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Receptor mediated gene delivery by folate conjugated *N*-trimethyl chitosan *in vitro*

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ABSTRACT

Folate conjugated *N*-trimethyl chitosan (folate-TMC) that was used for intracellular delivery of protein before was studied as a gene delivery carrier in this study using *N*-trimethyl chitosan (TMC) as a reference. MTT assay indicated that the two polymers were much less toxic than PEI. Agarose gel electrophoresis indicated that the two polymers effectively condensed pDNA. TMC/pDNA complex and folate-TMC/pDNA complex were nano-scale spherical particles confirmed by atomic force microscopy. Cellular uptake of the folate-TMC/pDNA complex containing YOYO-1 labeled pDNA in KB cells was enhanced compared with that of the TMC/pDNA complex and was inhibited by free folate (1 mM) in the medium. Transfection efficiency of the folate-TMC/pDNA complex in KB cells and SKOV3 cells (folate receptor over-expressing cell lines) increased with increasing N/P ratio and were enhanced up to 1.6-fold and 1.4-fold compared with those of the TMC/pDNA complexes, however, there was no significant difference between transfection efficiencies of the two complexes in A549 cells and NIH/3T3 cells (folate receptor deficient cell lines). It was concluded that the increase in transfection efficiencies of the folate-TMC/pDNA complexes were attributed to folate receptor mediated endocytosis. Subcellular distributions of both of the complexes at different time points in the process of cellular uptake were examined by confocal laser scanning microscope, which suggested that different intracellular trafficking pathways were employed by the two complexes.

1. Introduction

Recently, gene therapy has attracted considerable attentions and interest because it can not only treat devastating inherited diseases but also prevent, correct or modulate genetic and acquired diseases by introducing genes coding for therapeutic proteins (Kim et al., 2007). Nowadays, there are two groups of vectors for gene delivery: viral vector and non-viral vector. Though viral vectors have high transfection efficiencies, they have many drawbacks, like immunogenicity, possible recombination with wild-type viruses, limitations in the size of inserted DNA and oncogenicity (Schreier, 1994; Byrnes et al., 1995; Gorecki, 2001; McTaggart and Al-Rubeai, 2002). Compared with viral vectors, non-viral vectors such as polymers have significant advantages such as safety, low cost and lack of restraints on the size of DNA to be delivered (Curiel et al., 1991; Felgner and Rhodes, 1991).

Chitosan is a naturally occurring biocompatible and biodegradable polymer (Felt et al., 1998; Onishi and Machida, 1999). It consists of D-glucosamine and N-acetyl-D-glucosamine linked by a β-(1 → 4) glycosidic bond and can condense anionic DNA into a compact structure through electrostatic interaction (Hashimoto et al., 2006). Till now, it has been extensively studied as a vector for gene. However, transfection efficiency of chitosan/DNA complex is poor compared with other standard non-viral vectors due to the low solubility of chitosan at physiological pH (Lee et al., 2007; Yu et al., 2007). Among several water-soluble derivatives of chitosan,

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N-trimethyl chitosan (TMC), a permanently quaternized chitosan derivative, is of great interest because of its well-defined structure, improved solubility and easy preparation (Sieval et al., 1998). As TMC can more effectively condense DNA at physiological pH, transfection efficiency of TMC/DNA complex has been increased 30-fold than that of chitosan/DNA (Thanou et al., 2000). There are some studies about the ability of TMC with different degrees of quaternization (DQ) to transfect cancer cells (Thanou et al., 2002; Kean et al., 2005). TMC with DQ between 40 and 57% has higher transfection efficiency than that with lower or higher DQ. A definite trend is shown in previous studies that IC_{50} value of TMC decreased with increasing DQ, which means that toxicity of TMC increases with increasing DQ (Kean et al., 2005; Mao et al., 2007). Above all, TMC with DQ around 40% was used in this study.

An ideal gene carrier system should efficiently accumulate in specific target tissues with minimal toxicity to non-target tissues (Luten et al., 2008). In order to specifically increase transfection efficiency of polymer/DNA complex to tumor cell, various targeting ligands including antibodies, growth factors, peptides, transferrin and folate have been conjugated to several polymers (Coll et al., 1997; Lee et al., 2002; Hashimoto et al., 2006; Kim et al., 2007). Folate receptors are vastly overexpressed in a wide variety of human tumors, including ovarian, endometrial, colorectal, breast, lung, renal cell carcinomas, brain metastases derived from epithelial cancers and neuroendocrine carcinomas, but rarely are found on normal cell surface (Sudimack and Lee, 2000). Folate-chitosan was ever synthesized and folate-chitosan–DNA nanoparticle was prepared. Experiments both *in vitro* and *in vivo* suggested that this nanoparticle enhanced gene expression more than both naked DNA and chitosan–DNA nanoparticle (Fernandes et al., 2008). Besides, some other novel polymers have been synthesized like folate-polylysine (Mislick et al., 1995), folate-polyethylenimine (Guo and Lee, 1999) and folate-polyethylenimine-block-poly(L-lactide) (Wang and Hsiue, 2005), all of which specifically increased gene transfection in target cells compared with the non-targeted polymers.

In our laboratory, folate conjugated *N*-trimethyl chitosan (folate-TMC) was synthesized. Protein loaded nanoparticle was prepared by ionic cross-linking with sodium alginate using this novel polymer, which specifically increased intracellular uptake of protein in folate receptor over-expressing cancer cells compared with nanoparticle made of TMC (Zheng et al., 2009). As mentioned above, TMC is a promising gene delivery vector and now we extend the use of folate-TMC as a gene carrier. Two complexes, folate-TMC/pDNA and TMC/pDNA, were prepared and physicochemical characterization of the complexes was carried out. Cellular uptake and transfection *in vitro* were evaluated and compared using folate over-expressing cell lines (KB cells and SKOV3 cells) and folate receptor deficient cell lines (A549 cells and NIH/3T3 cells). In addition, intracellular trafficking of complexes was examined by investigating subcellular distributions with confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Green fluorescent protein plasmid DNA (pDNA) was purchased from Clontech (Mountain View, CA, USA). YOYO-1 was purchased from Molecular Probes Inc. (Eugene, OR, USA). DRAQ 5 was purchased from Biostatus Limited Inc. (Leicestershire, UK). Gel loading buffer was purchased from Invitrogen (CA, USA). Trypan blue, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum, RPMI 1640 and folate-free RPMI 1640 media were obtained from Gibco BRL (Grand Island, NY, USA). Cells were provided by the Cell Bank of the Chinese Academy of Science (Shanghai, China). Trimethyl chitosan (TMC) and folate conjugated *N*-trimethyl chitosan were synthesized as described earlier (Zheng et al., 2009).

2.2. Cell culture

KB cells (a human oral squamous cell carcinoma cell line) and SKOV3 cells (a human ovarian cancer cell line) were maintained in folate-free RPMI 1640 media supplemented with 10% fetal bovine serum. A549 cells (a human lung carcinoma cell line), L929 cells (a mouse connective tissue fibroblast cell line) and NIH/3T3 (a mouse embryo fibroblast cell line) were maintained in standard RPMI 1640 media supplemented with 10% fetal bovine serum. Cells were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. MTT assay

L929 cell line was selected to evaluate cytotoxicity as recommended by USP 26. L929 cells were seeded into 96-well plates at a density of 2×10^4 cells/well one day before treatment. Then, cell culture medium was aspirated and replaced by 200 μl serial dilutions of polymer stock solution in complete cell culture medium in each well. The cells were incubated with media containing the polymers for 4 h at 37 °C. Here, PEI was studied as a positive control. Then, the medium was replaced by 200 μl RPMI 1640 containing 0.5 mg/ml MTT in each well. After 4 h incubation at 37 °C in the dark, the MTT solution was replaced by 200 μl DMSO in each well to dissolve formazan crystals. Plates were read using a plate reader (Molecular Devices, CA, USA) at a wavelength of 570 nm. In the case of polymer/DNA complex, KB cell line was also selected for evaluation of toxicity since it was used for transfection. Seeding of KB cells was performed like that of L929 cells. The cells were incubated with 200 μl complete cell culture medium in each well containing the complexes at a ratio of polymer nitrogen to DNA phosphate (N/P ratio) of 20. The final polymer concentration in the medium was 120 μg/ml, which was the concentration used for transfection. The following experiments were carried out as outlined above. Relative cell viability compared with control cells without treatment was calculated using Eq. (1):

$$\text{relative cell viability}\,(\%) = \frac{[A]_{\text{test}}}{[A]_{\text{control}}} \times 100 \quad (1)$$

IC_{50} values, which are the concentrations of the polymers needed to inhibit cell growth by half, were calculated.

2.4. Preparation of complexes

pDNA in 50 μl of PBS (pH 7.4) with the concentration of 0.065 mg/ml was added to appropriate amount of the polymers (0.21–40 μg of TMC or 0.22–45 μg of folate-TMC) in 50 μl of the same buffer to obtain the desired polymer nitrogen to DNA phosphate ratio (N/P ratio). Complexes were mixed on a vortex mixer and incubated for 20 min at RT before use.

2.5. Agarose gel electrophoresis

Complexes were prepared as described in Section 2.4. 10% loading buffer was added and 20 μl of the complex solutions were applied to a 1% agarose in TAE (40 mM Tris/HCl, 1% acetic acid, 1 mM EDTA, pH 7.4) containing 0.6 μg/ml of ethidium bromide. Electrophoresis (Sunrise 96, Labrepco, USA) was carried out at a constant voltage of 70 V for 60 min. Ethidium bromide fluorescence

Table 1
IC_{50} curves of all the polymers had 10 concentrations in the range 1–10^3 μg/ml. Curves were fitted according to the dose–response curve performed by GraphPad Prism, and the IC_{50}s were calculated automatically.

Polymer	IC_{50} (μg/ml)	
	L929	KB
TMC	80.1 ± 16.1	166.3 ± 30.6
Folate-TMC	72.3 ± 10.6	147.2 ± 38.2
PEI	9.6 ± 4.1	14.3 ± 4.2

was detected using a gel documentation system (Micro Photonics Inc., USA).

2.6. Size and zeta potential

Particle sizes and zeta potentials of the complexes were measured as a function of N/P ratio by a zetasizer nano ZS90 (Malvern Instruments Ltd., USA). All measurements were carried out in triplicate.

2.7. Atomic force microscopy

Surface morphologies of the complexes at N/P ratio 20 were analyzed by atomic force microscopy (AFM). Freshly peeled mica was used as sample support. Samples were analyzed within 2 h after preparation. Microscopy was performed on a Dimension 3100 (Veeco Instruments, San Jose, USA) using commercial silicon tips attached to an Itype cantilever with a length of 125 μm. The scan size was 5 μm × 5 μm.

2.8. Cellular uptake

pDNA was labeled with YOYO-1 as describe before (Fernandes et al., 2008). Briefly, 10 μl of 1 mg/ml pDNA was mixed with 10 μl of 10 μM YOYO-1 and incubated at room temperature for 1 h in the dark. Complexes were prepared at N/P ratio 20 with 8 μg pDNA entrapped in each sample. 2.5×10^5 KB cells were incubated with the complexes at 37 °C for 4 h in complete culture medium with or without 1 mM folate. Then, the cells were washed with PBS once and incubated with 0.4% trypan blue solution to quench extracellular fluorescence. After that, the cells were washed three times with PBS and fixed in 1% paraformaldehyde solution for 30 min at 4 °C. The fixed cells were washed twice with PBS and stored in 0.1% paraformaldehyde. Complex uptake in KB cells was analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, USA).

2.9. Transfection experiment

KB cells, SKOV3 cells, A549 cells and NIH/3T3 cells were seeded on 6-well plate (2.5×10^5 cells/well). After 24 h of growth, medium was replaced by 960 μl RPMI 1640 or folate-free RPMI 1640 in each well. pDNA was diluted with PBS (pH 7.4) to 0.4 mg/ml and was added to appropriate amount of the polymers in the same buffer with equal volume to obtain desired N/P ratios. Complexes were mixed on a vortex mixer and incubated for 20 min at RT. Complexes in a final volume of 40 μl containing 8 μg pDNA were subsequently added to the wells. After 4 h incubation, medium was changed to complete culture medium again and the cells were incubated for an additional 48 h. After that, the cells were washed three times with PBS and fixed in 1% paraformaldehyde solution for 30 min at 4 °C. The fixed cells were washed twice with PBS and stored in 0.1% paraformaldehyde. The cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

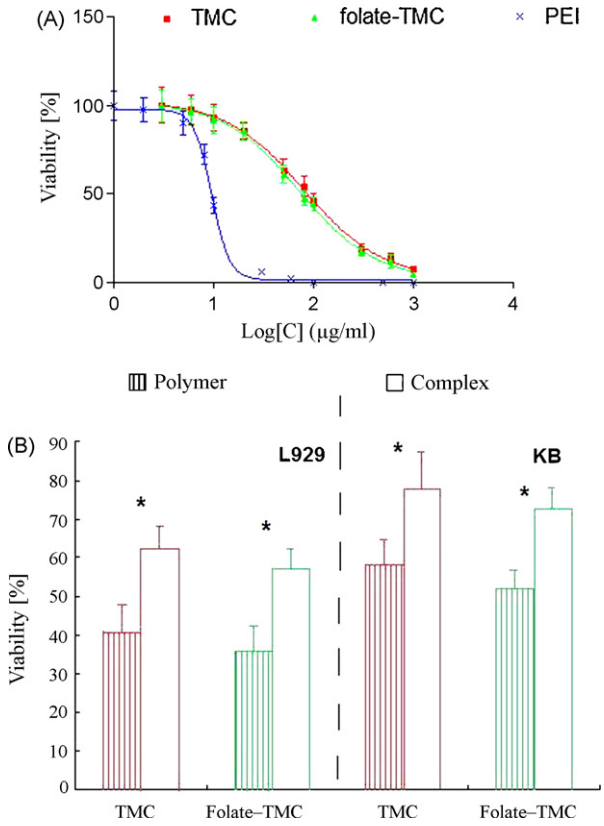

Fig. 1. (A) Viabilities of L929 cells treated by the polymers with serial concentrations and (B) viabilities of L9292 and KB cells treated by the polymers alone (0.12 μg/ml) or their complexes with pDNA at N/P ratio 20 applied with the polymer concentrations of o.12 μg/ml. Results were the mean of six independently experiments and statistically analyzed using one-way ANOVA with Bonferroni post-tests. $^*P < 0.05$.

2.10. Confocal laser scanning microscopy

The complexes were prepared at N/P ratio 20 with 8 μg YOYO-1 labeled pDNA entrapped in each sample. 2.5×10^5 KB cells were

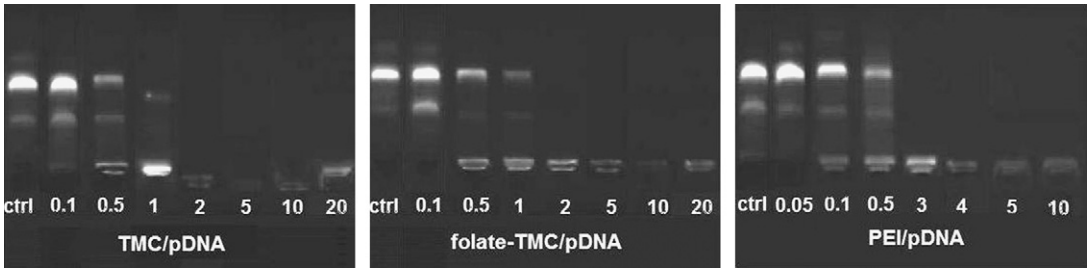

Fig. 2. Agarose gel electrophoresis of the TMC/pDNA complexes and the folate-TMC/pDNA complexes at N/P ratios ranging from 0.1 to 20 and PEI/pDNA complex at N/P ratios ranging from 0.05 to 7.

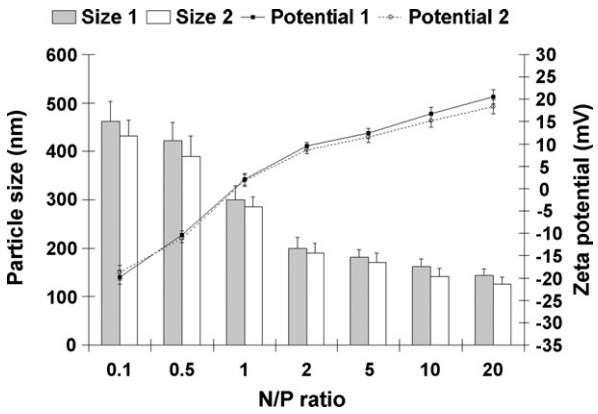

Fig. 3. Particle sizes and zeta potentials of the complexes at different N/P ratios. Size 1 and size 2 referred to the particle size of the TMC/pDNA complex and the particle size of the folate-TMC/pDNA complex, respectively. Potential 1 and potential 2 referred to the zeta potential of the TMC/pDNA complex and the zeta potential of the folate-TMC/pDNA complex, respectively.

incubated with the complexes at 37 °C for 5, 15, 60, 240 min in complete culture medium. Then, the cells were washed with PBS once and incubated with 0.4% trypan blue solution to quench extracellular fluorescence. After that, the cells were washed three times with PBS and fixed in 1% paraformaldehyde solution for 30 min at 4 °C. The fixed cells were washed twice with PBS and stored in 0.1% paraformaldehyde. The cells were stained by DRAQ 5 (a nuclear counterstain) and examined on a Zeiss LSM 510 META laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

3. Results and discussion

3.1. Cytotoxicity of the polymers and the complexes

Cytotoxicities of the polymers and the complexes were investigated by MTT assay, which evaluated metabolic activity of cells treated with the polymers or the complexes. 4 h was selected to mimic the transfection experiment *in vitro*. The IC_{50}s of both TMC and folate-TMC are higher than that of PEI suggesting that both TMC and folate-TMC are much less toxic than PEI, which is the gold standard for cationic polymer-based gene carriers (Table 1). The cell viabilities of folate-TMC at different concentrations are lower than those of TMC (Fig. 1A). Though there is no significant difference, the IC_{50} of folate-TMC is lower than that of TMC, which may be due to greater cellular uptake mediated by folate receptor and therefore greater effects on cells. Because of interaction with anionic component (sialic acid) of the glycoproteins on cell sur-

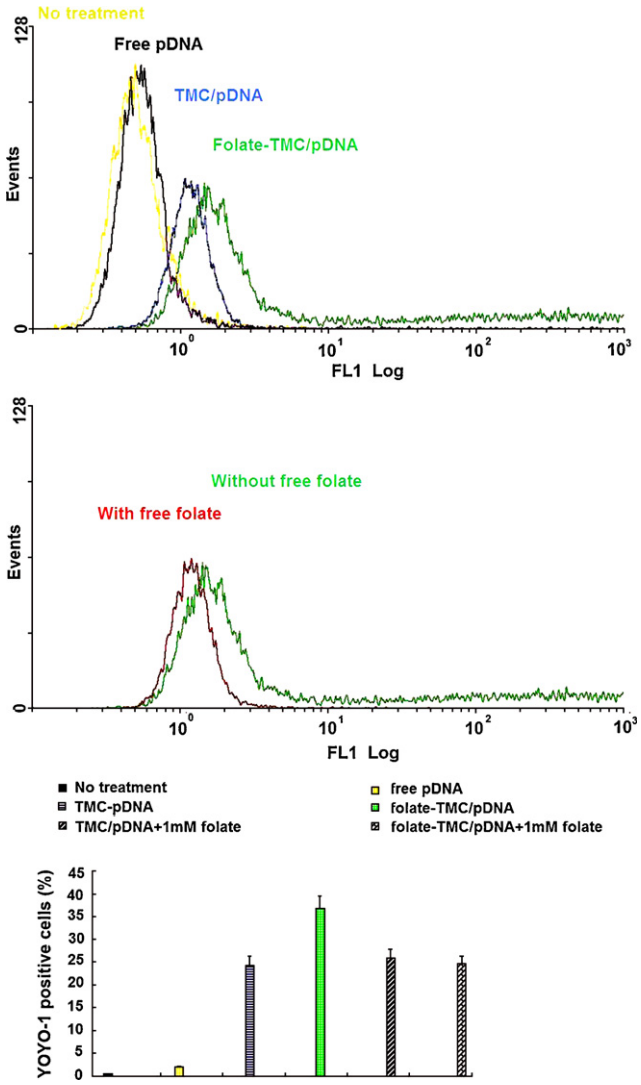

Fig. 5. Cellular uptake of the complexes in KB cells. (A) Cells were treated with the complexes at N/P ratio 20 entrapping YOYO-1 labeled pDNA, and cellular fluorescence was measured on a FACSCalibur flow cytometer. (B) Cells cultured in medium with or without 1 mM free folate were treated with the folate-TMC/pDNA complex at N/P ratio 20 for 4 h at 37 °C and cellular fluorescence was measured on a FACSCalibur flow cytometer. Results of flow cytometry are shown in histogram with the *X*-axis indicating the cellular fluorescence intensity and the *Y*-axis indicating the cell count. (C) Percentage of cells being positive for fluorescently labeled DNA measured on a FACSCalibur flow cytometer. Results were the mean of three independently experiments and statistically analyzed using one-way ANOVA with Bonferroni post-tests. $^{**}P < 0.01$.

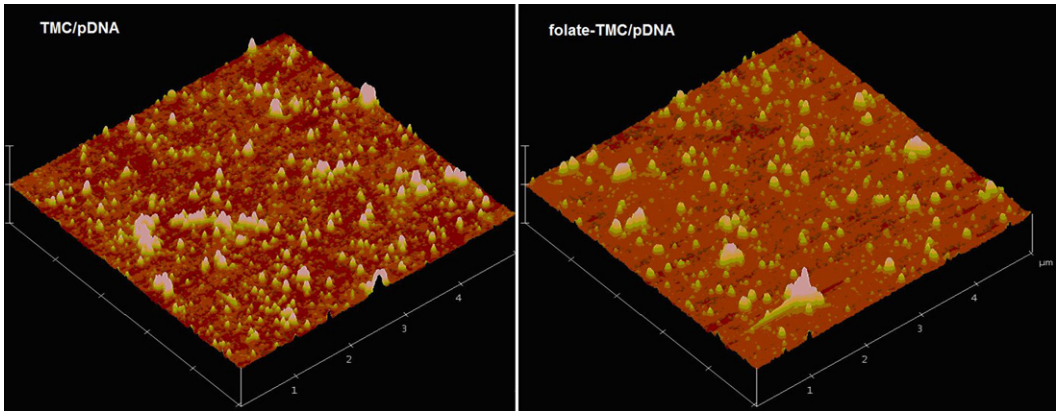

Fig. 4. AFM topography images of the complexes at N/P ratio 20. Edge length: 5 μm × 5 μm.

faces, TMC and folate-TMC have cytotoxicities like other cationic polymers such as PEI, poly(L-lysine), protamine, etc. (Mao et al., 2007). The effects of the polymers alone on cell viability were compared with those of the complexes (Fig. 1B). Both of the complexes showed decreases in toxicity on both L929 cells and KB cells because the cationic charges on the polymers are partially neutralized by complexation with pDNA and the conformations of the polymers have been changed (Koping-Hoggard et al., 2003). Cherng et al. and Mao et al. also reported that toxicities of complexes of TMC with macromolecules dramatically decreased compared with the intrinsic polymer (Cherng et al., 1996; Mao et al., 2005).

3.2. Physicochemical characterization of the complexes

Agarose gel electrophoresis was used to study and compare abilities of the two polymers to condense pDNA (Fig. 2). It turned out that both of them completely retarded DNA migration at N/P ratio 2, which agrees with previous study using TMC as a gene carrier (Germershaus et al., 2008).

Sizes of the complexes as a function of N/P ratio was shown in Fig. 3. The two complexes exhibited similar alteration tendencies of particle size. When N/P ratio <2, the particle sizes are very large indicating incomplete condensation of pDNA with the polymers. When N/P ratio ≥2, the sizes became much smaller because of formation of compact complexes. The alteration tendencies of the complex particle sizes resemble that of TMC/pDNA complex reported previously (Mao et al., 2007). The result of this assay is consistent with that of gel retardation assay. The particle sizes of the folate-TMC/pDNA complexes are smaller than those of the TMC/pDNA complexes due to the contribution of folate to suppression of self-aggregation of the complex. Folate conjugation in folate-chitosan impacted particle size of the folate-chitosan/pDNA complex in a similar way (Mansouri et al., 2006). Zeta potentials of the TMC/pDNA complexes and the folate-TMC/pDNA complexes at different N/P ratios were shown in Fig. 3. It was found that the two complexes exhibited similar alteration tendencies of the zeta potential and the zeta potentials of both complexes increased with increasing N/P ratios. The results are close to that of the TMC/pDNA complex reported before (Mao et al., 2007).

AFM confirmed formation of the spherical complexes (Fig. 4). And the particle sizes gained in this assay are similar to those given by the particle sizer.

3.3. Cellular uptake

Cellular uptakes of both of the complexes containing pDNA labeled with YOYO-1 were evaluated in KB cells using flow cytometry. It was found that level of uptake of the folate-TMC/pDNA complex was significantly higher than that of the TMC/pDNA complex ($p < 0.01$) and was blocked by excess free folate (1 mM) in the medium, indicating that enhancement of folate-TMC/pDNA uptake is due to folate receptor mediated endocytosis (Fig. 5). This experiment further verified assumption given in Section 3.1 that the increased cellular uptake of folate-TMC compared with TMC is due to folate receptor mediated endocytosis.

3.4. Transfection experiments

Transfection efficiencies of the two complexes at different N/P ratios from 1 to 20 were determined in our preliminary experiments (data not shown), however, only the complexes at N/P ratios between 5 and 20 had significant transfection of GFP pDNA. Transfection efficiencies of both of the complexes increased with increasing N/P ratio in not only folate over-expressing cell lines but also folate deficient cell lines (Fig. 6). Increased transfection efficiency following increased N/P ratio of TMC/DNA complex was also reported in previous studies (Thanou et al., 2000; Ghaghada et al., 2005). It was also reported that the transfection efficiencies of PEI/DNA complexes were enhanced with the increase of N/P ratio (Benns et al., 2002). The authors explained that the improved ionic interactions between the positively charged complexes and negatively charged plasma membrane driven by the increase in

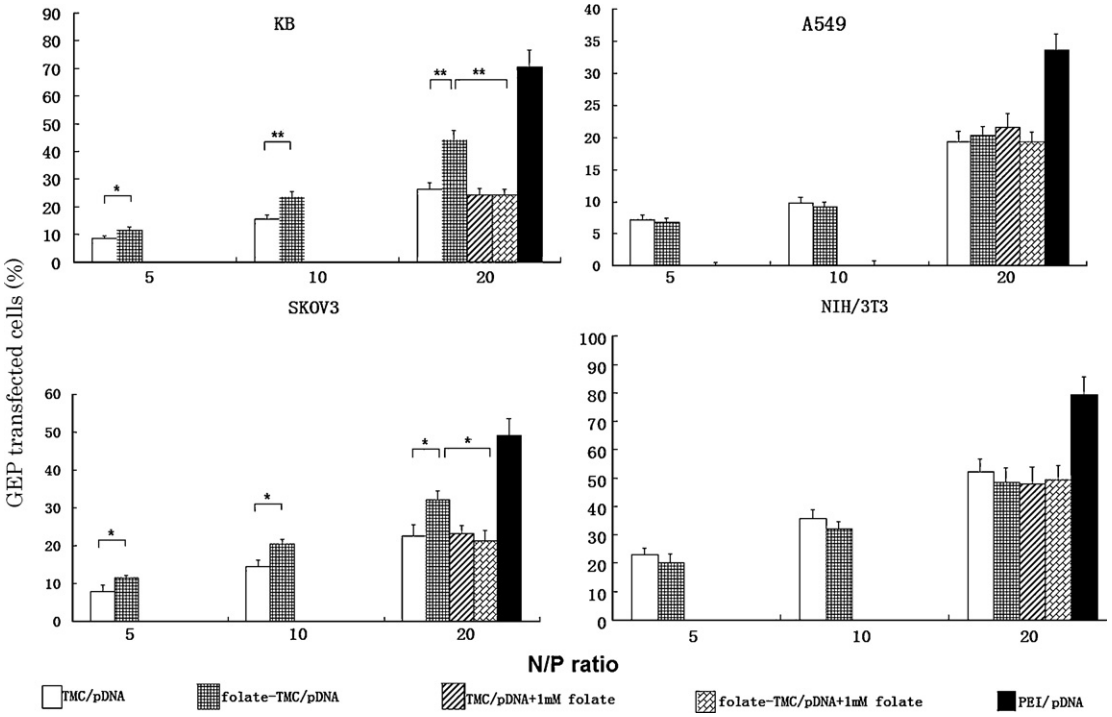

Fig. 6. GFP gene expression efficiency after treatment with the complexes at different N/P ratios in four cell lines. PEI/pDNA at N/P ratio 10 is used for comparison of transfection efficiency. Results were the mean of three independent measurements and statistically analyzed using one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$.

surface charge of the complexes accounted for the increased endocytosis and dominated the gene transfer of the complexes. Some other people drew a similar conclusion (Khalil et al., 2006). In our study, there were also ionic interactions between the TMC/pDNA complexes and plasma membrane due to the cationic character of the complexes. We speculate that the increase of N/P ratio causes the increase of ionic interactions between the complexes and the plasma membrane, which leads to the enhanced endocytosis and consequently results in the increased transfection efficiencies. Like PEI/DNA complexes, TMC/pDNA complexes, probably, mainly relied on this receptor-independent endocytosis to be internalized by cells.

As for the folate-TMC/pDNA complex, the increase of N/P ratio resulted in the increase of number of folate in the complex and consequently led to the increase of the number of folate ligand exposing on the surface of the complex. Ghahada et al. proposed and proved a theory that number of bond that can be formed between a carrier and receptor on cell surface depends on number of ligand on the surface of the carrier, and the increased bonds will ultimately increase cellular uptake of the carrier (Ghaghada et al., 2005). According to this theory, the increased N/P ratio resulted in the increased cellular uptake of the complex, which explained the increase in transfection efficiency with increasing N/P ratio. Though there was one more uptake pathway that contributed to the gene transfer of the folate-TMC/pDNA complexes, folate receptor mediated endocytosis, the transfection efficiencies of the folate-TMC/pDNA complexes only increased 1.6-fold or 1.4-fold in two folate receptor over-expressing cell lines compared with the TMC/pDNA complexes. There are probably several reasons for this. Firstly, the existence of receptor-independent endocytosis made the impact of folate receptor mediated endocytosis less significant. Secondly, in this study, folate was directly conjugated to TMC without any flexible polymer chain as tether. It was concluded in previous study that the presence of a tether

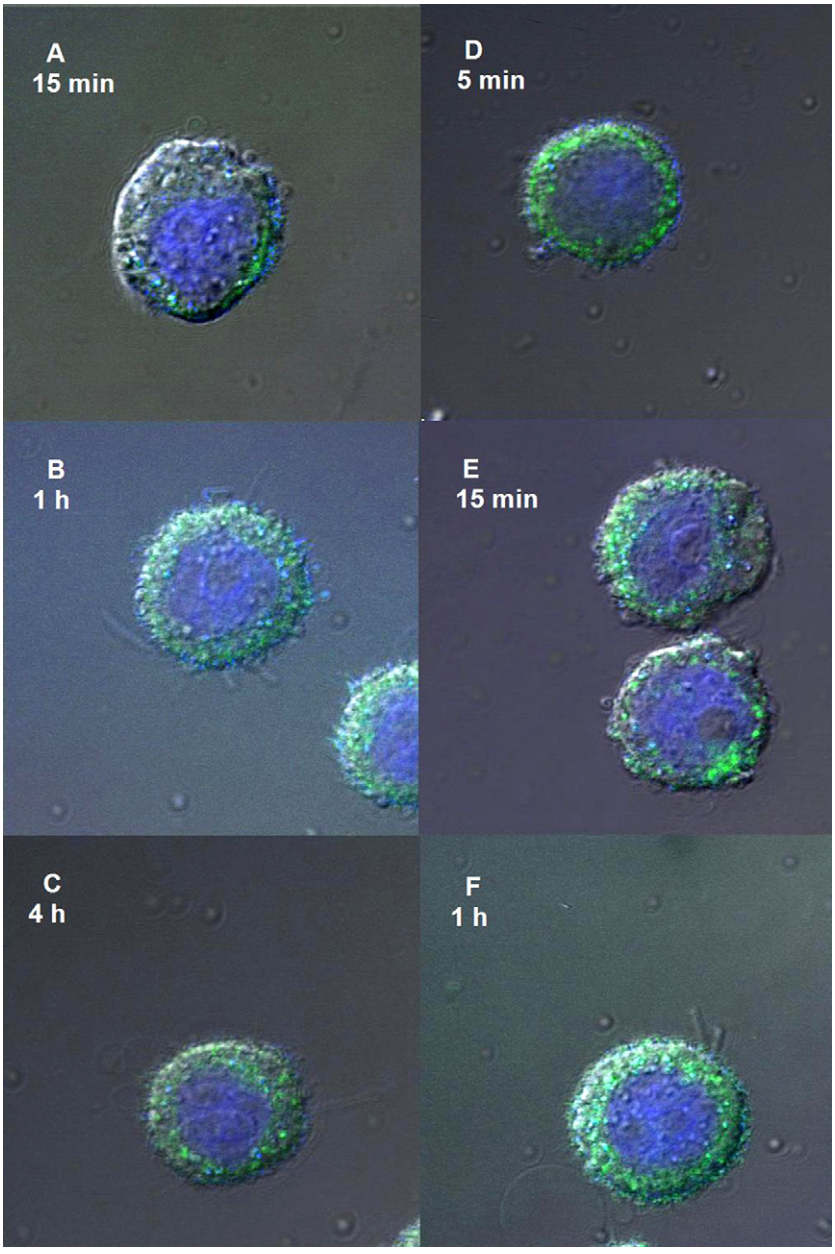

Fig. 7. Time courses of the subcellular localizations of the complexes in KB cells. The cells were incubated with the TMC/pDNA complex (A–C) and folate-TMC/pDNA (D–F) for 5 min (D), 15 min (A and E), 1 h (B and F) and 4 h (C).

might increase the spatial freedom afforded to the ligand at the distal end of the tether, and therefore facilitate the interaction between ligands and receptors (Ghaghada et al., 2005). Thirdly, cells were incubated with the complexes for 4 h. As it took less than 1 h for the folate-TMC/pDNA complexes or 4 h for the TMC/pDNA complexes to gather around nucleus in folate receptor over-expressing cells (shown in Section 3.5). So, shortening the incubation time will be helpful to see the difference in transfection efficiency between the two complexes. However, 4 h was adopted to obtain good transfection efficiencies. In previous study, folate-PEG-coated poly(2-dimethylaminoethylamine-*co*-diaminobutane) phosphazene (poly(DMAEA-co-BA) phosphazene)/DNA complex showed a similar increase (1.5-fold) in transfection efficiency compared with poly(DMAEA)/DNA complex (Luten et al., 2008). As excess free folate can competitively bind to folate receptor, after addition of folate (1 mM) in the medium, the transfection efficiency of folate-TMC/pDNA complex in KB cells and SKOV3 cells were close to that of TMC/pDNA complex. In order to further prove the effect of folate receptor on the transfection efficiencies of the complexes, the transfection experiment was conducted using another two cell lines that are deficient in folate receptor: A549 and NIH/3T3. The two complexes exhibited no difference in the transfection efficiency in both of the cell lines. Moreover, free folate in the medium did not affect the transfection efficiencies of the complexes. Above all, we conclude that the transfection efficiency of the folate-TMC/pDNA complex can be specifically enhanced in folate over-expressing cells through folate receptor mediated endocytosis. Note that the transfection efficiency of the TMC/pDNA complex in four cell lines varied. It was reported that cell type characteristics such as charge density on cell surface and differential internalization routes account for difference in transfection efficiency (Mao et al., 2007). In this case, folate receptor density on cell surface will also affect transfection efficiency of folate-TMC/pDNA complex, since it is one of the factors that determine bonds formed between a carrier and receptor on cell surface (Ghaghada et al., 2005). Both folate-TMC and TMC were less effective for gene transfection than PEI, which proved a correlation between toxicity and transfection efficiency for cationic polymer-based non-viral vectors (Germershaus et al., 2008).

3.5. Confocal laser scanning microscopy

By using confocal laser scanning microscopy, subcellular localizations of the two complexes in course of internalization by KB cells were examined and compared. The TMC/pDNA complex was absorbed on cell surface at 15 min, while the folate-TMC/pDNA complex was seen on cell surface and inside of cells at 5 min (Fig. 7). The folate-TMC/pDNA complex just took 15 min to gather around nucleus, while the TMC/pDNA complex took 4 h. It was also reported in previous research that the TMC/pDNA complex took 4 h to be taken up into cells (Germershaus et al., 2008). It has been proved in the experiment above that the cellular uptake of the folate-TMC/pDNA complex is attributed to folate receptor mediated endocytosis. Mechanism for cellular uptake of TMC/pDNA complex has not been reported till now, and it is probably similar to that of chitosan polyplex proposed by Huang et al. (2002). The mechanism is clathrin-mediated endocytosis and proved very slow by fluorescence microscopy (MacLaughlin et al., 1998; Issa et al., 2006). Hashimoto et al. reported that receptor-mediated endocytosis is more rapid than nonspecific endocytosis by employing different intracellular trafficking pathway in hepatocytes having asialoglycoprotein receptors (Hashimoto et al., 2006). In this study, difference in rates of traversing endocytic compartments between the two complexes may be also due to different intracellular trafficking pathways. As there is a good correlation between cellular uptake and transfection efficiency suggesting that cellular uptake precedes efficient transfection (Huang et al., 2005), the 1.6-fold and 1.4-fold increase in gene transfection of the folate-TMC/pDNA complex compared with that of the TMC/pDNA complex is a result of the difference in intracellular trafficking pathway. It was also reported in the study of Hashimoto et al. that increased intracellular trafficking rate of lactosylated chitosan/pDNA complex led to efficient gene transfection (Hashimoto et al., 2006).

4. Conclusion

As the novel polymer folate-TMC can enhance intracellular protein transport in cells over-expressing folate receptor and TMC itself is an ideal gene vector that has been extensively studied, folate-TMC was investigated as a gene vector in this study in the purpose of increasing gene expression in folate receptor over-expressing cells. It is much less toxic than PEI and the folate-TMC/pDNA complex showed a decrease in toxicity on cells. Folate conjugation increased the intracellular uptake of the complex in KB cells and SKOV3 cells (folate receptor over-expressing cell lines) through folate receptor mediated endocytosis examined by flow cytometry. The transfection efficiency of the folate-TMC/pDNA complex was increased compared with that of the TMC/pDNA complex in both KB cells and SKOV3 cells. By using confocal laser scanning microscopy, it was found that the folate-TMC/pDNA complex traversed endocytic compartments more rapidly than the TMC/pDNA complex because of employing different trafficking pathways, which accounts for the increased intracellular uptake and transfection efficiency. In conclusion, folate-TMC is a promising vector for gene transfection.

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