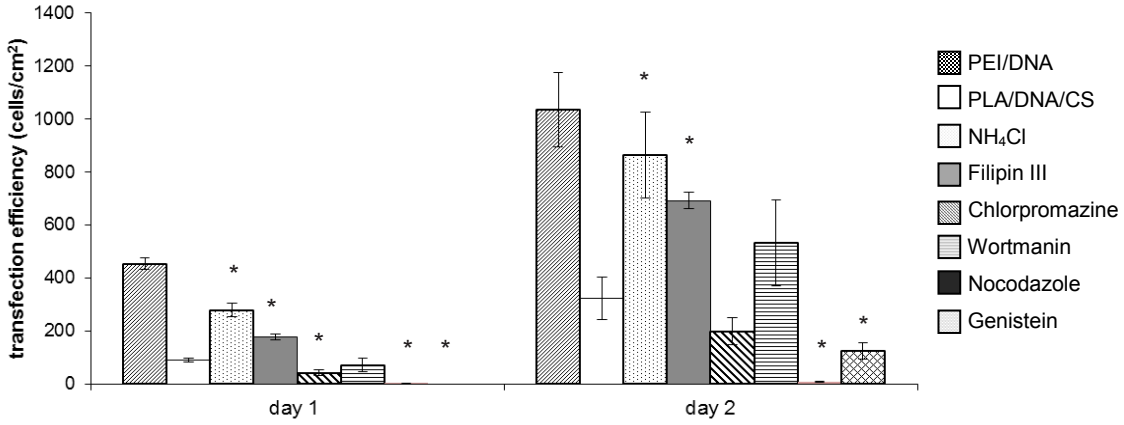


Figure 4. Effect of various endocytosis inhibitors on transfection efficiency.
Values shown are the means±SD of triplicate experiments (* indicates $p \leq 0.05$). *, $p<0.05$ vs control

## Discussion

In non-viral gene delivery, cellular uptake of the DNA nanoparticles can influence the overall gene expression. Successfully formed nanosized PLA/DNA/CS complexes were used as a good gene carrier comparing with PEI. PLA has been known to be an efficient gene transfer agent [2]. Even though transfection efficiency mediated with PLA/DNA/CS was not higher than that with PEI, it showed an impressive result. PLA can improve the gene transfer efficacy that mediates by CS/DNA complexes [6]. Moreover, we evaluated whether endocytosis inhibitors influence the transfection efficiency of PLA/DNA/CS. Clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis are the most important uptake pathways in mammalian cells [7]. Treatment with inhibitors, for example genistein, filipin III, chlorpromazine, wortmannin, and nocodazole are commonly used to evaluate mechanism uptake pathway of nanoparticles [3-5]. The results from the determination of endocytosis inhibitors affecting on transfection efficiency indicated that PLA/DNA/CS

may uptake via all major endocytosis pathway except for the pathway that needs cholesterol for endocytosis since filipin III can bind cholesterol in plasma membrane and lead to recycling defect. Furthermore, higher transfection level from the treatment of cells with ammonium chloride may cause by the prevention of endosome acidification. That may let the complexes trafficking to nucleus more [8]. Taken together, it could be said that ammonium chloride or filipin III might be potential as an attractive improved DNA delivery agent *in vitro*.

**Conclusion**

Our findings provide evidence that ammonium chloride and filipin III might be preferable for the enhancement of gene transfer, while chlorpromazine, nocodazole, genistein and wortmannin may reduce the gene transfer with their endocytosis inhibitory properties.

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**References**

1. MacLaughlin FC, Mumper RJ, Wang J, Tagliaferri JM, Gill I, Hinchcliffe M, Rolland AP. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J Control Release. 1998;56(1-3):259-72.
2. Hashimoto T, Mahara A, Kobori A, Murakami A, Yamaoka T. Various properties of polymeric carriers improved the transfection efficiency. Nucleic Acids Symp Ser (Oxf). 2006;50:333-4.
3. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. J Control Release. 2010;145(3):182-195. doi:10.1016/j.jconrel.2010.01.036.
4. Oh N, Park J-H. Endocytosis and exocytosis of nanoparticles in mammalian cells. Int J Nanomedicine. 2014;9(Suppl 1):51-63. doi:10.2147/IJN.S26592.
5. Cleal K, He L, Watson PD, Jones AT. Endocytosis, intracellular traffic and fate of cell penetrating peptide based conjugates and nanoparticles. Curr Pharm Des. 2013;19(16):2878-94.
6. Weecharangsan W, Opanasopit P, Ngawhirunpat T, Apirakaramwong A, Rojanarata T, Ruktanonchai U, Lee RJ. Evaluation of chitosan salts as non-viral gene vectors in CHO-K1 cells. Int J Pharm. 2008 Feb 4;348(1-2):161-8.
7. Conner SD, Schmid SL: Regulated portals of entry into the cell. Nature 2003;422:37-44.
8. Gillespie EJ, Ho CL, Balaji K, Clemens DL, Deng G, Wang YE, et al. Selective inhibitor of endosomal trafficking pathways exploited by multiple toxins and viruses. Proc Natl Acad Sci U S A. 2013;110(50):E4904-12. doi: 10.1073/pnas.1302334110.