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Investigation of gene transferring efficacy through nano-polyplex consisting of methylated *N*-(4-pyridinylmethyl) chitosan chloride and poly(ethylenimine) in human cell lines

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ABSTRACT

In this study, gene transfection efficiencies using nano-polyplex as gene carriers were investigated in a variety of human cell lines. Gene carriers were prepared using cationic polymer methylated *N*-(4-pyridinylmethyl) chitosan chloride (MPyMeChC) and/or poly(ethylenimine) (PEI) to form polyplex via electrostatic interaction with 1 μg of plasmid pGL-3-basic containing CMV promoter/enhancer at different weight ratios. Polyplex formations were confirmed by gel retardation assay. The result suggested that packaging of DNA in PEI-containing polyplexes occurred without interference of DNA integrity. Both size and zeta-potential were increased when PEI was incorporated in polyplex. *In vitro* transfection was performed in three different cell lines which are a human cervical cancer cell line (HeLa), a human lung adenocarcinoma epithelial cell line (A549) and a human neuroblastoma cell line (SH-SY5Y). The results revealed that transfection profiles were different among the three cell lines which indicated that transfection efficiency through MPyMeChC/PEI polyplex was cell-type dependent. A synergistic effect of MPyMeChC/PEI polyplex was found in HeLa cells with the maximum transfection efficiency at a weight ratio of 1/0.5/1 providing 95% cell viability, approximately. Our study demonstrated an alternative carrier in a non-viral gene delivery system which is able to promote high gene delivery efficiency with low cytotoxicity in human cell lines.

1. Introduction

Gene therapy has been considered as a promising approach for treating genetic defective diseases by delivering therapeutic genes to diseased cells (Tatum, 1966). However, the use of gene therapy in the clinical application has been limited due to the low ability in gene transferring efficiency and specificity, gene expression regulation and immunogenic response. In clinical trials, the dangers of using a viral vector as a gene carrier have been pointed out to be the cause of adverse effects on the immune system (Verma & Somia, 1997). Therefore, the use of a viral vector in gene therapy is of concern. Moreover, viral vectors are limited in size of the plasmid to be delivered. Recently, non-viral gene delivery has gained much attention as an alternative choice for a gene delivery system (Wong, Pelet, & Putnam, 2007). Polymeric vectors are regarded as safe, low immune response, inexpensive, easy to manipulate chemically, scalable, and stable upon storage (Anderson, 1998; Li & Huang, 2000). Therefore, several on-going studies about non-viral vectors have been intensively implemented to enhance gene delivery ability while addressing safety concerns.

A number of cationic polymers including poly(ethylenimine) (PEI), polylactic acid (PLA) and chitosan (Ch) have been verified for their potential uses as polymeric gene carriers (Kasturi, Sachaphibulkij, & Roy, 2005; Lee, 2007; Munier, Messai, Delair, Verrier, & Ataman-Onal, 2005; Wong et al., 2007). These polymers have the ability to neutralize negatively charged DNA to prevent repulsion against the anionic cell surface. Furthermore, the cationic polymers are able to condense DNA into a relatively small size which is crucial for cell internalization (Lv, Zhang, Wang, Cui, & Yan, 2006) and, simultaneously, DNA is protected from nucleus degradation (Lechardeur et al., 1999; Schaffer & Lauffenburger, 1998). Among cationic polymers, Ch and PEI are very well-known due to their potential in gene delivery applications. Ch is a natural polymer considered to be biocompatible, biodegradable and safe for medical use in humans (Shi et al., 2006). PEI is considered as the most effective cationic polymer due to its buffering capacity via its proton sponge effect helping DNA to escape from the endosome

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(Boussif et al., 1995). However, PEI is also known as a high cytotoxic transfection agent which is the major obstacle to applying PEI in human gene therapy (Kunath et al., 2003). Recently, a number of reports have demonstrated gene transferring using hybrid polymers of Ch and PEI (Jiang et al., 2007; Kim, Kim, Akaike, & Cho, 2005; Wong et al., 2006; Zhao et al., 2008). Hybrid polymer systems, both Ch/PEI-blending and -grafting were described for efficient gene delivery (Jiang et al., 2008). Hypothetically, the combination of Ch/PEI would synergistically exert their effects to diminish the drawback of each polymer in gene transfection.

Ch is recognized as a biodegradable polymer with biocompatibility, low cytotoxicity and low immunogenicity. Therefore, Ch derivatives have been continuously synthesized for many applications in medical uses especially for regenerative medicines including gene therapy (Shi et al., 2006; Varshosaz, 2007). Regarding our previous study (Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009), a methylated *N*-(4-pyridinylmethyl) chitosan chloride (MPyMeChC) was successfully synthesized with a degree of quaternization of 80% and molecular weight of 12 kDa. MPyMeChC was modified to improve water solubility and gene transfection efficiency of original Ch. *In vitro* transfection efficiency and cytotoxicity were previously determined in the human hepatoma cell (Huh7). The study revealed that MPyMeChC has the potential to be used as a gene carrier by forming a polymeric complex with DNA via electrostatic interaction resulting in polyplex with nano-sizes. Therefore, MPyMeChC was considered to be a novel carrier in a non-viral gene delivery system. However, the study remarked that transfection efficiency of MPyMeChC/DNA polyplex was low in HeLa. The results suggested that gene transfection via MPyMeChC was cell-type dependent due to the variation among cell types. The binding of nanoparticles and cell internalization may be facilitated or restricted by the cell membrane composition (Erbacher, Zou, Bettinger, Steffan, & Remy, 1998). The study results agreed well with previous studies reporting that transfection via Ch/DNA complex was cell-type dependent (Corsi, Chellat, Yahia, & Fernandes, 2003; Mao et al., 2001).

To improve gene transfection efficiency in a wide variety of cells, in this study, PEI was considered to be a supporting polymer used in combination with MPyMeChC. We aimed to investigate the role of MPyMeChC in the presence of PEI at different weight ratios for gene transfection in different human cell lines. Therefore, this study was conducted to investigate gene transfection efficacy of PEI-blended MPyMeChC polyplex. Different amounts of PEI were blended into the MPyMeChC/DNA complex to observe transfection results in three different human cell lines. Human cervical cancer cells (HeLa), a human lung adenocarcinoma epithelial cell line (A549) and the neuroblastoma cell line (SH-SY5Y) were selected for assessment of transfection efficiency.

2. Materials and methods

2.1. Materials

The chitosan, Ch, (Mw of 276 kDa) was purchased from Seafresh Chitosan Lab (Bangkok, Thailand). The branched poly(ethylenimine), PEI, (Mw of 25 kDa) was purchased from Aldrich (Milwaukee, USA). Plasmid pGL-3-basic containing CMV promoter/enhancer was used to monitor transfection efficiency (Tencomnao, Rakkhitawatthana, & Sukhontasing, 2008). The plasmid was propagated in *Escherichia coli* which was grown in Luria–Bertani broth (Molekula, UK) supplemented with ampicillin under shaking condition at 37 °C 250 rounds per minute. The plasmid was extracted using PureLink™ Hipure Plasmid DNA Purification (Invitrogen, USA). The extracted plasmid was observed by electrophoresis on 1.0% agarose gel. Then, plasmid purity and concentration were determined by measurement light absorbance at 260 and 280 nm using SpectraMax M2 microplate readers (MDS, Inc., Canada).

2.2. Preparation of a methylated N-(4-pyridinylmethyl) chitosan chloride (MPyMeChC)

N-(4-Pyridinylmethyl) chitosan (PyMeCh) were first prepared according to the previously reported procedure (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008). In brief, 1.0 g of Ch was dissolved in 70 mL of 1% (v/v) acetic acid. The solution was diluted with 70 mL of ethanol, and then 1.07 g of 4-pyridinecarboxaldehyde was added to the solution. The reaction mixture was stirred at 60 °C for 12 h. At this point the pH of the solution was adjusted to 5 by adding 15% (w/v) NaOH. Then, 1.54 g of $NaCNBH_3$ was added, and the resulting solution was stirred at room temperature for 24 h, followed by adjusting the pH to 7 with 15% (w/v) NaOH. The aqueous solution was dialyzed against de-ionized (DI) water for 3 days, followed by freeze drying. Finally, the PyMeCh were methylated by a single treatment with iodomethane, in the presence of sodium iodide, sodium hydroxide and *N*-methylpyrrolidone, yielding methylated *N*-(4-pyridinylmethyl) chitosan chloride (MPyMeChC) (Sajomsang et al., 2009).

2.3. Preparation of MPyMeChC/DNA and PEI/DNA polyplex

MPyMeChC and PEI were separately dispersed in sterile distilled water to prepare the stock solution at a final concentration of 1 mg/ml each. Different sets of polyplexes were prepared based on amount of used polymers. For complexes of MPyMeChC/DNA, w/w ratios of polymer per 1 μg DNA were 1/1, 5/1, 10/1 and 20/1. While for complex of PEI/DNA, w/w ratios were 0.5/1, 1/1, 5/1 and 10/1. The mixture of MPyMeChC or PEI with DNA were induced to form polyplexes through a self-assembly mechanism after pipetting, and subsequently incubated at room temperature for 15 min before use.

2.4. Preparation of MPyMeChC/PEI/DNA polyplex

For polyplex combined with both MPyMeChC and PEI, two different groups of complexes were prepared. The first group was prepared by constantly using 1 μg of PEI to generate polyplexes at weight ratios of MPyMeChC/PEI/DNA of 1/1/1, 5/1/1, 10/1/1 and 20/1/1. Another group was prepared by constantly using 1 μg of MPyMeChC yielding polyplexes at weight ratios of PEI/MPyMeChC/DNA of 0.5/1/1, 1/1/1, 5/1/1 and 10/1/1. For all mixture preparations, MPyMeChC and DNA were mixed together as above and followed by adding of PEI to coat the MPyMeChC/DNA surface by pipetting and incubation at room temperature for 15 min.

2.5. Size and zeta-potential measurement

The *Z*-average hydrodynamic diameter, polydispersity index (PDI) and surface charge of methylated Ch derivatives/DNA complexes were determined by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at room temperature. The complexes were prepared and combined to achieve 1 mL by DI water. All samples were measured in triplicate.

2.6. Atomic force microscopy analysis

The AFM images of polyplex were obtained by using a dynamic force microscope (DFM) mode (Seiko SPA4000, Japan). All samples were prepared by dropping the polyplex solution on a mica surface and left for air drying. All images were obtained with scanning speed 1.0 Hz over a 2 μm × 2 μm area.

2.7. Cell lines and cultivations

Three types of human cells were used for study in the gene transferring experiment. Human cervix epithelial carcinoma cells (HeLa) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA). Human neuroblastoma cells (SH-SY5Y) were maintained in a 1:1 mixture of Eagle's minimum essential medium (HyClone, USA) and Ham's F12 nutrient mixture (HyClone, USA) supplemented with 20% FBS and 1% non-essential amino acid (HyClone, USA). Human lung adenocarcinoma epithelial cells (A549) were grown in RPMI-1640 (HyClone, USA) containing 10% FBS. All cells were incubated at 37 °C and 5% CO_2–air for 24 h and periodically subcultured prior to transfection.

2.8. Gel retardation assay

The constant amount of plasmid 1 μg was incubated with different weight ratios of MPyMeChC or PEI for 15–30 min at room temperature. After the complexes were formed by self-assembly, loading dye was added and mixed to load into 1% agarose gel. Electrophoresis was carried out at 100 V for 60 min. Agarose gels were stained in 1 μg/ml of ethidium bromide. The presence of DNA was visible under UV transilluminator (Syngene, UK). The shifted bands corresponding to free plasmid were determined. Similar methods were applied to examine the gel retardation effect from the combination of MPyMeChC and PEI.

2.9. In vitro gene transfection and luciferase assay

For *in vitro* transfection, HeLa, A549 and SH-SY5Y cells were seeded in 96-well plate at densities of 3×10^5, 2×10^5 and 5×10^5 cells/well in 96-well plate. Prior to transfection, media was removed and rinsed with PBS twice then plated and incubated with serum-free DMEM. Cells were incubated with polyplex at 37 °C for 4 h in serum-free medium then replaced with growth medium. Twenty-four hours after transfection, luciferase activity was determined in accordance with the manufacture's recommendation (Promega, USA). Luciferase activity was quantified as relative light units (RLU) using a luciferase assay system (Promega, USA). Luciferase activity was normalized for protein concentration using the Bradford assay, and was measured to be controlled. Lipofectamine 2000™ (Invitrogen, USA) was used as a control carrier for gene transfection.

2.10. Evaluation of cytotoxicity

MTT assay was performed to evaluate cell viability after treating with polyplex. All cells were seeded as the same density applied for transfection. Cells were cultured at 37 °C under 5% CO_2 overnight. The assay was performed 24 h after transfection according to the manufacturer's recommendation. Percent cell viability was related to control untreated cells.

3. Results

3.1. Polyplexes formation

AFM analysis was employed to investigate polyplex formation including size and physical morphology. AFM images exhibited a spherical shape of polyplex in all studied ratios (Fig. 1). However, packing of DNA in MPyMeChC carrier was not densely formed at weight ratios 5 and 10 because groups of DNA strands appeared around particle surfaces (Fig. 1a and b). Particle forming was improved when 1 μg of PEI was supplemented (Fig. 1c and d). Particles exhibited more compact and round shapes. Morphology of MPyMeChC/DNA polyplex was further observed in the correlation with PEI at 0.5, 1 and 10 μg. AFM results indicated that the size of polyplex tended to increase according to the amount of PEI. This was probably due to the coating of PEI on MPyMeChC/DNA polyplex (Fig. 1e and f). This tendency corresponded well with size analyzed by nano-sizer (Fig. 2).

3.2. Size and zeta-potential analysis

The particle size and zeta-potential were determined at pH 7.4. The amounts of DNA were constantly kept at 1 μg to form complexes with MPyMeChC and/or PEI at different weight ratios. The polyplexes were prepared as described in Section 2. Every formulation was increased to 1 mL before measurement by zetasizer. The results indicated that polyplexes were formed as nano-sized particles which were expected to be a suitable gene carrier for transfection (Fig. 2). Sizes of PEI-blended complexes were generally larger than the sizes of non-blended ones. This may be the effect of the coating of PEI onto the MPyMeChC/DNA complex yielding larger and more spherical shapes. In the present study, the smallest polyplex obtained from MPyMeChC/DNA at w/w ratio of 5/1 was 191 nm in size. Zeta-potential measurement revealed that charge values of polyplexes ranged from −30 to 60 mV. Weight ratios of 0.5/1 and 1/1 of MPyMeChC/DNA complex resulted in a negative charge which implied that complexes were not completely formed and DNA strands were agglomerated around particle surfaces. This result suggests that addition of PEI was able to enhance the positive charge in responding to the amount of PEI until reaching the maximum capacity at 60 mV.

3.3. Gel retardation assay

A gel retardation assay was carried out to determine DNA binding affinity and stability of polyplex (Fig. 3). One microgram of plasmids was applied to form polyplexes with MPyMeChC and MPyMeChC/PEI at different weights. For the MPyMeChC/DNA polyplex (lanes 2–8), the results indicate that plasmid migrations were retarded along with the amount of supplemented polymers. The results suggest that plasmids were incorporated to form polyplexes via electrostatic interaction with MPyMeChC. Polyplexes were completely formed since weight ratio of MPyMeChC/DNA was 20/1. However, DNA integrity may be interfered with MPyMeChC because the trailing of DNA appeared in the gel.

In contrast, there was no free DNA remaining in the gel when 1 μg of PEI was added onto the MPyMeChC/DNA polyplex, suggesting that polyplex formation was completely formed (lanes 9–15). No DNA was detected from the gel because it was coated by PEI via electrostatic interaction. The obtained result agreed with zetasizer analysis which showed polyplexes with a positive zeta-potential (Fig. 2d).

3.4. Transfection efficiency and synergistic effect of combined polymers on DNA delivery into human cell lines

This study displayed the potential of MPyMeChC/PEI to be used as a transfection reagent in mammalian cells. To broaden the use of MPyMeChC in a variety of cells, therefore, this experiment selected a human cervical cancer cell line (HeLa), a human lung adenocarcinoma epithelial cell line (A549) and a human neuroblastoma cell line (SH-SY5Y) to investigate the *in vitro* transfection profile of MPyMeChC, PEI and MPyMeChC/PEI polyplexes at different weight ratios. The experiment was performed with 1 μg of plasmid DNA mixed with MPyMeChC and/or PEI at different weight ratios described in Section 2. To quantify transfection effectiveness, luciferases expressed in cells were deter-

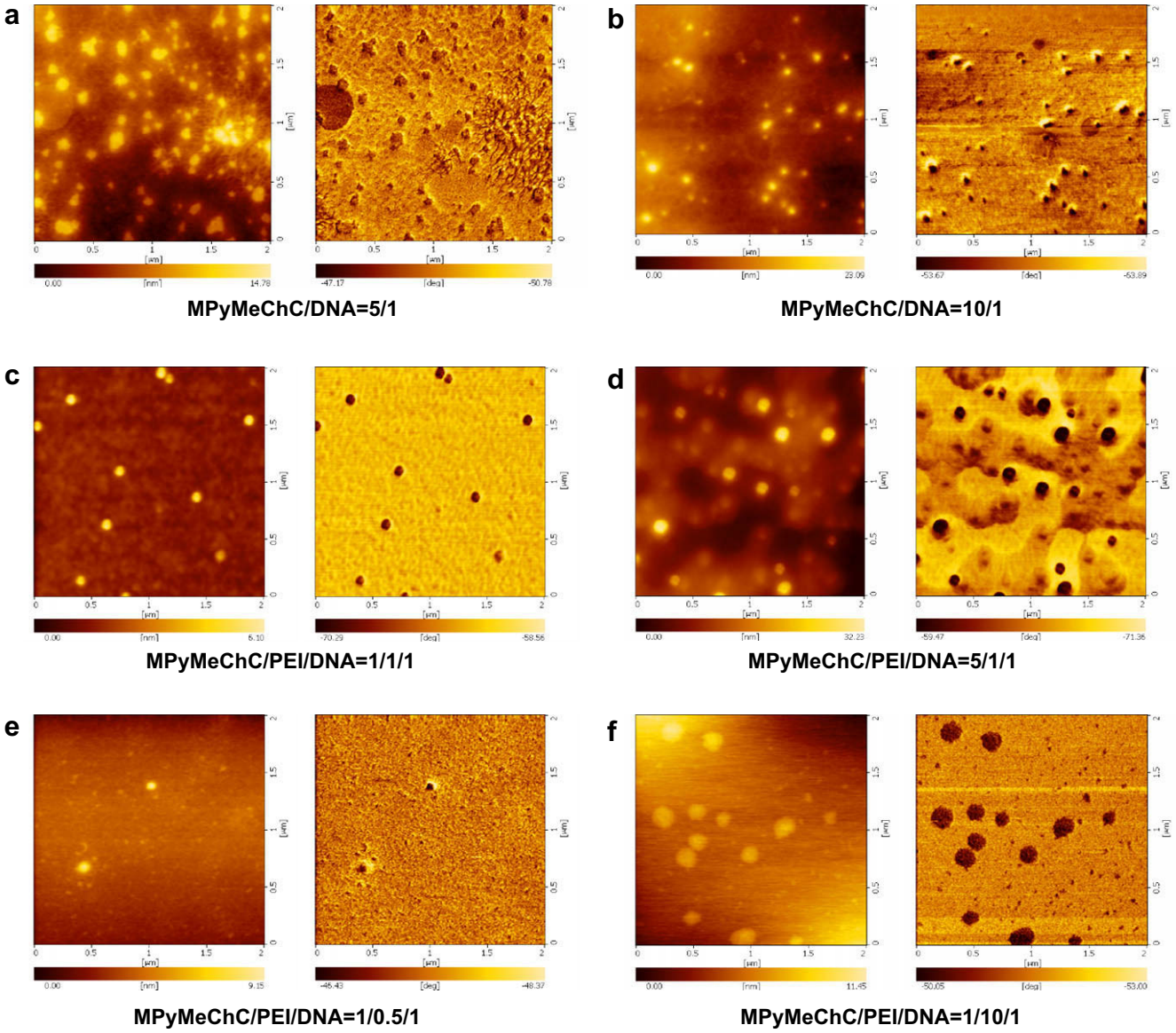

Fig. 1. Atomic force microscope (AFM) images topology (right) and phase contrast (left) of MPyMeChC forming complex with DNA at weight ratios 5/1 (a) and 10/1 (b), and MPyMeChC/PEI forming complex with DNA at weight ratios 1/1/1 (c), 5/1/1 (d), 1/0.5/1 (e) and 1/10/1 (f).

mined. Cells incubated with naked DNA were performed to be controls. The results reveal that transfection profiles in three types of cell lines were different (Fig. 4). MPyMeChC/DNA polyplexes induced gene transfection with very low efficiency in HeLa cells (less than 10^3 RLU/mg protein, Fig. 4a). This result corresponds to our previous study which demonstrated that MPyMeChC was not a good transfection reagent for this cell line (Sajomsang et al., 2009). This study also revealed that increasing the amount of MPyMeChC did not significantly impact on transfection ability. However, a synergistic activity of MPyMeChC and PEI clearly occurred in HeLa cells because transfection ability was significantly improved when 1 μg of PEI was added into MPyMeChC/DNA polyplexes. Supplementary PEI clearly promoted *in vitro* transfection efficacy of MPyMeChC/DNA in the range of 10^2- to 10^3-fold compared without addition of PEI. Transfection efficiency was highest at the weight ratio of MPyMeChC/DNA/PEI of 1/1/1. However, increasing the amount of MPyMeChC in a combination system resulted in slightly decreased gene transferring capability.

In order to optimize the amount of PEI applied to use in this polymer-blending system, we continued to analyze transfection results by constantly holding w/w of MPyMeChC/DNA polyplex at 1/1. Polyplexes were titrated with different amounts of PEI varying from 0.5 to 10 μg. The complexes of PEI/DNA were carried out for comparison. The results of gene expression suggest that 0.5–1 μg of PEI could enhance up to 10^3-fold higher than cells incubated with naked DNA. Nevertheless, adding excess PEI resulted in low transfection efficiency due to cytotoxicity of PEI.

We continued to examine the transfection level in other mammalian cell lines. In A549, gene transfection via MPyMeChC induced luciferase signal up to 10^5 RLU/mg protein with optimal weight ratio of MPyMeChC/DNA at 5/1 (Fig. 4b). Increasing MPyMeChC did not enhance gene transferring ability. Adding of 1 μg of PEI into MPyMeChC/DNA complex could enhance transfection efficiency in the range of 10^2- to 10^4-fold. Similar to A549, a synergistic effect of the MPyMeChC/PEI combination over PEI was not observed in SH-SY5Y. However, combined polymer could enhance transfection compared with using MPyMeChC alone. Adding 0.5–

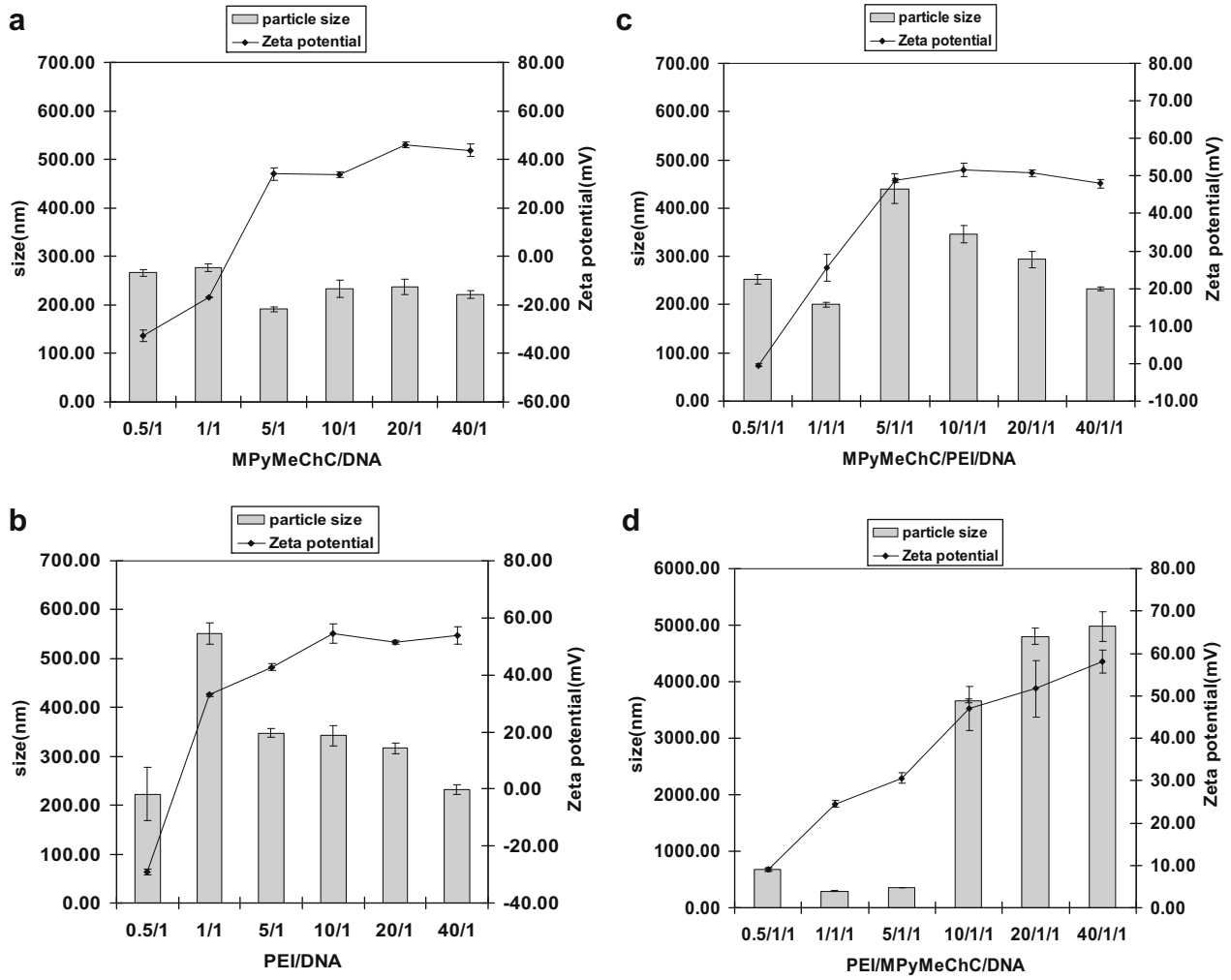

Fig. 2. The size and zeta-potential of MPyMeChC/DNA (a) and PEI/DNA (b) polyplexes at weight ratios of 0.5/1, 1/1, 5/1, 10/1, 20/1 and 40/1, MPyMeChC/PEI/DNA (c) and PEI/MPyMeChC/DNA (d) at weight ratios of 0.5/1/1, 1/1/1, 5/1/1, 10/1/1, 20/1/1 and 40/1/1.

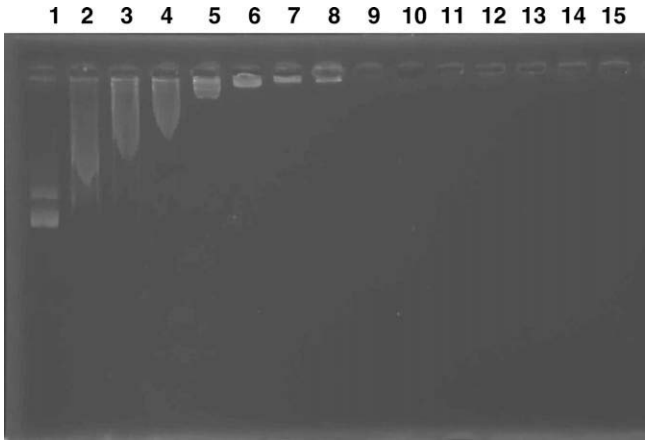

Fig. 3. Gel retardation assay. One microgram of DNA was formed into a complex with MPyMeChC or MPyMeChC/PEI. Lane 1 is free plasmid DNA. Lanes 2–8 are MPyMeChC/DNA complexes at weight ratios of 0.5/1, 1/1, 2.5/1, 5/1, 10/1, 20/1 and 40/1, respectively. Lanes 9–15 are MPyMeChC/PEI/DNA complexes at weight ratios of 0.5/1/1, 1/1/1, 2.5/1/1, 5/1/1, 10/1/1, 20/1/1 and 40/1/1, respectively.

1 μg of PEI onto MPyMeChC/DNA was able to enhance transfection efficiency 10^2- to 10^3-fold. Furthermore, it is likely that adding 0.5–1 μg of PEI into the MPyMeChC/DNA complex at w/w 1/1 is appropriate for transfection.

In neuroblastoma SH-SY5Y cells (Fig. 4c), no synergistic effect over PEI was found in this cell type. However, the addition of 1 μg of PEI enhanced transfection efficiency significantly by approximately 10-fold. MPyMeChC probably is not a suitable gene carrier for SH-SY5Y. In this study, PEI was found to be the most effective transfection reagent because transfection efficiency was induced at a rate of 10^2–10^3 higher than either MPyMeChC or PEI-blended MPyMeChC. The PEI/DNA complex at the 0.5/1 w/w ratio showed higher gene delivery capability of approximately 10^3- to 10^4-fold increasing from transfection by MPyMeChC/DNA complexes. Increasing of PEI resulted in decreasing of transfection ability. Clearly, adding either PEI or MPyMeChC to generate polyplexes in combination with MPyMeChC/PEI/DNA did not improve gene transferring efficiency, but caused defects in cells.

3.5. Cytotoxicity of MPyMeChC, PEI and their combination

The effect of polymers on HeLa, A549 and SH-SY5Y cells were evaluated to determine cell viability and biocompatibility after transfection using MTT assay. Cells were treated with polyplexes under identical conditions to transfection procedures. It was likely that the growth of all cell types was higher than in the controls (free cells treated with naked DNA) when cells were incubated with the MPyMeChC/DNA complex at w/w 1/1 (Fig. 5a–c). This is probably because MPyMeChC is able to promote cell growth of

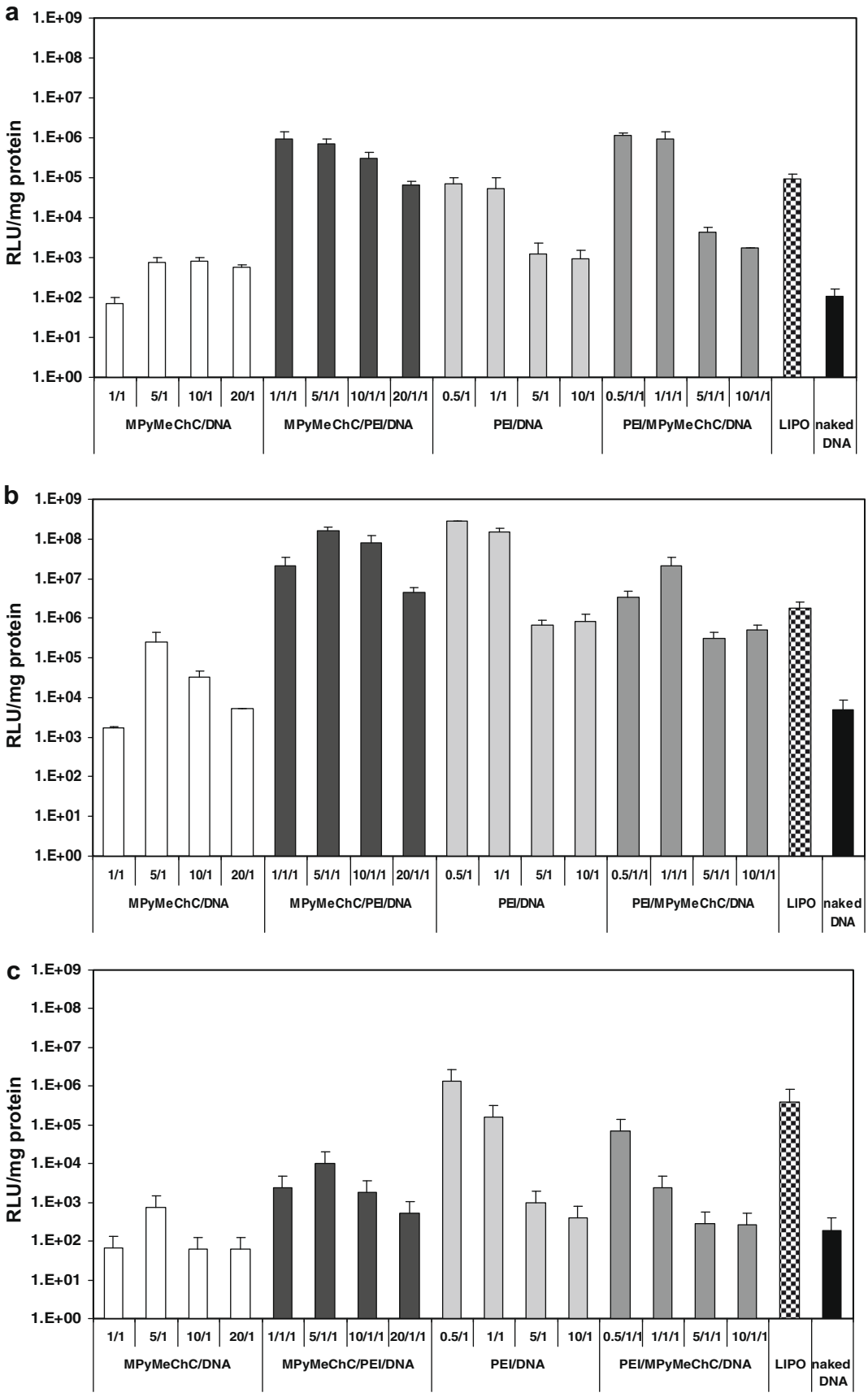

Fig. 4. Transfection efficiencies of MPyMeChC/DNA and MPyMeChC/PEI/DNA polyplexes in HeLa cells (a), A549 (b) and SH-SY5Y (c) at various weight ratios as indicated. The transfection efficiency was compared to positive control Lipofectamine 2000™ (Invitrogen, USA) (Lipo) and negative control (naked DNA; plasmid pGL-3-basic containing CMV promoter/enhancer).

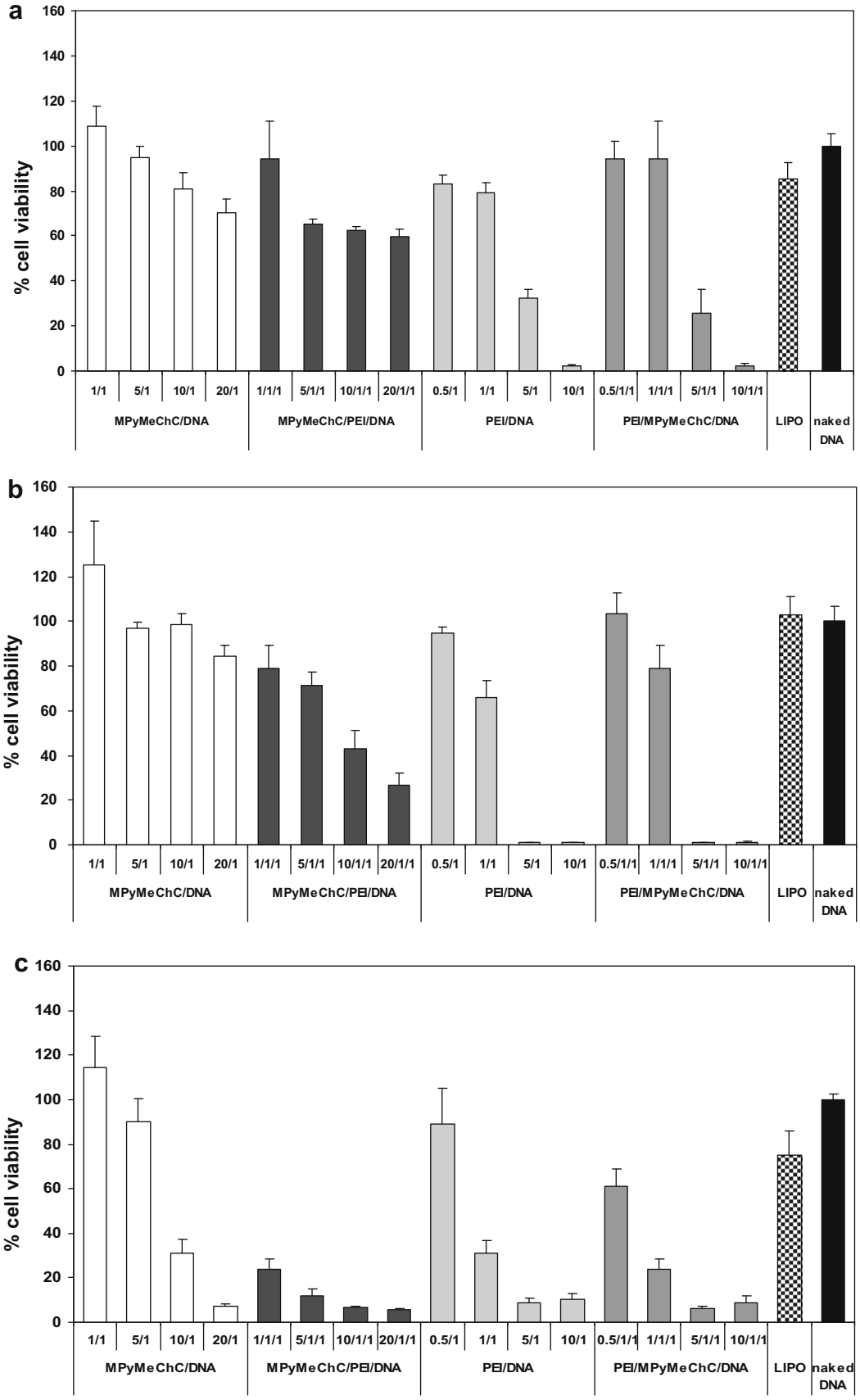

Fig. 5. Cell viability assay in HeLa cells (a), A549 (b) and SH-SY5Y (c). Cells were treated with MPyMeChC/DNA and MPyMeChC/PEI/DNA polyplexes at weight ratios as indicated. Cytotoxicity was determined by MTT assay. Percent viability of cells was expressed relative to cells incubated with naked DNA.

HeLa at low concentrations (Fig. 5a) in which polyplex was not formed completely. MPyMeChC, instead of being a gene carrier, may serve as a supplement for cells which is able to support the expression of extracellular matrix protein and finally promote survival and function of cells. However, the excess concentration of MPyMeChC yielded the opposite effect for cells. Too much MPyMeChC can induce cell membrane leakage due to the aggressive penetration of highly positive-charged nano-polyplexes. Clearly, adding MPyMeChC or PEI resulted in a substantial loss of cell viability in SH-SY5Y cells. The results imply that the neuronal SH-SY5Y cells are very sensitive, even exposed to a high biocompatibility polymer such as chitosan. Therefore, for SH-SY5Y, the more polymers that are applied, the more toxic they are to the cells. Meanwhile, the combination of MPyMeChC and PEI exhibited low cytotoxicity to HeLa and A549 compared to PEI used at the same weight ratio.

4. Discussions

Physical characteristics of the polyplex through AFM images indicated that PEI influenced packaging of DNA in the polyplex. According to the results, DNA was packed loosely in the MPyMeChC/DNA complex. In all cell types, the optimal weight ratio for gene transfection through MPyMeChC/DNA was 5/1. This ratio generated the smallest complex sizes (approximately 191 nm). However, transfection efficiencies were enhanced significantly when PEI was supplemented. This result indicated that even though size is one important factor for gene delivery based on the MPyMeChC/DNA transfection result, the presence of PEI has more influence in this system. In all cell types, transfection efficiencies through MPyMeChC/DNA polyplexes usually were lower than using PEI-blended polyplexes, even though the total charge surfaces were comparable. This may be because of the lack of the proton sponge effect of MPyMeChC. PEI contains the proton sponge effect, which assumes that PEI-based polyplexes are able to buffer the endolysosomal pH, and caused endosome disruption via osmotic swelling. Therefore, the presence of PEI in polyplexes is more effective in enhancing transfection efficiency. The result obtained from AFM images revealed that adding of PEI clearly promoted DNA entrapment when PEI was varied from 0.5, 1 and 10 μg and added into the MPyMeChC/DNA complexes. This result agreed with the previous hypothesis (Kim et al., 2005) which mentioned that PEI was probably coated on the surface of MPyMeChC/DNA complex by ionic interaction between the positive charge of PEI and the negative charge of DNA in MPyMeChC/DNA. Consequently, transformation efficiency was increased by the proton sponge effect of PEI.

Size and zeta-potential analysis indicated that sizes of PEI/DNA (Fig. 2b), MPyMeChC/PEI/DNA (Fig. 2c) and PEI/MPyMeChC/DNA (Fig. 2d) polyplexes were strongly dependent on the weight ratios. This may be explained by the ability of PEI to condense DNA into polyplex. Since PEI contains the amine group two times higher than MPyMeChC at the same amount of PEI and MPyMeChC, PEI has excellent capacity to bind with DNA via the interaction of amine and phosphate. In this study, adding PEI in the range of 0.5–40 μg was adequate to observe the tendency of size-dependent weight ratio, whereas this situation was not clearly observed in MPyMeChC/DNA polyplex (Fig. 2a). In contrast, the sizes of MPyMeChC/DNA polyplexes seem to be rather independent on MPyMeChC/DNA weight ratio. However, size-dependent weight ratio would probably be observed if an increased amount of MPyMeChC were continually added. The size analysis result revealed that PEI-blended polyplexes generally provide bigger sizes of polyplexes compared to with non-blended ones. It was also noted that polyplexes of PEI/DNA at a weight ratio of 1/1 have the biggest size (Fig. 2b). At this point, the condensation of polyplex between DNA and PEI is probably started and the particle might form loosely, while increasing the amount of PEI caused DNA to be more condensed. Moreover, the result shows the significant differences of changes in size by varying the amount of added PEI (Fig. 2d). Adding PEI in the range of 0.5–5 μg resulted in nanoscale polyplexes of PEI/MPyMeChC/DNA, whereas the sizes of these polyplexes were changed into microscale if more than 10 μg of PEI were supplied. An increase in particle size occurred when an additional amount of PEI was applied into the mixture. In this phase, the mixture composed of mixed population between free polyplexes and agglomerated polyplexes. Agglomerated polyplexes usually are the main population in a mixture. However, this situation did not occur in MPyMeChC/PEI/DNA polyplexes (Fig. 2c) where the amount of added PEI was limited at 1 μg. Therefore, MPyMeChC/PEI/DNA polyplexes were all in nanoscale at w/w 10/1/1, 20/1/1 and 40/1/1.

Gene transfection efficiency and cytotoxicity are the two most important factors that need to be considered in a gene delivery system (Shea, Smiley, Bonadio, & Mooney, 1999). Previously, we developed the chitosan derivative, MPyMeChC (Sajomsang et al., 2009). This derivative was proposed for use as a novel gene carrier in a non-viral gene delivery system. MPyMeChC provided high transfection efficiency and biocompatibility to Huh7 cells. However, low transfection efficiency was found in HeLa cells. This implies that the use of MPyMeChC is limited due to low transfection efficiency into a variety of cell types. Therefore, this study aimed to enhance gene transferring efficiency for further use of MPyMeChC as a polymeric-based non-viral carrier. Whereas MPyMeChC retains its biocompatibility as well as chitosan, PEI coating on the MPyMeChC/DNA complex was expected to facilitate gene delivery by improving DNA condensation and increasing the positive charge on the surface of polyplex. Gene transfection results revealed the advantage of adding PEI, which possesses the proton sponge effect after cell internalization. The synergistic effects of combined polymer on gene transfection were revealed in some other reports. The studies demonstrated that gene transfection through combined polymers such as PEI and cationic liposome (Sato, Kawakami, Yamada, Yamashita, & Hashida, 2001; Yamazaki et al., 2000) PEI and chitosan (Jiang et al., 2008; Zhao et al., 2008, 2009) provided effective transfection with low cytotoxicity. This study found that the blending of MPyMeChC and PEI could diminish the drawbacks of each polymer for transfection into HeLa and A549 cells, but not in SH-SY5Y cells. A MTT assay revealed the high cytotoxicity of PEI, especially if it was applied in the amount for more than 5 μg into the cells. However, PEI cytotoxicity to HeLa and A549 could be decreased in combination with MPyMeChC. The results indicated that MPyMeChC somehow neutralized the toxicity of PEI resulting in high transfection efficiency with low toxicity. On the other hand, PEI-containing MPyMeChC appeared to be very toxic to SH-SY5Y, more than using MPyMeChC or PEI alone. SH-SY5Y cells seem to be very sensitive to the amount of polymer used as a gene carrier. Therefore, MPyMeChC/PEI is not a good system for this cell line; only PEI is more suitable. Correspondingly, PEI has been considered as the polymer of choice for gene therapy in brain cells (Wu et al., 2004). A number of research studies reported the use of PEI for transfection into neuronal cells (Abdallah et al., 1996; Bergen, Park, Horner, & Pun, 2008; Suk et al., 2006; Wang, Ma, Gao, Yu, & Leong, 2001). However, according to the results, our study demonstrated high transfection efficiency with low cytotoxicity via MPyMeChC forming a complex with PEI. This system is simply prepared and has the potential to be applied for a wide variety of uses in gene delivery into a variety of cells, especially HeLa and A549.

Different cells displaying significant differences in transfection profile found in the study were probably due to the two different

mechanisms of the transfection process, including interaction of polyplex with the cell culture medium and the subsequent cellular barriers (cell membrane, endosome–lysosome, cytoplasm and nuclear envelope) (Guillem & Aliño, 2004). In the step of polyplex incubation with cells, polyplex interaction with elements of different cell culture medium (ions, anionic proteins from serum) can originate structural changes in size and surface charge that in turn can affect transfection efficiency. Furthermore, it can be considered that *in vitro* transfection begins with polyplex interaction with the cell membrane. The type of interaction depends on the cell type involved in transfection. Different forms of membrane interaction can be different including non-specific interaction with receptors or other components of the cell membrane (such as proteoglycans), and specific interaction with membrane receptors (Godbey & Mikos, 2001). Therefore, the obtained result in this study suggests that, using MPyMeChC and PEI-blending system, there is a need to match these with cell type, gene transferring ability and amount of harmful reagents.

5. Conclusions

Previously, accomplishment of gene delivery via MPyMeChC was likely to be limited in specific cells. Therefore, to expand the use of MPyMeChC for gene delivery applications in mammalian cells, we used PEI incorporated into the complex formation of MPyMeChC and DNA to enhance transfection efficacy into mammalian cells. Polyplexes were formed at different weight ratios to examine physical characteristics of particles. MPyMeChC polyplexes showed potential to be used as a gene carrier. This study demonstrated that an MPyMeChC/PEI-blending system provided high transfection efficacy with low cytotoxicity in general cells. In particular, the synergistic effect of two polymers was found in HeLa cells showing the potential of the system to be used as an alternative carrier for gene therapy.

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References

Abdallah, B., Hassan, A., Benoist, C., Goula, D., Behr, J. P., & Demeneix, B. (1996). A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: Polyethylenimine. *Human Gene Therapy, 7*, 1947–1954.

Anderson, W. F. (1998). Human gene therapy. *Nature, 392*, 25–30.

Bergen, J. M., Park, I. K., Horner, P. J., & Pun, S. H. (2008). Nonviral approaches for neuronal delivery of nucleic acids. *Pharmaceutical Research, 25*, 983–998.

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., et al. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 7297–7301.

Corsi, K., Chellat, F., Yahia, L., & Fernandes, J. C. (2003). Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan–DNA nanoparticles. *Biomaterials, 24*, 1255–1264.

Erbacher, P., Zou, S., Bettinger, T., Steffan, A. M., & Remy, J. S. (1998). Chitosan-based vector/DNA complexes for gene delivery: Biophysical characteristics and transfection ability. *Pharmaceutical Research, 15*, 1332–1339.

Godbey, W. T., & Mikos, A. G. (2001). Recent progress in gene delivery using non-viral transfer complexes. *Journal of Controlled Release, 72*, 115–125.

Guillem, V. M., & Aliño, S. F. (2004). Transfection pathways of nonspecific and targeted PEI-polyplexes. *Gene Therapy and Molecular Biology, 8*, 369–384.

Jiang, H. L., Kim, Y. K., Arote, R., Nah, J. W., Cho, M. H., Choi, Y. J., et al. (2007). Chitosan-graft-polyethylenimine as a gene carrier. *Journal of Controlled Release, 117*, 273–280.

Jiang, H. L., Kim, T. H., Kim, Y. K., Park, I. Y., Cho, M. H., & Cho, C. S. (2008). Efficient gene delivery using chitosan–polyethylenimine hybrid systems. *Biomedical Materials, 2*, 25013.

Kasturi, S. P., Sachaphibulkij, K., & Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. *Biomaterials, 26*, 6375–6385.

Kim, T. H., Kim, S. I., Akaike, T., & Cho, C. S. (2005). Synergistic effect of poly(ethylenimine) on the transfection efficiency of galactosylated chitosan/DNA complexes. *Journal of Controlled Release, 105*, 354–366.

Kunath, K., von Harpe, A., Fischer, D., Petersen, H., Bickel, U., Voigt, K., et al. (2003). Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: Comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *Journal of Controlled Release, 89*, 113–125.

Lechardeur, D., Sohn, K. J., Haardt, M., Joshi, P. B., Monck, M., Graham, R. W., et al. (1999). Metabolic instability of plasmid DNA in the cytosol: A potential barrier to gene transfer. *Gene Therapy, 6*, 482–497.

Lee, K. Y. (2007). Chitosan and its derivatives for gene delivery. *Macromolecular Research, 15*, 195–201.

Li, S., & Huang, L. (2000). Non viral gene therapy: Promises and challenges. *Gene Therapy, 7*, 31–34.

Lv, H., Zhang, S., Wang, B., Cui, S., & Yan, J. (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. *Journal of Controlled Release, 114*, 100–109.

Mao, H. Q., Roy, K., Troung-Le, V. L., Janes, K. A., Lin, K. Y., Wang, Y., et al. (2001). Chitosan–DNA nanoparticles as gene carriers: Synthesis, characterization and transfection efficiency. *Journal of Controlled Release, 70*, 399–421.

Munier, S., Messai, I., Delair, T., Verrier, B., & Ataman-Onal, Y. (2005). Cationic PLA nanoparticles for DNA delivery: Comparison of three surface polycations for DNA binding, protection and transfection properties. *Colloids and Surfaces B: Biointerfaces, 43*, 163–173.

Sajomsang, W., Ruktanonchai, U., Gonil, P., Mayen, V., & Opanasopit, P. ((2009). Methylated *N*-aryl chitosan derivative/DNA complex nanoparticles for gene delivery: Synthesis and structure–activity relationships. *Carbohydrate Polymers, 78*, 743–752.

Sajomsang, W., Tantayanon, S., Tangpasuthadol, V., & Daly, W. H. (2008). Synthesis of methylated chitosan containing aromatic moieties: Chemoselectivity and effect on molecular weight. *Carbohydrate Polymers, 72*, 740–750.

Sato, A., Kawakami, S., Yamada, M., Yamashita, F., & Hashida, M. (2001). Enhanced gene transfection in macrophages using mannosylated cationic liposome–polyethylenimine–plasmid DNA complexes. *Journal of Drug Targeting, 9*, 201–207.

Schaffer, D. V., & Lauffenburger, D. A. (1998). Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *Journal of Biological Chemistry, 273*, 28004–28009.

Shea, L. D., Smiley, E., Bonadio, J., & Mooney, D. J. (1999). DNA delivery from polymer matrices for tissue engineering. *Nature Biotechnology, 17*, 551–554.

Shi, C., Zhu, Y., Ran, X., Wang, M., Su, Y., & Cheng, T. (2006). Therapeutic potential of chitosan and its derivatives in regenerative medicine. *Journal of Surgical Research, 133*, 185–192.

Suk, J. S., Suh, J., Choy, K., Lai, S. K., Fu, J., & Hanes, J. (2006). Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. *Biomaterials, 29*, 5143–5150.

Tatum, E. L. (1966). Molecular biology, nucleic acids, and the future of medicine. *Perspectives in Biology and Medicine, 10*, 19–32.

Tencomnao, T., Rakkhitawatthana, V., & Sukhontasing, K. (2008). Evaluation of a novel luciferase reporter construct: A positive control plasmid for reporter gene assay. *African Journal of Biotechnology, 7*, 2124–2127.

Varshosaz, J. (2007). The promise of chitosan microspheres in drug delivery systems. *Expert Opinion on Drug Delivery, 4*, 263–273.

Verma, I. M., & Somia, N. (1997). Gene therapy – Promises, problems and prospects. *Nature, 389*, 239–242.

Wang, S., Ma, N., Gao, S. J., Yu, H., & Leong, K. W. (2001). Transgene expression in the brain stem effected by intramuscular injection of polyethylenimine/DNA complexes. *Molecular Therapy, 3*, 658–664.

Wong, S. Y., Pelet, J. M., & Putnam, D. (2007). Polymer systems for gene delivery – Past, present, and future. *Progress in Polymer Science, 32*, 799–837.

Wong, K., Sun, G., Zhang, X., Dai, H., Liu, Y., He, C., et al. (2006). PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. *Bioconjugate Chemistry, 17*, 152–158.

Wu, K., Meyers, C. A., Bennett, J. A., King, M. A., Meyer, E. M., & Hughes, J. A. (2004). Polyethylenimine-mediated NGF gene delivery protects transected septal cholinergic neurons. *Brain Research, 1008*, 284–287.

Yamazaki, Y., Nango, M., Matsuura, M., Hasegawa, Y., Hasegawa, M., & Oku, N. (2000). Polycation liposomes, a novel nonviral gene transfer system, constructed from cetylated polyethylenimine. *Gene Therapy, 7*, 1148–1155.

Zhao, Q. Q., Chen, J. L., Han, M., Liang, W. Q., Tabata, Y., & Gao, J. Q. (2008). Combination of poly(ethylenimine) and chitosan induces high gene transfection efficiency and low cytotoxicity. *Journal of Bioscience and Bioengineering, 105*, 65–68.

Zhao, Q. Q., Chen, J. L., Lv, T. F., He, C. X., Tang, G. P., Liang, W. Q., et al. (2009). N/P ratio significantly influences the transfection efficiency and cytotoxicity of a polyethylenimine/chitosan/DNA complex. *Biological & Pharmaceutical Bulletin, 32*, 706–710.