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RESEARCH PAPER

Insight into Polycation Chain Length Affecting Transfection Efficiency by *O*-Methyl-Free *N,N,N*-Trimethyl Chitosans as Gene Carriers

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ABSTRACT

Purpose The structure–function relationship and mechanism of polycations as gene carriers have attracted considerable research interest in recent years. The present study was to investigate the relationship between polycation chain length and transfection efficiency ($R_{CL\text{-}TE}$), and the corresponding mechanism by *O*-methyl-free *N,N,N*-trimethyl chitosans (TMCs) as gene carriers.

Methods Four TMCs with various chain lengths were synthesized and used to evaluate the $R_{CL\text{-}TE}$. To investigate the details of $R_{CL\text{-}TE}$, a number of factors such as cytotoxicity, cellular uptake efficiency, cellular uptake pathway and intracellular trafficking, were evaluated.

Results In comparison to short chain length TMCs (S-TMCs), long chain length ones (L-TMCs) mediated higher gene expression. The polyplexes formed by L-TMCs and pDNA showed higher stability. The cellular uptake pathway and intracellular trafficking of these TMC/pDNA polyplexes were different. These above factors are probably the key ones in $R_{CL\text{-}TE}$ rather than polycation–DNA binding affinity, polyplex particle size in water, zeta potential, serum, cytotoxicity, and cellular uptake efficiency.

Conclusions For rational design of chitosan-based polycations as gene carriers, polycations with relative long chain lengths are more favorable and more attention should be paid to polyplex stability, function of uncomplexed polycation chains, cellular uptake pathway, and intracellular trafficking.

KEY WORDS gene carrier · mechanism · polycation chain length · transfection efficiency · trimethyl chitosan

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INTRODUCTION

The use of polycations as gene delivery carriers has attracted a great deal of research interest over the last decade. To date, the polycations that have been explored in detail include polyethylenimine (PEI) (1) and chitosan (2), among others. As a naturally derived polysaccharide, chitosan is a promising polycation for use in gene delivery, given the lower immunogenicity, better biocompatibility, non-allergenicity, and high mucoadhesion of chitosan compared with other synthetic polycations (3). However, chitosan is insoluble under physiological conditions, and the transfection efficiency (TE) of chitosan-based polyplexes is relatively low. Thus, numerous efforts have been made to modify chitosan to improve its solubility and TE, such as *N,N,N*-trimethyl chitosan (TMC) (4,5). In most cases (4–8), TMCs are synthesized according to the methods developed by Domard (9) or Sieval (10). The drawbacks of these methods include the occurrence of chain scission under harsh reaction conditions and uncontrollable 3,6-*O*-methylation as a side reaction (11). Because these may affect the physicochemical properties of TMCs (12), the synthesis and evaluation of well-defined TMCs featuring regioselective 2-*N*-methylation as gene carriers are of utmost importance.

Many factors are known to affect the transfection efficiency, such as the physical and chemical properties of the polycations (e.g., chain length, modification, lipophilicity, and binding affinity with DNA or RNA), formulations (e.g., N/P ratio and the components of the solvent and additive), properties of the polyplexes (e.g., particle size, zeta-potential, and stability), and interactions between the polyplexes and cells (e.g., cytotoxicity, interaction with the extracellular matrix, cellular uptake pathway, and intracellular trafficking). The structure of the polycation is a fundamental consideration when synthesizing an efficient gene carrier because the structure affects the other properties of the carrier. Chain length is one such important structural parameter that must be addressed. Generally, polycations with longer chain length mediate higher TE, such as quaternized cellulose (13) and poly(β-

amino ester) (14). However, in the case of chitosan and its derivatives, no consensus has been reached on the relationship between chain length and TE ($R_{CL\text{-}TE}$) to date. On one hand, chitosan with a short chain length (chitosan oligomer) functions better than chitosan with a long chain length (2,15). On the other hand, *N,N,N*-trimethyl chitosans (TMCs) synthesized from both chitosan oligomers (<20 monomer units (5); 3 kDa to 6 kDa (6)) and chitosans with high molecular weight (~100 kDa; (6) ~400 kDa;(4) 30, 100, and 200 kDa (7)) exhibit high TE. Note that in these studies, besides the severe side reactions (3,6-O-methylation) of the TMCs, the final chain lengths of the obtained TMCs have yet to be characterized, resulting in difficulty in establishing the $R_{CL\text{-}TE}$. Moreover, to date, although some studies have addressed the gene delivery mechanism mediated by chitosan and its derivatives (3), detailed investigation of the effects of specific factors on the $R_{CL\text{-}TE}$ is still lacking.

In the present study, four *O*-methyl-free TMCs of various chain lengths were synthesized and used as gene carriers (Fig. 1a) to examine the $R_{CL\text{-}TE}$. We found that longer chain length TMCs (L-TMCs) mediate higher TE than shorter chain length TMCs (S-TMCs). We then investigated a variety of factors to ascertain their effect on the higher TE mediated by L-TMCs. The results suggest that the key factors include polyplex stability, polycation free chain function, the cellular uptake pathway, and intracellular trafficking.

MATERIALS AND METHODS

Materials

Chitosans were purchased from Golden-shell Biochemical Co., Ltd. (Zhejiang, China), and the degree of deacetylation (DDA) was determined by ^{1}H NMR. Plasmid luciferase was amplified in *E. coli* and purified by Plasmid ezFilter maxi kits (Biomiga, USA). Wortmannin and Bafilomycin A1 were from J&K Scientific Co., Ltd.. PEI25k (branched, with average Mw and Mn around 25,000 and 10,000, respectively), heparin sodium salt (≥ 180 USP units/mg), chlorpromazine hydrochloride, monodansylcadaverine, methyl-β-cyclodextrin, Filipin III obtained from Streptomyces filipinensis, genistein, nocodazole, and chloroquine diphosphate salt were from Sigma-Alderich Co. LLC (USA). CCK-8 was purchased from Dojindo Laboratorise, Japan. Label IT tracker™ intracellular nucleic acid localization kits (Cy3 and Cy5) were from Mirus Bio LLC (USA). LysoTracker® Probes labeling kit were supplied by Invitrogen (USA). Other reagents were commercially available and used without further purification if not specified.

Synthesis and Characterization of *O*-Methyl-Free TMCs

O-Methyl-free TMCs were synthesized and characterized as a previous report (11) with some modifications. The details are shown in Supplementary Materials.

Preparation of TMC-Ps

Polyplexes were formed by adding equal volume (200 μl) of polycations to pDNA solution at various N/P ratios (the N/P ratio was calculated as the ratio of the number of amines in the polycation to the number of phosphates in the pDNA), and then the dispersion was mixed by vortex immediately for 15 s. The polyplexes were incubated for at least 30 min at room temperature prior to other experiments. Chitosan was

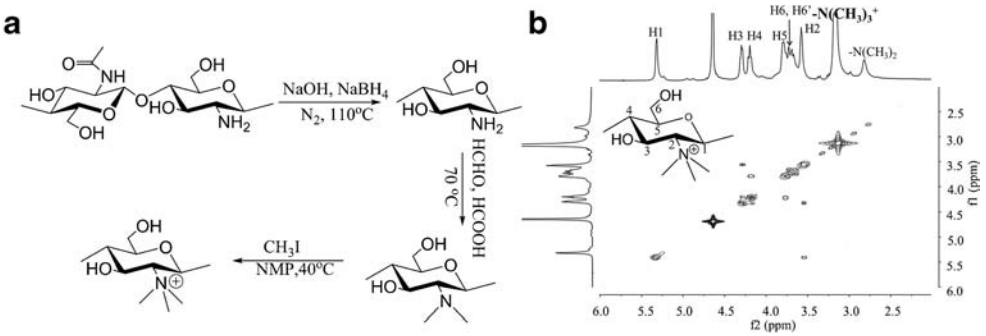

Fig. 1 Synthesis and characterization of *O*-methyl-free TMC. (**a**) Synthetic route for *O*-methyl-free TMC. (**b**) H-H COSY spectra of TMC-2 in D_2O at 293 K. (**c**) Characterization of TMCs synthesized in this study. *DDA* degree of deacetylation, *DDM* degree of dimethylation, *DTM* degree of trimethylation, *Mw* weight average molecular weight, *PDI* polydispersity index.

c

Sample	Raw material	DDA%	DDM%	DTM%	Mw (kDa)	PDI
TMC-1	Chitosan-1	94.1	10.1	85.4	71	2.1
TMC-2	Chitosan-2	100	12.1	89.1	67	2.1
TMC-3	Chitosan-3	93.2	12.9	84.1	27	1.6
TMC-4	Chitosan-4	100	18.5	84.9	19	1.3

dissolved in 20 mM acetate buffering solution (pH 5.5), while TMC and PEI with molecular weight of 25 kDa (PEI25k) were dissolved in deionized water. The final concentration of pDNA was 50 μg/ml.

Gel-Shift Assay

DNA binding affinity was evaluated by gel-shift assay. Polyplexes with various N/P ratios were mixed with 10× loading buffer and then loaded on a 1% (w/v) agarose gel containing ethidium bromide in TAE buffer. The amount of pDNA in each well was 1 μg with a total volume of 40 μl, and the experiment was carried out at 80 V for 1 h. Finally, the DNA bands were imaged using electrophoresis image analysis system (Shanghai Furi Science & Technology Co., Ltd., China). To test the stability of polyplexes in the presence of heparin, various concentration of heparin was added to 40 μl polyplex (containing 1 μg pDNA), and then the mixture was incubated for 2 h at room temperature prior to electrophoresis.

Particle Size and Zeta-Potential

The zeta-potential and particle size of polyplexes were measured at 25°C with a Zetasizer Nano instrument (Malvern Inst. Ltd., UK) equipped with a standard capillary electrophoresis cell and dynamic light scattering (DLS) (He − Ne laser, 633 nm wavelength), respectively. The concentration of pDNA was 5 μg/ml and the total volume was 1 ml. For analysis of particle size, the Stokes-Einstein relationship was used, while for zeta-potential, the Smuloschowshi model was used. To test the stability of polyplexes in DMEM, 800 μl serum-free DMEM was added to 200 μl polyplex (containing 5 μg pDNA), and the hydrodynamic sizes of polyplexes were monitored in time. The measurements were performed in triplicate. Transmission electron microscopy (TEM) samples were prepared by placing the polyplexes on a copper grid, and then the excess liquid was removed with a piece of filter paper. After stained by uranyl acetate and air dried, the samples were visualized with a FEI Tecnai G^2 instrument at 200 kV.

Cell Culture

COS-7 cell was from the cell bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C using a humidified 5% CO_2 incubator.

Luciferase Expression of TMC-Ps

Cells were seeded in 96-well plates at 1×10^4 cells/well (5×10^4 cells/well in 24-well plates) and transfected the next day at 70–80% confluence. Prior to transfection, the media were removed and cells were rinsed twice with 100 μl serum-free DMEM. Cells were then replenished with 100 μl serum-free DMEM (400 μl in 24-well plates) containing polyplexes at various N/P ratios (0.5 μg pDNA/well). At 4 h post-transfection, the transfection media containing test polyplexes were removed, and then the wells were refilled with 200 μl serum-containing DMEM (800 μl in 24-well plates). At 48 h post-transfection, the cells were treated cell lysis buffer after rinse with PBS twice, followed by evaluation of luciferase expression (Promega) and the content of protein (BCA method, Biomega) according to the protocols. As a result, relative light unit per milligram of luciferase protein (RLU/mg) was thus calculated. Luciferase expression in the presence of serum was evaluated in the same procedures as those in the absence of serum, except that serum-containing DMEM was used instead of serum-free DMEM at 0–4 h. To evaluate the effect of incubation time periods of TMC-Ps in DMEM on TE (1 μg pDNA/well in 24-well plates), the TMC-Ps were incubated in DMEM (80 v%) for various time periods prior to addition to cells. To investigate the effect of treating time periods between addition and removal of TMC-Ps on TE (1 μg pDNA/well in 24-well plates), the TMC-Ps were added to cells at 0 h and removed at various time points. To investigate the effect of polycation free chain on luciferase expression (0.5 μg pDNA/well in 96-well plates), the free chains of polycations were added at −4 h prior to or simultaneously with or at 4 h post addition of chitosan-3/DNA polyplex (chitosan-3-P, N/P=6), with total interaction time of 4 h, i.e. the free chains of polycations were removed at 0, 4 and 8 h respectively. In the case of free chains added post addition of polyplexes, the polyplexes were removed at 8 h post-transfection, while in the other two cases, the polyplexes were removed at 4 h post-transfection.

Cytotoxicity of TMC-Ps

Cells were cultured in the same procedures as those evaluating the luciferase expression. At 4 post-transfection or 48 h post transfection, the culture media were replaced with fresh serum-free DMEM. CCK-8 was added in dark according to the protocol, and A450 were tested using an Elx 800 instrument (Biotek, USA) after incubation for 2 h.

Cellular Uptake Efficiency by FCM

Plasmid luciferase was labeled using a Cy5 Label kit according to the protocol. For quantification of cellular uptake efficiency using FCM (BD FACSCalibur), COS-7 cells were seeded in

24-well plates and TMC/Cy5-pDNA polyplexes were used at 1 μg pDNA/well. At various time points post-transfection, the transfection media were removed, followed by washing twice with PBS containing 0.001% SDS to remove the polyplexes remained outside of the cells (16). Afterwards, the cells were detached using trypsin/EDTA, washed twice by PBS containing 0.001% SDS and then once with only PBS. At last, the suspensions of cells were measured on a FCM with excitation at 635 nm, and 10,000 viable cells were evaluated in each experiment. This experiment was carried out in triplicates.

TE in the Presence of Various Inhibitors

The working concentration of inhibitors were as follows: wortmannin, 200 nM; chlorpromazine, 10 μg/ml; monodansylcadaverine, 50 μM; methyl-β-cyclodextrin, 5 mM; genistein, 200 μM; filipin, 0.7 μg/ml; nocodazole, 7.5 μg/ml, which were reported efficient (17,18). For transfection, COS-7 cells were seeded on 24-well plates at 5×10^4 cells/well. At cell density of 70–80% confluence, the cells were washed twice with serum-free DMEM, and then replenished with 400 μl serum-free DMEM containing inhibitors (deemed as −1 h). The cells without treatment with inhibitors were used as control. At 0 h, polyplexes were added at a concentration of 1 μg pDNA/well, followed by incubation for another 2 h in the presence of inhibitors. At 2 h, the transfection media containing test polyplexes were replaced with 400 μl serum-containing DMEM. At 24 h, the expression of luciferase and the content of protein were measured. The cytotoxicity was evaluated under the same conditions at 2 h post-transfection using a CCK-8 method. The transfection in the presence of chloroquine (various concentrations) and bafilomycin (200 nM) was carried out as a previous report (19) in a 24-well plate, and the results were evaluated at 48 h post transfection.

Colocalization by LSCM

For colocalization of TMC-Ps with lysosome, COS-7 cells were seeded on a 35-mm glass bottom culture dish at 2×10^5 cells/well. At ~50% cell confluence, the media was removed and the cells were rinsed twice with transfection media, and then the cells were replenished with 2 ml transfection media containing TMC/Cy3-pDNA polyplexes (2 μg pDNA/well). After 0.5 h, lysotracker was added according to the protocol. At 1 h post-transfection, the media were removed. The cells were washed several times by PBS, followed by refilling the wells with 2 ml serum-free DMEM. Afterwards, the cells were imaged using laser scanning confocal microscope (LSCM, Olympus Fluoview FV1000).

Buffering Capacity by Acid–Base Titration

Buffering capacities for the polycations in solution were analyzed at room temperature by acid–base titration on a Schott Titroline Easy instrument (Germany). The concentration of the polycations was 10 mM based on molecular weight of structural repeating unit. The solution was initially set to pH value of 4.0, and then the solution was gradually titrated with 0.1 M NaOH solution (10 μl per drop). The proton buffering capacity was compared by the volume of NaOH consumed to increase the pH value from 4.5 to 7.2.

Data Analysis

Statistical differences between mean values were investigated using one-way ANOVA analysis and Tukey's test. Differences between group means were considered significant at $p<0.05$ (*) and $p<0.01$ (**).

RESULTS

Synthesis and Characterization of *O*-Methyl-Free TMCs

O-Methyl-free TMCs were successfully synthesized, as characterized by NMR in Fig. 1b. These TMCs were synthesized from chitosan, with *N*,*N*-dimethyl chitosan (DMC) as intermediate. To synthesize TMCs with uniform structures, two chitosan samples (chitosan-2 and chitosan-4) were completely deacetylated, as certified by ^{1}H NMR spectra, where no peak was observed at 2.0 ppm to 2.1 ppm (Fig. S1a). As an intermediate for TMC synthesis, DMCs were obtained by subjecting the chitosans to the Eschweiler-Clarke reaction. In a previous study, this reaction was conducted for 118 h (11). After a detailed investigation of the degree of dimethylation (DDM) *vs.* reaction time in the present work, the highest DDM was found to occur after 48 h, as shown in Fig. S1b. Thus, the TMCs were synthesized from the DMCs obtained at 48 h. Detailed information on the TMCs are shown in Fig. 1c. The four TMCs displayed similar DTM, and their chain lengths decreased in the following order: TMC-1 > TMC-2 > TMC-3 > TMC-4.

Characterization of Polycation/pDNA Polyplexes

Polyplexes were formed by mixing positively charged polycations and negatively charged pDNAs together. As shown in Fig. 2a, pDNA migration was retarded by all of the TMCs at N/P around one, suggesting that the TMCs have similar binding affinities to pDNA, which are stronger than their original chitosan (Fig. S2) and independent of chain length. By contrast, in the case of chitosans, the minimum

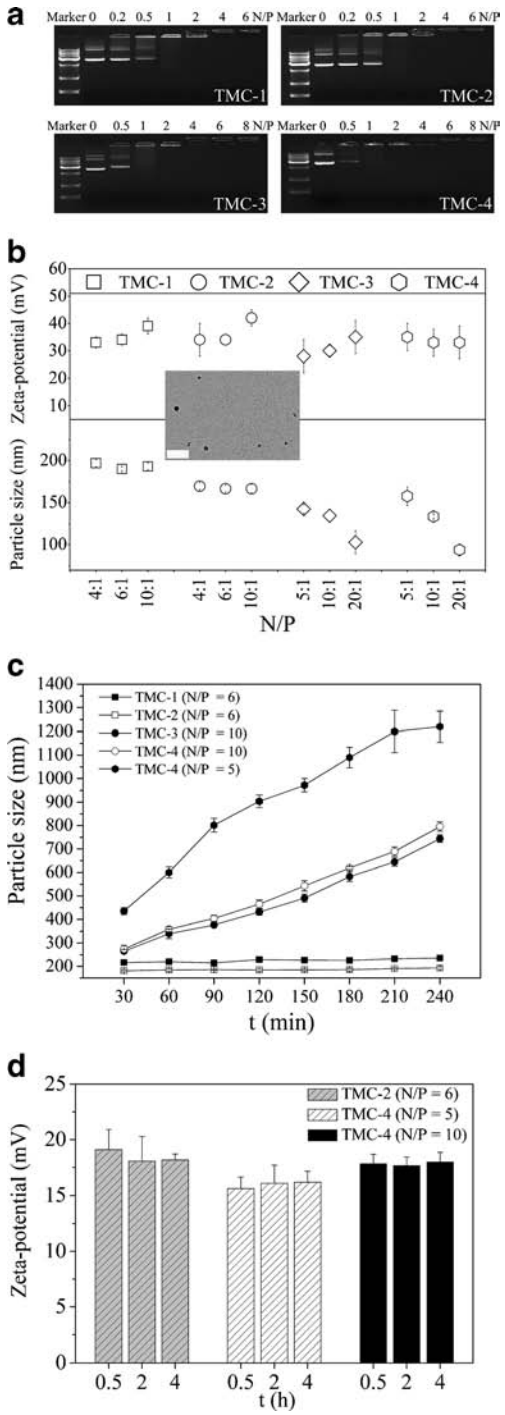

Fig. 2 Physicochemical properties of TMC-Ps. (**a**) DNA binding affinity of TMCs by gel-shift assay. (**b**) Z-Average Particle size as measured by means of DLS and zeta-potential at various N/P ratios (mean ± SD, $n = 3$). Inset in (**b**): TEM image of TMC-2/pDNA polyplex at N/P = 6 (bar = 200 nm). (**c**) Z-Average polyplex sizes *versus* time in serum-free DMEM (80 v%) (mean ± SD, $n = 3$). (**d**) Zeta-potential *versus* time in serum-free DMEM (80 v%) (mean ± SD, $n = 3$).

N/P ratio required to retard the migration of pDNA increased as the chain lengths decreased, which agrees well with the results of previous reports (15,20).

The particle size and zeta-potential were investigated at the N/P ratios used for evaluation of cytotoxicity and TE (Figs. 3 and 4). In comparison with longer chain lengths TMC/pDNA polyplexes (L-TMC-Ps, i.e. TMC-1-P and TMC-2-P), shorter chain length TMC/pDNA polyplexes (S-TMC-Ps, i.e. TMC-3-P and TMC-4-P) were evaluated at higher N/P ratios because their optimal N/P ratios were higher. The particle sizes of L-TMC-Ps are generally greater than those of S-TMC-Ps by DLS, particularly at high N/P ratios (Fig. 2b). Moreover, the particle sizes of the L-TMC-Ps were similar in all the test N/P ratios, whereas the particle sizes of the S-TMC-Ps decreased as the N/P ratio increased. TEM imagery (Fig. 2b inset) shows that the particle size of TMC-2-P is around 50 nm, much smaller than that measured using DLS (around 170 nm) because the dewatering process led to the shrinkage of the TEM samples. Additionally, similar zeta-potentials are observed for both L-TMC-Ps and S-TMC-Ps.

Polyplex stability was investigated in terms of particle size *vs.* time. In Fig. 2c, the particle sizes of S-TMC-Ps in the serum-free Dulbecco's modification of Eagle's medium (DMEM) (i.e., the condition used to evaluate the TE) increased over time as a result of swelling and/or aggregation of polyplexes, whereas the L-TMC-Ps were relatively stable under the same conditions. These results suggest that L-TMC-Ps are more stable than S-TMC-Ps. Moreover, TMC-4-P was more stable at N/P = 10 than at N/P = 5, suggesting that stability increases as the N/P ratio increases. Additionally, the zeta-potentials of both L-TMC-P and S-TMC-P were still similar in DMEM (Fig. 2d), although lower in comparison with those in water due to the interaction between polyplexes and negative-charged components in DMEM.

Cytotoxicity and TE *In Vitro*

The cytotoxicity of TMC-Ps was investigated in serum-free DMEM at both 4 h (Fig. 3a) and 48 h (Fig. 3b) post-transfection, that is, when the polyplexes were removed from the solution and when the TE was evaluated, respectively. The cytotoxicity of TMC-Ps was found to increase with increasing chain length at 4 h post-transfection, presumably because a polycation with a long chain length tends to interact with biological membranes more strongly than one with a short chain length, leading to inhibition of crucial cellular processes (21). Cytotoxicity also increased as the N/P ratio increased. At 48 h post-transfection, cell viability was similar in all cases, indicating that the cells recovered from the initial cytotoxicity at 4 h. This recovery suggested that some cells were possibly not dead but with poor status (less activity of mitochondrion) and less response to CCK-8 at 4 h, but the cell status may change to good after removing the polyplex. Further increase of the dose of polyplexes or the incubation time period may result in the real death of the cells. Therefore,

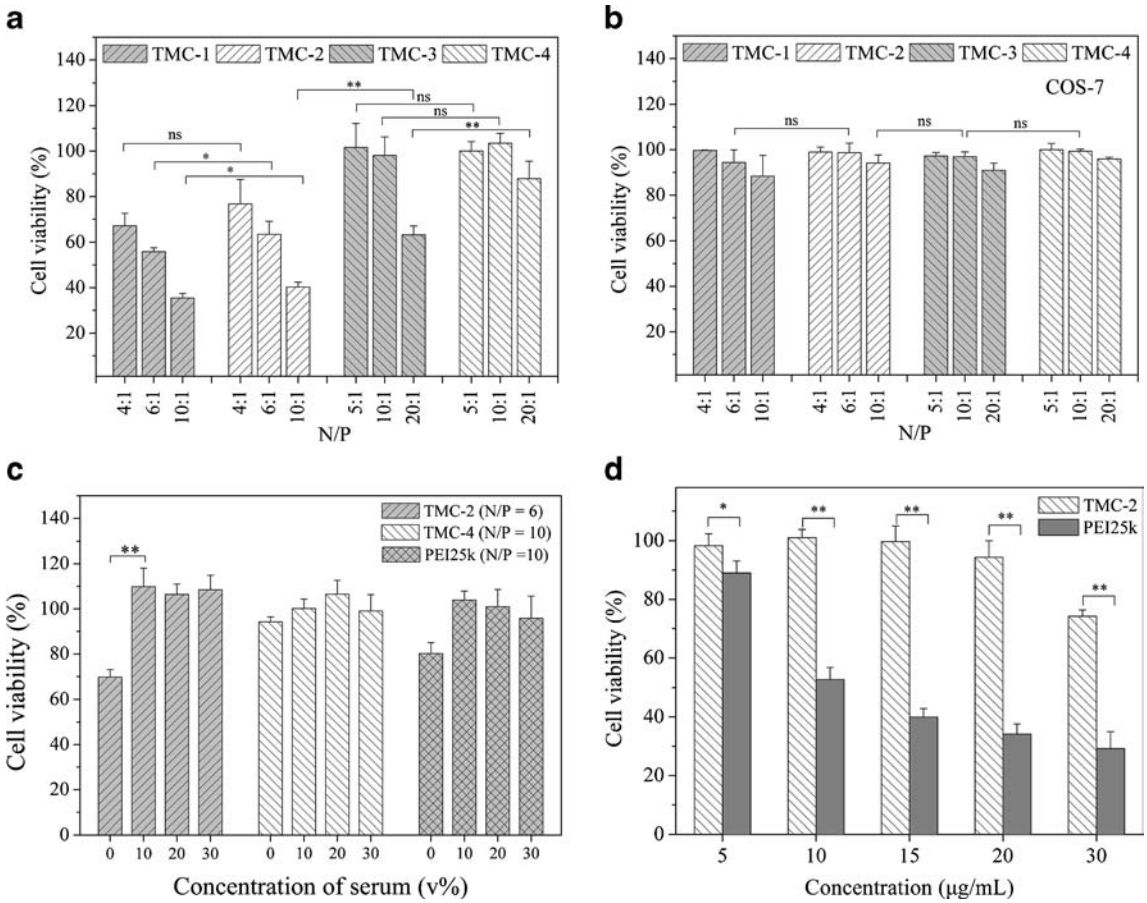

Fig. 3 Cytotoxicity determined using CCK-8 assays in COS-7 cells. (**a**) Cytotoxicity of TMC-Ps in serum-free DMEM at 4 h post-transfection. (**b**) Cytotoxicity of TMC-Ps in serum-free DMEM at 48 h post-transfection. (**c**) Cytotoxicity of TMC-Ps in serum-containing DMEM at 4 h post-transfection. (**d**) Cytotoxicity of TMC-2 and PEI25k in COS-7 cell after incubation for 4 h. Data are expressed as mean ± SD ($n = 4$). Statistical differences are expressed as: *, $p < 0.05$; **, $p < 0.01$; *ns* no significant difference, $p > 0.05$.

cytotoxicity should be tested immediately after removing the polyplexes from the solution, and be evaluated in this way hereafter. The cytotoxicity of the DMEM containing serum apparently decreased in comparison with that in the serum-free DMEM, particularly for L-TMC-P (Fig. 3c). Moreover, the cytotoxicities of TMC-2 and PEI25k were evaluated after incubation with cells for 4 h (Fig. 3d). TMC-2 generally exhibits lower cytotoxicity than PEI25k at the same concentrations, but this does not mean that TMC-2 is better than PEI25k in terms of cytotoxicity because the polycation concentrations required to reach the optimal TE are not identical. In this study, at their optimal N/P for TE, the TMC-2-Ps (N/P=6; 21.3 μg polycation/ml) exhibited cytotoxicity no lower than the cytotoxicity of the PEI25k/pDNA polyplex (PEI25k-P; N/P=10; 6.5 μg polycation/ml).

The TE of the TMC-Ps was evaluated in COS-7 cells. As shown in Fig. 4a, in serum-free DMEM, the TE decreases in the following order: PEI25k>TMC-1≈TMC-2>TMC-3>TMC-4, indicating that a longer chain length is better than a short one. This trend is also confirmed by the luciferase expression in the NCI-H1299 cell and the 293T cell, and by the enhanced green fluorescent protein (EGFP) expression in the 293T cell (Fig. S3). Moreover, the TE of the TMC-Ps is cell line-dependent, as evidenced by the observation that luciferase expression and optimal N/P ratio vary among the cell lines. This result is consistent with those of previous reports (4,6). In addition, compared with TMC-2, the higher cytotoxicity of TMC-1 may explain the similar TE observed between TMC-1- and TMC-2-mediated gene delivery, suggesting that further increases in chain length may result in severe cytotoxicity and even decreased TE. In serum-containing DMEM, the TE of the polyplexes, except for TMC-2 at lower serum concentrations, generally decrease as serum concentration increases (Fig. 4b). The negative effect of serum on the TE increases in the following order: TMC-2-P <TMC-4-P <PEI25k-P. In the case of TMC-2 at lower serum concentrations, the TE is not apparently affected by serum, possibly as a result of decreased cytotoxicity (Fig. 3d).

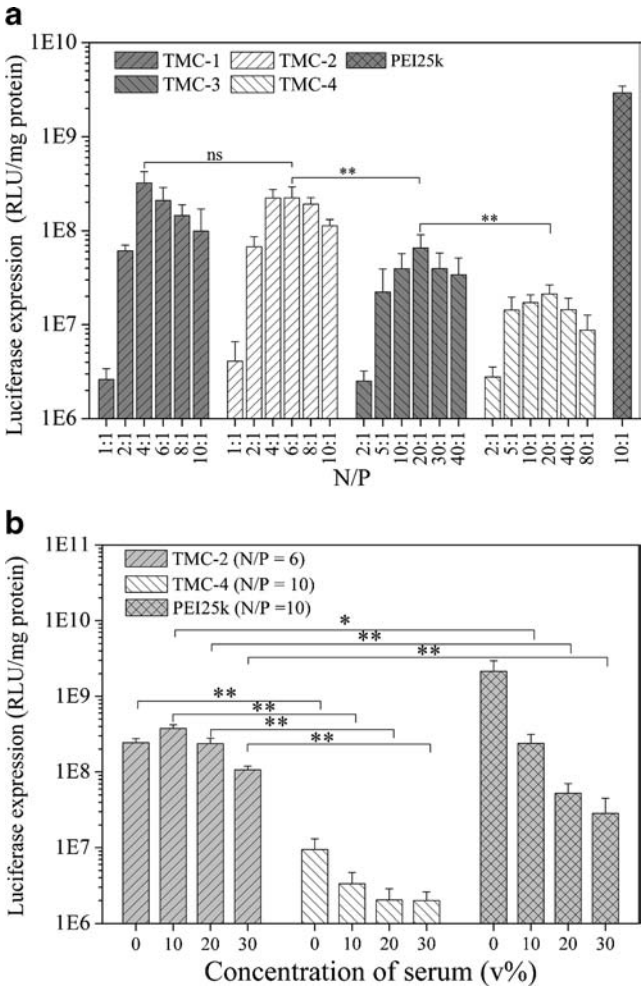

Fig. 4 TE of TMC-Ps in COS-7 cells. (**a**) Luciferase expression in serum-free DMEM at 48 h post-transfection. (**b**) Luciferase expression in serum-containing DMEM at 48 h post-transfection. Data are expressed as mean ± SD ($n = 4$). Statistical differences are expressed as: *, $p < 0.05$; **, $p < 0.01$; *ns* no significant difference, $p > 0.05$.

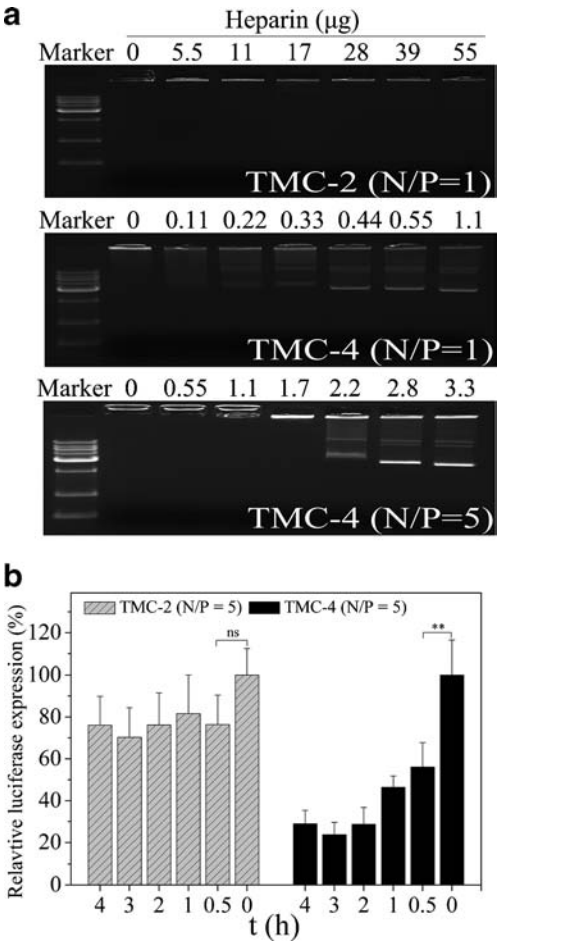

Fig. 5 Effect of TMC-Ps stability on TE. (**a**) TMC-Ps stability in the presence of heparin measured by gel-shift assay. (**b**) Effect of incubation time of TMC-Ps in DMEM on TE (mean ± SD, $n = 3$). The polyplexes were incubated in DMEM for various time periods prior to transfection. The TE was evaluated at 48 h post-transfection and the results were normalized to the luciferase expression of TMC-P without incubation ($t = 0$). Statistical differences are expressed as: **, $p < 0.01$; *ns* no significant difference, $p > 0.05$.

Effect of Polyplex Stability on TE

TMC-P stability was evaluated by means of heparin replacement, given that heparin is capable of displacing pDNA from polyplexes with high efficiency (4). As shown in Fig. 5a, DNA migration is observed in TMC-4-P (N/P = 1) in the presence of 0.22 μg heparin, whereas no migration is observed in TMC-2-P (N/P = 1) even in the presence of 55 μg heparin, indicating that TMC-2 can form a more stable polyplex with pDNA than TMC-4. This result is in good agreement with the stability of TMC-Ps in DMEM (Fig. 2c), where TMC-2-P is more stable than TMC-4-P. Moreover, DNA migration is observed in TMC-4-P (N/P = 5) with heparin amounts exceeding 1.7 μg, suggesting greater amounts of polyanions are required to replace pDNA in polyplexes at higher N/P ratios. Furthermore, luciferase expression was evaluated by incubating polyplexes for various time periods in DMEM prior to transfection. As shown in Fig. 5b, no evident decrease in luciferase expression is observed in TMC-2-P after incubation in DMEM for 4 h prior to transfection, whereas an apparent decrease is observed in TMC-4-P after incubation in DMEM for only 0.5 h, implying the importance of polyplex stability in efficient gene transfection.

Effect of Polycation Free Chains on TE

In the present study, the effects of free chains on the TE was investigated by adding TMC-2, TMC-4, and PEI25k free chains to a same polyplex at various time points. Here, a chitosan/pDNA polyplex, chitosan-3-P (N/P = 6, i.e. the minimum N/P ratio required to completely neutralize and condense pDNA) (Fig. S3) was chosen to make a distinction between polyplex and polycation free chain. As shown in Fig. 6, all of the free chains can increase the TE of chitosan-

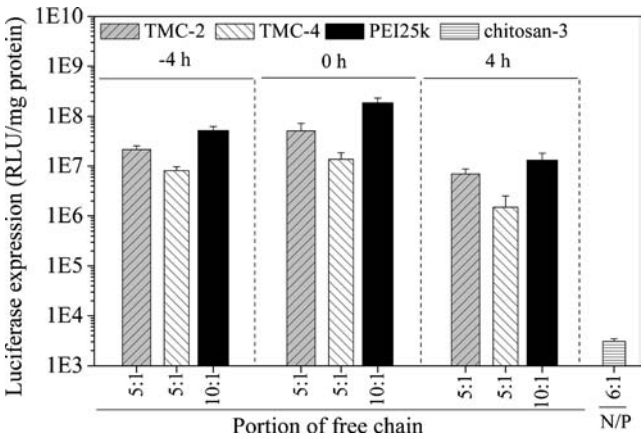

Fig. 6 Effect of polycation free chains on TE of chitosan-P. The time point of the addition of polyplex was designated as 0 h. TMC free chains were added at −4, 0, and 4 h, and then removed after treating cells for 4 h. Luciferase expression was evaluated at 48 h post-transfection. Data are expressed as mean ± SD, $n = 4$.

3-P regardless of the time of addition. The highest TE increase is observed in the simultaneous addition (0 h) of the free chains to chitosan-3-P. Addition of the free chains at −4 h and 4 h results in smaller TE increases. Moreover, in all of the cases, the free chains function in the order of PEI25k>TMC-2> TMC-4, which shares the trend of the TE of their polyplexes (Fig. 4).

Effect of Cellular Uptake Efficiency of Polyplex on TE

The cellular uptake efficiency of TMC-Ps was evaluated by flow cytometry (FCM) (Fig. 7a). The mean fluorescence intensity of the plasmid-labeled TMC-Ps increases over time, and is higher for TMC-2-P than in TMC-4-P at each time point. This is also confirmed by laser scanning confocal microscopy (LSCM) images (Fig. S4). Figure 7b shows the relative luciferase expression at various treatment time periods between the addition and removal of the TMC-Ps on TE. The TE of the TMC-Ps removed at $t = 4$ h is higher than that of the TMC-Ps removed at $t = 0.5$ h, indicating that incubation time and cellular uptake efficiency may affect TE. However, different from cellular uptake efficiency, no further increase in TE is found after the removal of TMC-2-P at t≥1 h and of TMC-4-P at t≥2 h, suggesting that the polyplexes internalized after a certain time no longer enhance gene transfection.

Investigation of Cellular Uptake Pathway and Intracellular Trafficking of TMC-Ps

Luciferase expression was evaluated in the presence of various inhibitors to determine the differences between L-TMC-Ps and S-TMC-Ps in terms of cellular uptake pathways and intracellular trafficking. Considering that cytotoxicity of

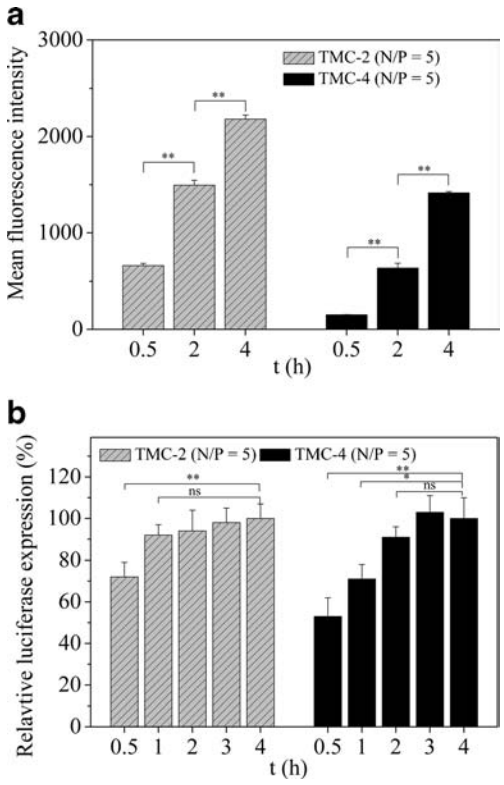

Fig. 7 Effect of cellular uptake efficiency of TMC-Ps on TE. (**a**) Mean fluorescence intensity of TMC/Cy5-labeled pDNA polyplexes at various time points post-transfection in COS-7 cells, measured by FCM (mean ± SD, $n = 3$). (**b**) Effect of treatment time periods between addition and removal of TMC-Ps on TE (mean ± SD, $n = 3$). Polyplexes were added at 0 h and removed at various time points, followed by the addition of serum-containing DMEM. Luciferase expression was evaluated at 48 h post-transfection and the results were normalized to the luciferase expression of TMC-P removed at $t = 4$ h. Statistical differences are expressed as: *, $p < 0.05$; **, $p < 0.01$; *ns* no significant difference, $p > 0.05$.

polyplexes could increase in the presence of an inhibitor (22), the cytotoxicity of a polyplex plus an inhibitor was measured under the same conditions. Figure 8a shows the following results: 1) Using wortmannin (to inhibitor macropinocytosis (17)), the TE of TMC-2-P and TMC-4-P is similar to that without inhibitors, and no apparent difference is observed between TMC-2-P and TMC-4-P. 2) Using chlorpromazine and monodansylcadaverine (to inhibit clathrin-mediated endocytosis endocytosis (CME) (17)), the TE of TMC-2-P increases, whereas that of TMC-4-P decreases. The increase of TE in the presence of CME inhibitors was also observed in literature (26), suggesting that the uptake pathways of polyplexes are probably interchangeable, i.e. inhibition of one pathway may increase the utilization of the others. 3) Using methyl-β-cyclodextrin (to inhibit lipid raft-mediated endocytosis (LME) (17)), filipin III and genistein (to inhibit caveolae-mediated endocytosis (CvME) (17,23)), the TE of both TMC-Ps decreases and no apparent difference was observed between them (in the case of filipin, the higher cytotoxicity of TMC-2-P should be considered). 4) Using

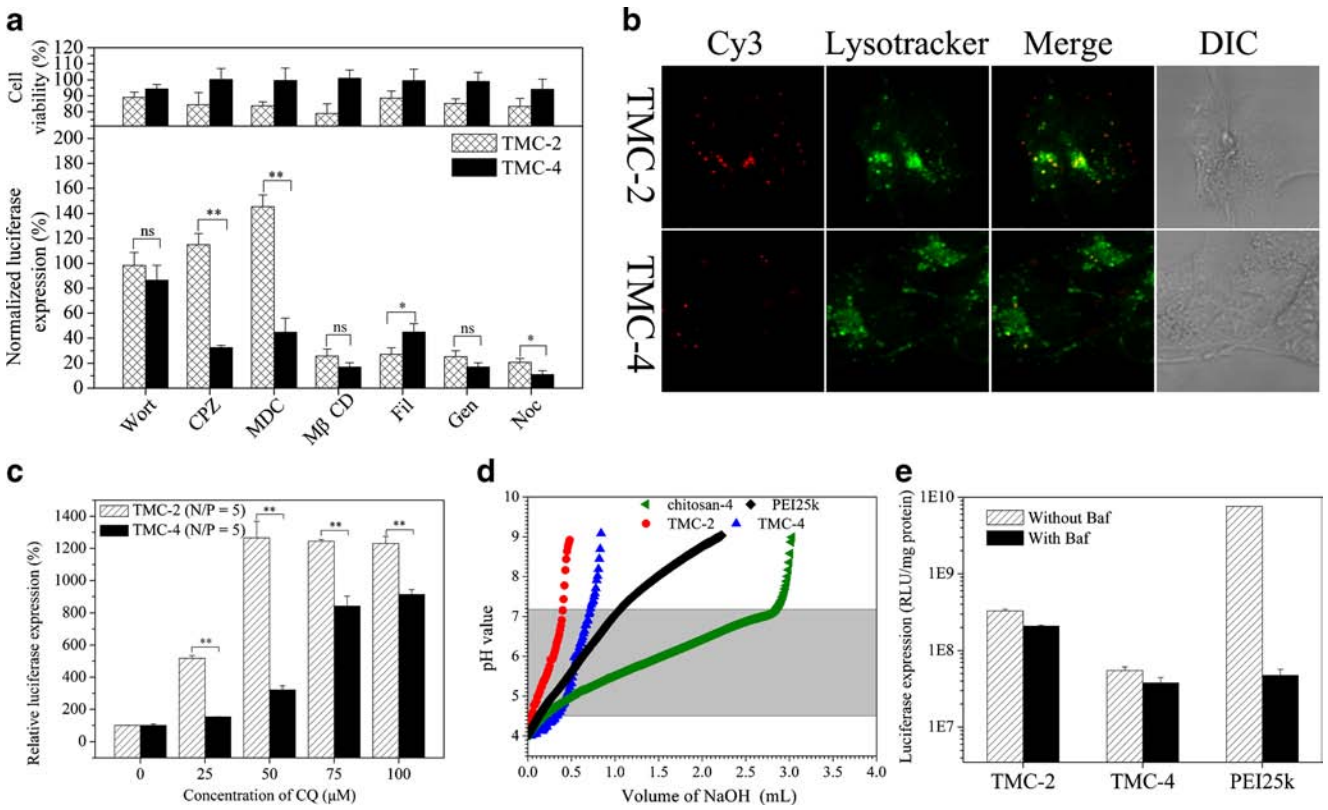

Fig. 8 Investigation of cellular uptake pathway and intracellular trafficking. (**a**) Cell viability and relative luciferase expression of TMC-Ps in the presence of various inhibitors. Among the inhibitors, wortmannin (Wort) was used to inhibit macropincocytosis; chlorpromazine (CPZ) and monodansylcadaverine (MDC) to inhibit CME; methyl-β-cyclodextrin (MβCD) to inhibit LME; filipin (Fil) and genistein (Gen) to inhibit CvME; nocodazole (Noc) to inhibit formation of microtubule. Cell viability was evaluated at 2 h post-transfection using cells without adding polyplex and inhibitors as control. The luciferase expression was evaluated at 24 h post-transfection, and the results were normalized to the expression mediated by the polyplex without the presence of inhibitors. (**b**) Colocalization of TMC-Ps with lysosome by LSCM. *Red*: Cy3-labeled pDNA; *green*: lysotracker-labeled lysosome. (**c**) TE in the presence of chloroquine (CQ). The results were normalized to that without addition of CQ. (**d**) Buffering capacity of polycations determined by acid–base titration. (**e**) Inhibitory effect of bafilomycin (Baf) on luciferase expression. Data are expressed as mean ± SD, $n = 3$. Statistical differences are expressed as: *, $p < 0.05$; **, $p < 0.01$; *ns* no significant difference, $p > 0.05$.

nocodazole (to inhibit microtubule formation (24)), the TE of both TMC-Ps decreases; however, the effect on the TE of TMC-2-P is less than on that of TMC-4-P.

To further investigate cell trafficking of TMC-Ps, live cells at 1 h post-transfection were imaged using LSCM. As shown in Fig. 8b, a part of the polyplexes are co-localized with the lysosome in both the cases of TMC-Ps, indicating that TMC-Ps could be trapped in lysosome regardless of the polycation chain length. In the presence of CQ (chloroquine, a fusogenic agent to buffer acidic vesicles and promote endolysosomal release (19)), gene expression significantly increased for both TMC-Ps and its effect was better for TMC-2-P than TMC-4-P (Fig. 8c), indicating that more TMC-2-Ps are presumably trapped in lysosome and fail to deliver the DNA cargo to the nucleus. In previous reports (19,25), a "proton sponge" mechanism (the buffering capacity of polycations leads to osmotic swelling and the rupture of endolysosomes) is used to explain the release of the trapped polyplexes from the endolysosome that was formed from polycations with high buffering capacity. Herein, buffering capacity was tested by acid–base titration. In the pH range between 4.5 and 7.2, both TMCs showed similar and limited buffering capacity, significantly lower than PEI25k (Fig. 8d). This result indicates that buffering capacity does not account for the less trapped TMC-4-P in the endolysosome. Moreover, only slight decrease of luciferase expression in the presence of Bafilomycin (a vacuolar proton pump inhibitor (19)), was observed for both TMC-2 and TMC-4 in comparison with PEI25k (Fig. 8e), further confirming that the less trapped TMC-4-P is not related to the "proton sponge" mechanism. Additionally, because the polyplexes internalized by CvME are possibly trafficked to Golgi and ER rather than trapped in the endolysosome (26), we evaluated the route through ER by LSCM. As shown in Fig. S5, a number of polyplexes are trafficked to ER, suggesting this route as a possible route for efficient gene delivery.

DISCUSSION

Among the numerous polycations used as gene carriers, ones with relatively long chain lengths are advantageous for gene expression (14). However, it seems that chitosan and TMC are

exceptions (2,4–7,15). In the case of chitosan, we presumed that this is due to the solubility of chitosan under the test conditions (cell culture media at pH around 7.2). The solubility of chitosans, particularly that with long chain lengths, is governed by the protonation of the amino groups. Thus, these chitosans are only soluble under acidic conditions. By contrast, chitosans with short chain lengths (chitosan oligomers) are soluble at pH around 7.2 (2,27). In the case of TMC, it is readily soluble in water over the whole pH range (4,5). Thus, TMC is an ideal material to investigate the $R_{CL\text{-}TE}$. However, most of the previous studies were based on TMCs with poorly defined structure such as a partial O-methylation (4,5,7), which negatively contributed to establishing a clear structure–function relationship. For example, both TMCs synthesized from chitosan with long chain length (~100 kDa) and short chain length (3–6 kDa) were reported to exhibit high TE, and the latter was even better in COS-7 cell (6), but the structure of TMCs was poorly characterized (without characterization of O-methylation and the molecular weight of TMC), and all the experiments including the control group PEI25k were conducted at the same ratio (10:1 w/w) of polycation to DNA (the optimal ratio of polycation to DNA may change as the TMC chain length varies, such as shown in Fig. 4a and Fig. S3 in this work). In the present study, well-defined O-methyl free TMCs with four chain lengths were synthesized (Fig. 1) and used as gene carriers. These TMCs have similarly high degree of trimethylation and low degree of acetylation. In two cases (i.e., TMC-2 and TMC-4), TMCs were from completely deacetylated chitosan to avoid the possible effects of acetylation on TE. These two TMCs were then used to investigate the factors affecting $R_{CL\text{-}TE}$. The TE mediated by L-TMCs is higher than that mediated by S-TMCs (Fig. 4a and Fig. S3), confirming that a relatively long chain length facilitates gene transfection.

To elucidate $R_{CL\text{-}TE}$, various factors were investigated to ascertain the key ones. The interactions among factors and their effects on one another, such as the stability of polyplexes probably affects the polyplex size and cellular uptake pathway, were also taken into consideration.

Polycation–DNA Binding Affinity (Fig. 2a)

The binding affinity is evaluated by gel-shift assay, where the migration of pDNA can be completely retarded if a stable complex is formed between polycation and pDNA. In the present work, all the TMCs can retard the migration of pDNA at N/P around 1, indicating that polycation–DNA binding affinity is not a key factor in $R_{CL\text{-}TE}$.

Particle Size in Water (Fig. 2b)

Particle size can affect a series of physiological processes and small size is generally believed to benefit for a rapid entry into cells (28,29). Herein, S-TMC-Ps exhibit smaller particle sizes than L-TMC-Ps in water. Considering that the TE of S-TMC-Ps is lower, this smaller size of S-TMC-Ps is not a key factor in the transfection performance. The particle size of S-TMC-Ps is also noted to decrease as the N/P ratio increases. In turn, such decrease of particle size does not result in the changes of TE, further confirming that the particle size in water is not significantly related to the terminal TE. Moreover, the particle size of polyplexes may vary in different solvents (29). By evaluating the stability of polyplexes in DMEM (Fig. 2c), we found that the sizes of S-TMC-Ps become larger over time, whereas those of L-TMC-Ps do not. The size of S-TMC-Ps in DMEM, rather than that in water, may be more important for biophysical process in these *in vitro* experiments. Additionally, L-TMC-Ps show higher cellular uptake efficiency than S-TMC-Ps (Fig. 7a), indicating that the size of S-TMC-Ps in water does not contribute to efficient cellular uptake and further confirming the importance of the polyplex size in DMEM.

Zeta-Potential (Fig. 2b and d)

A positive charge is essential for nontargeting polyplexes because polyplexes associate with cells through electrostatic interactions with anionic cell surface proteoglycans (30). Both of L-TMC-Ps and S-TMC-Ps have similar positive charges in either water or DMEM, suggesting that zeta-potential is not a key factor in $R_{CL\text{-}TE}$. Furthermore, we speculated that the charge potential mainly influences the initial interaction between polyplex and cell membranes, but for efficient cellular uptake, the particle size may play a dominant role, because with similar zeta-potential in DMEM, the cellular uptake efficiency of L-TMC-P is evidently higher than S-TMC-P (Fig. 7).

Stability of Polyplex (Figs. 2c and 5)

High stability of polyplex means that the binding force between polycation and pDNA is strong enough to prevent substitution by other charged macromolecules, and that the polyplex is stable to prevent swelling and aggregation. High stability is important to ensure that the DNA stays protected from degradation and has high cellular uptake efficiency (31). It should be noted that the high binding force reflected by stability of polyplexes is not the same as high polycation-DNA binding affinity, because the former means that the polyplex is stable enough to resist the external environment whereas the later merely means that the DNAs are successfully assembled into the polyplex. By testing particle size *vs.* time in DMEM and the replacement effect of pDNA in the presence of heparin, we found that L-TMC-Ps are more stable than S-TMC-Ps, suggesting that polyplex stability may be a key factor in $R_{CL\text{-}TE}$. Moreover, this stability, aside from its protective

effect on the DNA, potentially affects TE through the polyplex size given that the polyplex size of S-TMC-Ps changes in DMEM due to swelling and aggregation. Additionally, although balancing between protection and release of DNA is believed to be essential for efficient gene delivery (32), the polyplex should be stable before cellular uptake at least. Thus, L-TMC-Ps may be more favorable for further applications.

Cytotoxicity (Fig. 3)

Low cytotoxic and highly efficient gene carriers are the aim of numerous state-of-the-art polymers designed in the field. Longer chain length generally associates with high cytotoxicity (33), which is also the case in this work. However, this association is apparently not a key factor in $R_{CL\text{-}TE}$ because higher cytotoxicity only negatively contributes to the TE. Moreover, the high cytotoxicity of polycation with a long chain is expected to be caused by its high charge density (21), and this high cytotoxicity can be counteracted by linking the short chain polycations together (34), further implying that cytotoxicity is not a key factor. Additionally, in the presence of serum, the cytotoxicity of L-TMC-Ps decreases and becomes similar to that of S-TMC-Ps, while the TE of L-TMC-Ps were still higher than that of S-TMC-Ps, also confirming that cytotoxicity is not a big issue in $R_{CL\text{-}TE}$.

Serum (Fig. 4b)

Serum possibly inhibits the TE of gene carriers, e.g. PEI (35). Herein, the gene expression mediated by PEI25k (N/P = 10) is also found to decrease in the presence of serum. In the case of TMCs, L-TMC-Ps retain higher TE than S-TMC-Ps in the presence of serum, indicating that serum may be an insignificant factor. Moreover, the TE of L-TMC-Ps is less affected by serum than S-TMC, which may be associated with its higher stability.

Polycation Free Chains (Fig. 6)

The N/P ratio of polycations to pDNA greatly affects the TE of the polyplexes, as shown in Fig. 4. In most cases, pDNA can be neutralized and condensed by polycations at relatively low N/P ratios, but efficient TE is obtained only at high N/P ratios (2,36,37). Excess polycation chains are unbound from the polyplex and remain free in the solution (38,39). These free chains may affect the TE and the corresponding mechanism (1,33,40,41). Herein, the TMC free chains remarkably enhance the TE of chitosan-P, and the effect of L-TMC is stronger than S-TMC. A number of interactions (such as replacement of chitosan by TMC to form a more stable polyplex and adsorption of TMC on the swelled and/or aggregated chitosan-Ps in DMEM) between chitosan and TMC free chains cannot be ruled out in the case of free chains added at 0 h and at 4 h. However, a similar trend of enhanced TE is observed in the case of the free chains added at −4 h and removed at 0 h during which no direct interaction could happen. These results suggest that the function of free chains is very crucial in $R_{CL\text{-}TE}$. In view of polycation/pDNA complexes composed of polyplexes and free polycations, polycations as gene carriers are better understood as follows: Free chains enhance the stability of polyplexes, which is confirmed by the fact that TMC-4-P is more stable at higher N/P ratio and an increased number of heparin is required to replace pDNA from TMC-4-P at high N/P ratio. Unlike the complexed ones, free chains, particularly the long ones, lead to strong cytotoxicity as claimed in previous reports (33,42). Moreover, these free chains promote the cellular uptake, but the enhanced cellular uptake efficiency does not directly contribute to TE (1,33). Furthermore, free chains may affect intracellular trafficking and facilitate the release of polyplexes from endolysosome (28,33,41). Nevertheless, systematic investigations are still required to completely understand the function of free polycations, particularly their interaction with polyplexes and their effects on organelles. Also the *in vivo* performance of free chains should be carefully evaluated because free chains may be a key factor in determining the different behavior of polyplexes between *in vitro* and *in vivo*, particularly those with optimal TE at high N/P ratio *in vitro*.

Cellular Uptake Efficiency (Fig. 7)

Although the cellular uptake efficiency of L-TMC-Ps is higher than that of S-TMC-Ps at the same time points, this higher uptake efficiency of L-TMC-Ps is not essentially related to its higher TE, because with the similar uptake efficiency (e.g. TMC-2-P at $t = 2$ h and TMC-4-P at $t = 4$ h), the TE of the former is significantly higher than that of the latter. This is consistent with a previous study (33). Moreover, the polyplexes internalized after certain time points no longer contribute to gene expression. It should be noted that these time points are before that with the highest cellular uptake efficiency. This result indicates that the ability to efficiently use a certain type of polyplex may be "saturated" (presumably due to the limitation of intracellular trafficking), and/or that the polyplexes internalized after these times are difficult to be efficiently used intracellularly (possibly due to large polyplex size caused by swelling and aggregation in DMEM).

Cellular Uptake Pathway and Intracellular Trafficking (Fig. 8)

Particles are internalized by cells through pinocytosis and phagocytosis, and primarily through the former in non-phagocytes (28). Pinocytosis entails CME and clathrin-independent endocytosis, and the latter is further classified into CvME, macropinocytosis, and caveolae-/clathrin-

independent endocytosis (28). Given that many cellular uptake pathways, such as CvME, are associated with the lipid-raft domain in the cell membrane, El-Sayed and Harashima (43) suggested to classify the endocytic pathways based on the membrane regions that make up the primary endocytic vesicles. Among these cellular uptake pathways, macropinocytosis, CME, and CvME are commonly evaluated for polyplexes (22,26,44–46). In the present study, these three cellular uptake pathways were investigated by evaluating the TE in the presence of various inhibitors. CME and CvME, rather than macropinocytosis, are associated with TE. Interestingly, unlike the TE of L-TMC-Ps, the TE of S-TMC-Ps is more affected by CME. We then investigated intracellular routes because polyplexes internalized by different cellular uptake pathways may vary in terms of intracellular trafficking (23). In previous studies (47,48), internalized polyplexes are found to use microtubule motors for intracellular trafficking. In this work, the transport of both L-TMC-Ps and S-TMC-Ps is found to be related to microtubules. The transport of S-TMC-Ps has a more significant correlation to microtube compared to that of L-TMC-Ps, suggesting their difference in intracellular trafficking. The intracellular fate of the clathrin-coated vesicles through CME are as follows: early endosome (pH 6.1–6.8); maturation into late endosome (pH 4.8–6); and finally maturation into lysosome (pH ~4.5) (23,28,43). The vesicles are acidified by proton pumps during the maturation process from early to late endosome, and during this acidification, some of the cargoes are recycled back to the cell exterior through recycling endosomes. Some gene carriers with fine buffering capacity, such as PEI, are believed to escape from the endolysosome by means of a "proton sponge" mechanism (25,46). However, this is apparently not the case for TMCs because both L-TMC and S-TMC show similarly low buffering capacity in the pH range of 4.5 to 7.2 and the luciferase expression of TMC-Ps is only slightly inhibited by bafilomycin in comparison with PEI25k, but S-TMC-Ps utilize CME as a productive cellular uptake pathway whereas L-TMC-Ps do not. Thus, the escape from endolysosomes may be due to other reasons. Actually, this "proton sponge" mechanism is also challenged in previous reports (16,49). As for the vesicles formed through CvME, their following intracellular fate is yet not completely clear, but the issue has attracted intense research attention owing to the ability of these vesicles to bypass endolysosomes, such as transferring the cargoes loaded in these vesicles to the ER (26,28). In this study, both L-TMC-Ps and S-TMC-Ps utilize CvME as a productive cellular uptake pathway and at least part of their pDNA cargo is transported to the ER (Fig. S5). Combining these results, these differences in cellular uptake pathway and intracellular trafficking of TMC-Ps may play an important role in $R_{CL\text{-}TE}$, although the detailed mechanism for escaping from and/or utilizing these vesicles remains to be addressed. In our view, this probably involves polyplex stability in cell culture media (i.e. the actual size and charge intensity of the internalized polyplexes including aggregates) and the function of free chains.

CONCLUSION

We have synthesized four well-defined TMCs and used them as gene carriers. L-TMCs generally mediate higher TE than S-TMCs. We have also investigated a number of factors affecting the $R_{CL\text{-}TE}$. Although some factors, such as cytotoxicity and cellular uptake efficiency, significantly influence the performance of polycations as gene carriers, they are apparently not crucial to determine the higher TE of L-TMC-Ps compared with that of S-TMC-Ps. The key factors include polyplex stability, function of polycation free chains, cellular uptake pathway, and intracellular trafficking, rather than polycation-DNA binding affinity, polyplex particle size in water, zeta-potential, serum, cytotoxicity, and cellular uptake efficiency. By clarifying these factors, the present work provides insight into the rational design of chitosan-based polycations as gene carriers.

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REFERENCES

1. Yue Y, Jin F, Deng R, Cai J, Chen Y, Lin MCM, *et al*. Revisit complexation between DNA and polyethylenimine — Effect of uncomplexed chains free in the solution mixture on gene transfection. J Control Release. 2011;155:67–76.
2. Strand SP, Lelu S, Reitan NK, de Lange Davies C, Artursson P, Vårum KM. Molecular design of chitosan gene delivery systems with an optimized balance between polyplex stability and polyplex unpacking. Biomaterials. 2010;31:975–87.
3. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev. 2010;62:12–27.
4. Germershaus O, Mao S, Sitterberg J, Bakowsky U, Kissel T. Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: establishment of structure-activity relationships in vitro. J Control Release. 2008;125:145–54.
5. Thanou M, Florea BI, Geldof M, Junginger HE, Borchard G. Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. Biomaterials. 2002;23:153–9.
6. Kean T, Roth S, Thanou M. Trimethylated chitosans as non-viral gene delivery vectors: cytotoxicity and transfection efficiency. J Control Release. 2005;103:643–53.
7. Zhao X, Yin L, Ding J, Tang C, Gu S, Yin C, *et al*. Thiolated trimethyl chitosan nanocomplexes as gene carriers with high in vitro and in vivo transfection efficiency. J Control Release. 2010;144:46–54.

8. Mao S, Shuai X, Unger F, Wittmar M, Xie X, Kissel T. Synthesis, characterization and cytotoxicity of poly(ethylene glycol)-graft-trimethyl chitosan block copolymers. Biomaterials. 2005;26:6343–56.
9. Domard A, Rinaudo M, Terrassin C. New method for the quaternization of chitosan. Int J Biol Macromol. 1986;8:105–7.
10. Sieval AB, Thanou M, Kotze AF, Verhoef JC, Brussee J, Junginger HE. Preparation and NMR characterization of highly substitutedN-trimethyl chitosan chloride. Carbohyd Polym. 1998;36:157–65.
11. Verheul RJ, Amidi M, van der Wal S, van Riet E, Jiskoot W, Hennink WE. Synthesis, characterization and in vitro biological properties of O-methyl free *N*, *N*,*N*-trimethylated chitosan. Biomaterials. 2008;29:3642–9.
12. Jintapattanakit A, Mao S, Kissel T, Junyaprasert VB. Physicochemical properties and biocompatibility of N-trimethyl chitosan: effect of quaternization and dimethylation. Eur J Pharm Biopharm. 2008;70:563–71.
13. Song Y, Wang H, Zeng X, Sun Y, Zhang X, Zhou J, *et al*. Effect of molecular weight and degree of substitution of quaternized cellulose on the efficiency of gene transfection. Bioconjug Chem. 2010;21:1271–9.
14. Eltoukhy AA, Siegwart DJ, Alabi CA, Rajan JS, Langer R, Anderson DG. Effect of molecular weight of amine end-modified poly(β-amino ester)s on gene delivery efficiency and toxicity. Biomaterials. 2012;33:3594–603.
15. Köping-Höggård M, Vårum KM, Issa M, Danielsen S, Christensen BE, Stokke BT, *et al*. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. Gene Ther. 2004;11:1441–52.
16. Forrest ML, Pack DW. On the kinetics of polyplex endocytic trafficking: implications for gene delivery vector design. Mol Ther. 2002;6:57–66.
17. Ivanov AI. Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? Methods Mol Biol. 2008;440:15–33.
18. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, *et al*. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem. 1987;262:5592–5.
19. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med. 2005;7:657–63.
20. Romøren K, Pedersen S, Smistad G, Evensen Ø, Thu BJ. The influence of formulation variables on in vitro transfection efficiency and physicochemical properties of chitosan-based polyplexes. Int J Pharm. 2003;261:115–27.
21. Mastrobattista E, Hennink WE. Polymers for gene delivery: charged for success. Nat Mater. 2012;11:10–2.
22. von Gersdorff K, Sanders NN, Vandenbroucke R, De Smedt SC, Wagner E, Ogris M. The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther. 2006;14:745–53.
23. Xiang S, Tong H, Shi Q, Fernandes JC, Jin T, Dai K, *et al*. Uptake mechanisms of non-viral gene delivery. J Control Release. 2012;158:371–8.
24. Peterson JR, Mitchison TJ. Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton. Chem Biol. 2002;9:1275–85.
25. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA. 1995;92:7297–301.
26. Reilly MJ, Larsen JD, Sullivan MO. Polyplexes traffic through Caveolae to the Golgi and endoplasmic reticulum en route to the nucleus. Mol Pharm. 2012;9:1280–90.
27. Pillai CKS, Paul W, Sharma CP. Chitin and chitosan polymers: chemistry, solubility and fiber formation. Prog Polym Sci. 2009;34:641–78.
28. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. J Control Release. 2010;145:182–95.
29. Gebhart CL, Kabanov AV. Evaluation of polyplexes as gene transfer agents. J Control Release. 2001;73:401–16.
30. Mintzer MA, Simanek EE. Nonviral vectors for gene delivery. Chem Rev. 2009;109:259–302.
31. Green JJ, Langer R, Anderson DG. A combinatorial polymer library approach yields insight into nonviral gene delivery. Acc Chem Res. 2008;41:749–59.
32. Grigsby CL, Leong KW. Balancing protection and release of DNA: tools to address a bottleneck of non-viral gene delivery. J R Soc Interface. 2010;7:S67–82.
33. Yue Y, Jin F, Deng R, Cai J, Dai Z, Lin MCM, *et al*. Revisit complexation between DNA and polyethylenimine — Effect of length of free polycationic chains on gene transfection. J Control Release. 2011;152:143–51.
34. Deng R, Yue Y, Jin F, Chen Y, Kung H, Lin MCM, *et al*. Revisit the complexation of PEI and DNA — How to make low cytotoxic and highly efficient PEI gene transfection non-viral vectors with a controllable chain length and structure? J Control Release. 2009;140:40–6.
35. Jiang H, Kim Y, Arote R, Nah J, Cho M, Choi Y, *et al*. Chitosan-graft-polyethylenimine as a gene carrier. J Control Release. 2007;117:273–80.
36. Nam HY, Nam K, Lee M, Kim SW, Bull DA. Dendrimer type bioreducible polymer for efficient gene delivery. J Control Release. 2012;160:592–600.
37. Hu W, Syu W, Chen W, Ruaan R, Cheng Y, Chien C, *et al*. Use of biotinylated chitosan for substrate-mediated gene delivery. Bioconjug Chem. 2012;23:1587–99.
38. Boeckle S, von Gersdorff K, van der Piepen S, Culmsee C, Wagner E, Ogris M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. J Gene Med. 2004;6:1102–11.
39. Ma PL, Buschmann MD, Winnik FM. One-step analysis of DNA/chitosan complexes by field-flow fractionation reveals particle size and free chitosan content. Biomacromolecules. 2010;11:549–54.
40. Dai Z, Gjetting T, Mattebjerg MA, Wu C, Andresen TL. Elucidating the interplay between DNA-condensing and free polycations in gene transfection through a mechanistic study of linear and branched PEI. Biomaterials. 2011;32:8626–34.
41. Thibault M, Astolfi M, Tran-Khanh N, Lavertu M, Darras V, Merzouki A, *et al*. Excess polycation mediates efficient chitosan-based gene transfer by promoting lysosomal release of the polyplexes. Biomaterials. 2011;32:4639–46.
42. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Control Release. 1999;60:149–60.
43. El-Sayed A, Harashima H. Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis. Mol Ther. 2013;21:1118–30.
44. Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Med. 2005;12:468–74.
45. van der Aa M, Huth U, Häfele S, Schubert R, Oosting R, Mastrobattista E, *et al*. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. Pharm Res. 2007;24:1590–8.
46. Thomas JJ, Rekha MR, Sharma CP. Unraveling the intracellular efficacy of dextran-histidine polycation as an efficient nonviral gene delivery system. Mol Pharm. 2011;9:121–34.
47. Suh J, Wirtz D, Hanes J. Efficient active transport of gene nanocarriers to the cell nucleus. Proc Natl Acad Sci USA. 2003;100:3878–82.
48. Kulkarni RP, Wu DD, Davis ME, Fraser SE. Quantitating intracellular transport of polyplexes by spatio-temporal image correlation spectroscopy. Proc Natl Acad Sci USA. 2005;102:7523–8.
49. Suk JS, Suh J, Choy K, Lai SK, Fu J, Hanes J. Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. Biomaterials. 2006;27:5143–50.