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RESEARCH PAPER

Ternary Polymeric Nanoparticles for Oral siRNA Delivery

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ABSTRACT

Purpose Poor stability and inefficient absorption in the intestinal tract are major barriers confronting oral delivery of siRNA. We aimed to uncover if ternary polymeric nanoparticles (cationic polymer/siRNA/anionic component) can overcome these obstacles through changing the formulation-related parameters.

Methods Ternary polymeric nanoparticles were prepared by ionic gelation of chitosan, N-trimethyl chitosan (TMC), or thiolated trimethyl chitosan (TTMC) with tripolyphosphate (TPP) or hyaluronic acid (HA), and siRNA was simultaneously encapsulated. Structural stabilities and siRNA protection of these nanoparticles were assessed in simulated intestinal milieu. Their transport across *ex vivo* rat ileum, macrophage uptake, *in vitro* gene silencing, and *in vivo* biodistribution after oral administration were investigated.

Results Ternary polymeric nanoparticles formed by TTMC, siRNA, and TPP (TTMC/siRNA/TPP nanoparticles) showed suitable structural stability and siRNA protection in the intestinal tract, good permeability across *ex vivo* rat ileum, superior cellular uptake and gene silencing efficiency in Raw 264.7 cells, and high systemic biodistribution after oral administration.

Conclusions TTMC/siRNA/TPP nanoparticles demonstrated efficient gene silencing *in vitro* and systemic biodistribution *in vivo*, therefore, they were expected to be potential vehicles for oral siRNA delivery.

KEY WORDS biodistribution · gene silencing · oral delivery · polymeric nanoparticles · siRNA

ABBREVIATIONS

CF	colonic fluids
CMH	colonic mucosa homogenates
CTH	colonic tissue homogenates
DF	duodenal fluids
DMH	duodenal mucosa homogenates
DTH	duodenal tissue homogenates
FAM-NC siRNA	FAM-labeled NC siRNA
HA	hyaluronic acid
IF	ileal fluids
IMH	ileal mucosa homogenates
ITH	ileal tissue homogenates
JF	jejunal fluids
JMH	jejunal mucosa homogenates
JTH	jejunal tissue homogenates
LPS	lipopolysaccharide
NC siRNA	negative control siRNA
RNAi	RNA interference
Scr	scrambled si*IL-6*
si*IL-6*	interleukin-6 siRNA
siRNA	small interfering RNA
TAMRA-NC siRNA	TAMRA-labeled NC siRNA
TMC	N-trimethyl chitosan
TPP	tripolyphosphate
TTMC	thiolated trimethyl chitosan.

INTRODUCTION

RNA interference (RNAi) induced by small interfering RNA (siRNA) holds great promises in the therapeutic settings, because it can effectively inhibit the expression of disease-

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specific genes (1,2). However, the effective delivery of siRNA meets considerable challenges due to rapid degradation by nucleases in physiological environment and poor cellular uptake (3). It is well-recognized that successful RNAi therapeutics demands an efficient delivery of siRNA to the cytoplasm of the target cells. Among the various administration routes, oral delivery has undeniably been preferred owing to high patient compliance and low healthcare cost (4). Moreover, the large mucosal surface area of the intestinal tract and the highly vascularized epithelium provide siRNA with a convenient access to the systemic circulation (5).

Previous studies have showed that both thioketal nanoparticles and nanoparticles-in-microsphere oral systems (NiMOS) could deliver siRNA to the colonic inflammatory sites following oral administration and exert therapeutic effects locally (6,7). Aouadi *et al.* (8) have reported that the β1,3-D-glucan particles (GeRPs) through delivering siRNA to the systemic circulation could silence gene expression in murine macrophages after oral administration. To the best of our knowledge, no polymer-based nanoparticles have been applied for systemic delivery of siRNA via the oral route. Among the cationic polymers used for gene delivery, chitosan and its derivatives seem to be more appealing because of their proven properties of versatility, biocompatibility, and biodegradability, as well as their efficiency in delivering genetic materials (9–12). The nanoparticles based on chitosan and its derivatives, e.g., N-trimethyl chitosan (TMC) and thiolated trimethyl chitosan (TTMC), have already been exploited to effectively deliver siRNA *in vitro* (9,13). Chitosan/siRNA nanoparticles can mediate specific gene silencing *in vivo* through intraperitoneal (i.p.) administration (14). For oral administration, comparatively speaking, chitosan, TMC, and TTMC nanoparticles offer great advantages by virtue of their unique muco-adhesion and enhanced transcellular transport across intestinal epithelium (15), as evidenced by the improved oral absorption of protein drugs encapsulated by them (16–19).

To achieve nanoparticles with compact and stable structures, ionic gelation technique has been employed for preparing siRNA loaded nanoparticles with cationic chitosan and its derivatives in the presence of an anionic component (20,21). Accordingly, ternary polymeric nanoparticles (cationic polymer/siRNA/anionic component) have been developed. Tripolyphosphate (TPP) is a known food additive approved by FDA and hyaluronic acid (HA) is a natural component in the extracellular matrix. Due to their high biocompatibility and low immunogenicity, they have been adopted as the anionic components for the preparation of ternary polymeric nanoparticles based on chitosan and its derivatives (1,13).

Orally delivered siRNA faces several challenges including instability in the intestinal tract, poor permeability across the intestinal epithelium, and restrictive cellular uptake. Ternary polymeric nanoparticles may overcome these challenges, if their formulation-related parameters are suitably changed. In this study, different types of polymers (chitosan, TMC, and TTMC) as well as anionic components (TPP and HA) were adopted to form various ternary polymeric nanoparticles by ionic gelation. Particle size, ζ potential, and siRNA association efficiency of the nanoparticles were evaluated. The structural stability and siRNA integrity of the nanoparticles in the intestinal tract were investigated. Using *ex vivo* rat ileum models, transport across the intestinal epithelium of the nanoparticles was determined. *In vitro* assessment of the cellular uptake and the gene silencing efficiency of the nanoparticles were carried out in Raw 264.7 cells. Finally, their biodistribution in mice after oral gavage of the nanoparticles was investigated.

MATERIALS AND METHODS

Materials

Chitosan (85% deacetylation degree, molecular weight of 200 kDa) was obtained from Golden-shell Biochemical Co., Ltd. (Zhejiang, China). TPP and HA (molecular weight of 20 kDa) were provided by Shanghai Experimental Reagent Co., Ltd. (Shanghai, China) and Zhenjiang Dong Yuan Biotech Co., Ltd. (Jiangsu, China), respectively. Lipofectamine 2000 as a proprietary transfection reagent was from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide (LPS) (obtained from *E.coli*) was purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

Interleukin-6 siRNA (si*IL-6*), scrambled si*IL-6* (Scr), and negative control siRNA (NC siRNA) duplexes were supplied by Genepharma (Shanghai, China) and dissolved in DEPC-treated water before use. The si*IL-6* contained the sequences of sense 5′-CUACCAAACUGGAUAUAAUdTdT-3′ and antisense 5′-AUUAUAUCCAGUUUGGUAGdTdT-3′. The Scr contained the scrambled sequences of sense 5′-GCUAUAUAACGAACAAUCUdTdT-3′ and antisense 5′-AGAUUGUUCGUUAUAUAGCdTdT-3′. The NC siRNA contained the sequences of sense 5′-UUCUCC GAACGUGUCACGUTT-3′ and antisense 5′-ACGUGA CACGUUCGGAGAATT-3′. FAM-labeled NC siRNA (FAM-NC siRNA) was used for *in vitro* siRNA quantification. TAMRA-labeled NC siRNA (TAMRA-NC siRNA) was used for *ex vivo* and *in vivo* siRNA quantification.

Cell Line and Animals

Raw 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS).

Male Sprague–Dawley (SD) rats (200 ± 20 g) and female Kunming mice (20–22 g) were obtained from the Animal Center of Fudan University. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Fudan University, China.

Preparation of TMC and TTMC

TMC and TTMC were synthesized as previously described (18,22). In brief, chitosan was reacted with methyl iodide in methyl-2-pyrrolidone/NaOH solution for 120 min at 65°C to obtain TMC. TMC reacted with cysteine at pH 5.0 and 1:2 (*w*/*w*) for 5 h at room temperature as mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS), and TTMC was then obtained. The amount of the immobilized sulphydryl was determined with Ellman's reagent (18).

Preparation and Characterization of Ternary Polymeric Nanoparticles

The siRNA loaded nanoparticles were prepared based on the ionic gelation as previously described (23). Chitosan was dissolved in acetate buffer solution (pH 4.5) while TMC, TTMC, HA, TPP, and NC siRNA were dissolved in DEPC-treated water. As for TPP-contained nanoparticles, 0.2 mL of siRNA solution (0.2 mg/mL) was mixed with 0.68 mL of TPP solution (1 mg/mL) at the siRNA/TPP weight ratio of 1:17. The cationic polymers (2 mg/mL) were added drop-wise into the mixture under magnetic stirring at the polymer/TPP weight ratio of 6:1, 8:1, and 10:1, respectively, followed by constant magnetic stirring for 30 s. The resultant nanoparticles were incubated at 37°C for 30 min before use, and termed as "cationic polymer/siRNA/TPP(m) nanoparticles, wherein m was the weight ratio of cationic polymer/TPP". As for HA-contained nanoparticles, 0.6 mL of siRNA solution (0.2 mg/mL) was mixed with 1.2 mL of HA solution (1 mg/mL) at the siRNA/HA weight ratio of 1:10. The cationic polymers (6 mg/mL) were added drop-wise into the mixture under magnetic stirring at the polymer/HA weight ratio of 5:1, 7:1, and 9:1, respectively, followed by constant magnetic stirring for 30 s. The resultant nanoparticles were incubated at 37°C for 30 min before use, and termed as "cationic polymer/siRNA/HA(m) nanoparticles, wherein m was the weight ratio of cationic polymer/HA".

The particle size and ζ potential of the nanoparticles were determined with Zetasizer Nano (Malvern, UK).

Stability of Nanoparticles Against Dilution, High Ionic Strength, and pH Alteration

Stability of nanoparticles against dilution, high ionic strength, and pH alteration was evaluated in terms of particle size and ζ potential. Chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles containing 400 ng of NC siRNA were diluted with DEPC-treated water 10, 100, 250, and 500 folds, respectively. NaCl solution (1 mol/L) was used to adjust ionic strength of nanoparticle suspension to 0.01, 0.02, 0.05, 0.1, 0.15, and 0.2 mol/L, respectively. The pH of nanoparticle suspension was modulated to 1.2, 5.8, 6.8, 7.4, and 7.8 using 1 mol/L HCl or NaOH, respectively. To mimic the pH alteration of the gastrointestinal tract, the pH of each nanoparticle suspension was adjusted to 1.2 using 1 mol/L HCl, and after the suspension was left standing for 1 min at room temperature, its pH was readjusted to pH 7.4 using 1 mol/L NaOH.

Preparation of Intestinal Fluids and Homogenates

The intestinal fluids and homogenates from freshly sacrificed male SD rats were collected as described by Yamagata *et al.* (24). Briefly, 20 mL of pH 5.8, pH 6.8, pH 7.4, and pH 7.8 phosphate buffered saline (PBS) was used to rinse the duodenum, jejunum, ileum, and colon, respectively. The solution was centrifuged at 13,800 g (12,000 rpm) for 20 min at 4°C, and the supernatants were collected as duodenal fluids (DF), jejunal fluids (JF), ileal fluids (IF), and colonic fluids (CF), respectively. To obtain the mucosa homogenates, the mucosa of duodenum, jejunum, ileum, and colon was scraped off and homogenized with cold corresponding medium. The homogenates were centrifuged at 13,800 g (12,000 rpm) for 20 min at 4°C, and the supernatants were collected as duodenal mucosa homogenates (DMH), jejunal mucosa homogenates (JMH), ileal mucosa homogenates (IMH), and colonic mucosa homogenates (CMH), respectively. After the mucosa was scraped off, the duodenal, jejunal, ileal, and colonic tissues were further homogenized with cold corresponding medium. The homogenates were centrifuged at 13,800 g (12,000 rpm) for 20 min at 4°C, and the supernatants were collected as the tissue homogenates, named as DTH, JTH, ITH, and CTH, respectively. The protein contents of obtained intestinal fluids and homogenates were determined by Lowry method. Intestinal fluids and homogenates were diluted with corresponding medium to achieve the protein content of 400–500 μg/ml, and then stored at −20°C before use.

Stability of Naked siRNA in Intestinal Fluids and Homogenates

To assess the stability of naked siRNA in intestinal environment, solution of naked NC siRNA (0.2 μg/μL) was incubated with equal volume of intestinal fluids or homogenates at 37°C for 5 min, 30 min, and 2 h. Then the mixture was heated at 80°C for 5 min to inactivate the enzyme. The

integrity of siRNA was subsequently evaluated on 2% agarose gel electrophoresis.

Stability of siRNA Encapsulated into Nanoparticles in Intestinal Fluids and Homogenates

To assess the stability of encapsulated siRNA in intestinal environment, chitosan/siRNA/TPP(8), TMC/siRNA/TPP (8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles containing 400 ng of NC siRNA or naked NC siRNA were mixed with equal volume of DF, DTH, DMH, and CMH at 37°C for 6 h, respectively. The mixtures were heated at 80°C for 5 min, followed by the addition of heparin sodium to dissociate siRNA. The integrity of siRNA was subsequently evaluated on 2% agarose gel electrophoresis. Naked NC siRNA incubated with DEPC-treated water served as a control.

To further quantitatively evaluate the protection of siRNA integrity provided by nanoparticles, chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles containing 1 μg of NC siRNA were mixed with equal volume of DF. The alteration in absorbance at 260 nm ($OD_{260\ nm}$) was continuously monitored at 37°C for 30 min using a VARioSKAN Flash microplate reader (Thermofisher®, USA). Naked NC siRNA treated with DF served as a control.

siRNA Permeation Across the Ileum *Ex Vivo*

The transport of siRNA across rat ileum *ex vivo* was monitored as described by Yin *et al.* (25). Briefly, after the rat was sacrificed, the ileum were collected, washed with Kreb's-Ringer buffer (glucose 2.0 g/L, KCl 0.4 g/L, NaCl 6.6 g/L, $MgSO_4$ 0.1 g/L, $CaCl_2$ 0.1 g/L, $NaHCO_3$ 1.4 g/L, $Na_2HPO_4 \cdot 12H_2O$ 3.6 g/L and KH_2PO_4 0.2 g/L), cut into 4 cm segments, and tied at both ends to form a sack. Five hundred microliters of nanoparticles loaded with TAMRA-NC siRNA and naked TAMRA-NC siRNA solution were syringed into the sack, respectively, which was further incubated in 6 mL of Kreb's-Ringer buffer at 37°C under shaking. At each predetermined interval, an aliquot of 200 μL was collected from the buffer to quantify the amount of transported TAMRA-siRNA by a microplate reader (λ_{ex}=544 nm, λ_{em}=576 nm), and another 200 μL of fresh buffer was added to make a constant volume. After the 4-h transport study, P_{app} was calculated according to the following equation:

$$P_{app} = \frac{dQ/dt}{A \times C_0}$$

where dQ/dt is the permeation rate (μg/s), C_0 is the initial concentration of TAMRA-NC siRNA, and A is the surface area of the intestinal sack.

Cellular Uptake

Raw 264.7 cells were seeded on 24-well plates at 5×10^4 cells/well and incubated for 24 h. The nanoparticles loaded with FAM-NC siRNA and naked FAM-NC siRNA solution were added at 0.4 μg siRNA/well. After incubation for 4 h, the cells were washed with PBS three times, and lysed with 0.5% SDS (*w*/*v*, pH 8.0). The cell lysate was quantified for FAM-NC siRNA by a microplate reader (λ_{ex}=480 nm, λ_{em}=520 nm) and protein content was measured by the Lowry method. Uptake levels were expressed as the amount of FAM-NC siRNA associated with 1 mg of cellular protein.

IL-6 Knockdown in LPS-Activated Raw 264.7 Cells

Raw 264.7 cells were seeded on 24-well plates at 4×10^4 cells per well and incubated for 24 h. The culture medium was replaced by the serum-free DMEM. The nanoparticles loaded with si*IL-6*, Lipofectamine 2000/si*IL-6* complexes, and TTMC/siRNA/TPP(8) nanoparticles loaded with Scr (TTMC/Scr/TPP(8)) were added at a siRNA dose of 600 ng per well. After incubation for 4 h, the culture medium was replaced by serum-containing DMEM. The cells were further cultured for 20 h before LPS (1 μg/mL) stimulation for 6 h. The supernatant of culture medium was collected for the quantification of extracellular IL-6 production with mouse IL-6 ELISA kit (Mingrui, China).

To investigate the influence of serum on the gene silencing efficiency, the culture medium was the serum-containing DMEM prior to adding si*IL-6* loaded TTMC/siRNA/TPP (8) nanoparticles and Lipofectamine 2000/si*IL-6* complexes and throughout the transfection experiment.

Biodistribution

Female Kunming mice were given a gavage of TAMRA-NC siRNA loaded nanoparticle suspensions and naked TAMRA-NC siRNA solution containing 4 μg of TAMRA-NC siRNA. At 2 h, 6 h, and 12 h post administration, blood was collected from the orbital sinus of mice and plasma was isolated via centrifugation at 13,800 g (12,000 rpm) for 4 min at 4°C. Mice were sacrificed and major organs including heart, liver, spleen, lung, kidney, and intestine were taken, weighed, and homogenized with RIPA lysis buffer. The homogenate was centrifuged at 900 g (3,000 rpm) for 15 min at 4°C, and the amount of TAMRA-NC siRNA in the supernatant as well as plasma was quantified with a microplate reader (λ_{ex}=544 nm, λ_{em}=576 nm). The results were expressed as percentage of total amount of TAMRA-NC siRNA delivered.

Statistical Analysis

Data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test and differences were judged to be significant at $P<0.05$.

RESULTS AND DISCUSSION

The overall goal of this study was to develop ternary polymeric nanoparticles, composed of cationic polymer, siRNA, and anionic component. They should have preferable stability in the intestinal tract, enhanced intestinal permeation, and improved cellular uptake, when delivered orally. Special attention has been given to understanding the effects of the differences of components in the nanoparticle formulation on their physicochemical characteristics, behaviors in the intestinal tract, *in vitro* gene silencing, and *in vivo* delivery efficiency, which will facilitate the rational design of oral siRNA delivery systems.

Preparation and Characterization of Ternary Polymeric Nanoparticles

Among the siRNA carriers investigated, chitosan has attracted considerable interests because it can bind anionic siRNA to form nanoparticles that facilitate endocytosis via adhering to the negatively charged cell membrane and maintain siRNA activity through shielding the restriction site of siRNA (9,11). However, chitosan is insoluble at neutral and alkaline environments, thereby limiting its biomedical applications. To improve the solubility of chitosan over a wide pH range, TMC and TTMC have been developed (26,27). They are soluble at neutral pH and have proved to be potent permeation enhancers for hydrophilic macromolecules across the gut (16,18). In this study, TMC with molecular weight of 200 kDa and 34% trimethylation degree were synthesized. TTMC was achieved via amide bond formed between the residual primary amino groups on TMC and carboxyl groups on cysteine as mediated by EDC/NHS, yielding TTMC conjugate with a free sulphydryl content of 119.4 ± 1.5 μmol/g and a disulfide content of 146.5 ± 1.3 μmol/g as quantified with Ellman's reagent.

Chitosan, TMC, and TTMC were employed to efficiently encapsulate siRNA in the presence of TPP or HA via ionic gelation, which resulted in the formation of ternary polymeric nanoparticles. Ionic gelation occurs spontaneously in aqueous solution through electrostatic interaction between cationic polymers and anionic TPP or HA without sonication or heating, which can avoid the accessibility of siRNA to toxic organic solvent and preserve its biological activity (13). It is known that the weight ratios of the cationic polymer/anionic component and the anionic component/siRNA affect the physicochemical characteristics of the nanoparticles. In this study, the TPP-contained nanoparticles were prepared at the TPP/siRNA weight ratio of 17:1 according to a previously published study (23). Gel retardation was used to evaluate the association efficiency of siRNA in the nanoparticles. As shown in Fig. S1, HA-contained nanoparticles exhibited stronger binding affinity for siRNA at the HA/siRNA weight ratio of 10:1 as compared to that of 15:1. The particle size, the polydispersity index, and the ζ potential of the nanoparticles varied at the different weight ratios of cationic polymer/anionic component (Table I). In general, to form nanoparticles with a smaller particle size, lower polydispersity index, and stronger binding affinity for siRNA, the optimal weight ratios of cationic polymer/TPP and TPP/siRNA were chosen as 8:1 and 17:1, respectively, while the weight ratios of cationic polymer/HA and HA/siRNA were adopted as 7:1 and 10:1, respectively. Consequently, six kinds of ternary polymeric nanoparticles (chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), chitosan/siRNA/HA(7), TMC/siRNA/HA(7), and TTMC/siRNA/HA(7)) were chosen for subsequent evaluation.

TTMC/siRNA/TPP(8) and TTMC/siRNA/HA(7) nanoparticles exhibited comparatively smaller particle sizes, because they had a compact structure resulting from the disulfide bonds formed between the thiol groups on TTMC (28). TMC/siRNA/TPP(8) and TMC/siRNA/HA(7) nanoparticles possessed a higher ζ potential, owing to the elevated positive charges caused by the trimethylation modification of chitosan (26). HA-contained nanoparticles showed a larger particle size and higher ζ potential than the TPP-contained ones. Due to the strong steric hindrance and few macromolecular coils (29), the long chain of HA would hide the charged groups into its inner parts, yielding a less compact structure and larger particle size.

Stability in the Simulated Physiochemical Milieu of Gastrointestinal Tract

The structural stability of nanoparticles is crucial for the effective protection of their payload. Orally delivered nanoparticles will be highly dispersed by the voluminous digestive fluids, and encounter a physiochemical milieu with high ionic strength and dramatic pH alterations, thereby damaging their desirable structure. Therefore, the stability of nanoparticles against massive dilution, high ionic strength, and pH alteration were particularly examined in terms of their particle size and ζ potential. In this investigation, based on the criteria of smaller particle size and lower polydispersity index, chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles were applied to elucidating the impact of the types

Table I Particle Size and ζ Potential of Different Positively Charged Nanoparticles Based on Chitosan and Its Derivatives. Indicated Values were Mean ± SD (n=3)

Ternary polymeric nanoparticles (polymer/anionic component weight ratio)	Particle size (nm)	Polydispersity index	ζ Potential (mV)
[a]chitosan/siRNA/TPP(6)	214.8 ± 10.6	0.311 ± 0.060	29.8 ± 2.7
[a]chitosan/siRNA/TPP(8)	173.3 ± 3.9	0.240 ± 0.007	32.7 ± 1.1
[a]chitosan/siRNA/TPP(10)	178.2 ± 8.0	0.241 ± 0.044	42.5 ± 1.1
TMC/siRNA/TPP(6)	precipitation	–	–
TMC/siRNA/TPP(8)	178.8 ± 7.6	0.030 ± 0.026	39.0 ± 1.4
TMC/siRNA/TPP(10)	163.5 ± 4.6	0.275 ± 0.027	25.7 ± 3.0
TTMC/siRNA/TPP(6)	precipitation	–	–
TTMC/siRNA/TPP(8)	159.0 ± 10.2	0.167 ± 0.029	25.9 ± 1.6
TTMC/siRNA/TPP(10)	152.0 ± 4.2	0.242 ± 0.044	23.2 ± 3.0
[a]chitosan/siRNA/HA(5)	424.4 ± 39.4	0.313 ± 0.147	22.7 ± 2.1
[a]chitosan/siRNA/HA(7)	216.2 ± 23.6	0.152 ± 0.024	36.4 ± 1.6
[a]chitosan/siRNA/HA(9)	293.9 ± 15.5	0.509 ± 0.071	37.8 ± 5.3
TMC/siRNA/HA(5)	291.8 ± 37.4	0.522 ± 0.235	24.3 ± 6.1
TMC/siRNA/HA(7)	180.1 ± 3.2	0.184 ± 0.041	40.3 ± 3.4
TMC/siRNA/HA(9)	170.2 ± 13.3	0.217 ± 0.042	49.1 ± 3.8
TTMC/siRNA/HA(5)	234.4 ± 22.5	0.570 ± 0.123	5.1 ± 0.7
TTMC/siRNA/HA(7)	174.4 ± 9.2	0.086 ± 0.038	34.3 ± 3.1
TTMC/siRNA/HA(9)	147.5 ± 6.3	0.420 ± 0.050	29.3 ± 2.4

[a]Samples were prepared at pH 4.5, and other samples were prepared at pH 6.0.

of the cationic polymers and the anionic components on their stability. As shown in Fig. 1a, a significant increase in particle sizes of chitosan/siRNA/TPP(8), TTMC/siRNA/HA(7), and TTMC/siRNA/TPP(8) was noticed after 100-fold, 250-fold, and 500-fold dilution, respectively. These results are in accordance with a previous study that the electrostatic complexes required a critical association concentration below which the complexes would be disassembled (30). Compared with chitosan/siRNA/TPP(8) nanoparticles, TTMC/siRNA/TPP(8) and TTMC/siRNA/HA(7) nanoparticles displayed less variation in particle size, which might be due to the disulfide bonds formed between the thiol groups on TTMC (28). The ζ potentials of the nanoparticles were decreased after 500-fold dilution, because dilution with DEPC-treated water, which contains counter-ions, could weaken the electrostatic interaction between oppositely charged molecules. The particle size of TMC/siRNA/TPP(8) nanoparticles only negligibly changed, due to their higher positive charge and their ζ potential of approximately 30 mV even after 500-fold dilution.

To assess the role of an anionic component in maintaining the structural stability, the binary polymeric nanoparticles (cationic polymer/siRNA) were prepared, and their structural stability against massive dilution was evaluated. Compared to the corresponding ternary polymeric nanoparticles tested, the particle size and ζ potential of these binary polymeric nanoparticles dramatically changed after 250-fold dilution (Fig. 1a and Fig. S2). This suggested that the participation of an anionic component could improve the structural stability of the nanoparticles. The formation of ternary polymeric nanoparticles seemed to be governed not only by the electrostatic interactions between the siRNA and the cationic polymer, but also by that between the anionic component (TPP or HA) and the cationic polymer. Since the latter was responsible for the controlled gelation of the cationic polymer in the nanoparticles (23), the ternary polymeric nanoparticles were more compact and stable than those binary polymeric nanoparticles.

Digestive fluids are hypertonic (5). Therefore, how ionic strength affects nanoparticle stability was also evaluated. As shown in Fig. 1b, elevating the ionic strength up to 0.2 mol/L exerted negligible effects on particle size and only a slight decrease in the ζ potential of the TPP-contained nanoparticles. However, compared with the initial data as shown in Table I, the fact that a 4-fold increment in the particle size and 22.8 mV decrease in the ζ potential of TTMC/siRNA/HA(7) nanoparticles were observed, suggesting that the involvement of the TPP rather than the HA might improve the structural stability of the nanoparticles against high ionic strengths. This is in agreement with a previously published study indicating that the turbidity of polyampholyte (poly(L-lysine)-graft-HA) solution was enhanced under high ionic strength conditions (31). The addition of NaCl could reduce the electrostatic attraction between the oppositely charged polyelectrolytes by providing counterions, which resulted in increased particle size and decreased ζ potential (16,32).

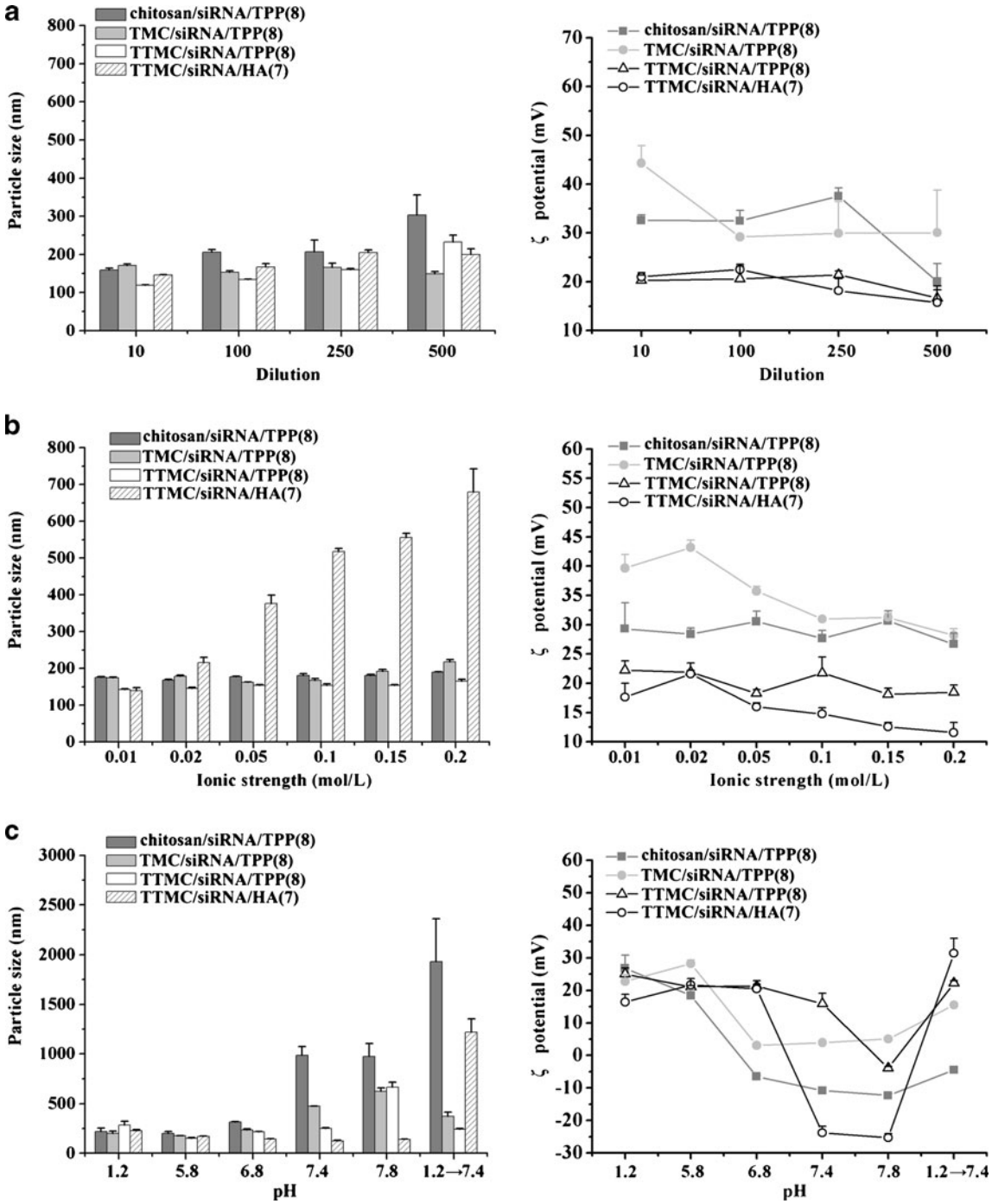

Fig. 1 Particle size (column) and ζ potential (scatter) of chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles after (**a**) dilution, (**b**) ionic strength elevation, and (**c**) pH alterations. Indicated values were mean ± SD (n=3).

Orally delivered nanoparticles encounter drastic pH alteration when they pass through the gastrointestinal tract (5). Therefore, the stability of the ternary polymeric nanoparticles was evaluated by adjusting the pH values of the nanoparticle suspensions to 1.2 (in stomach), 5.8 (in duodenum), 6.8 (in jejunum), 7.4 (in ileum), and 7.8 (in colon), respectively, to mimic the pH conditions in different segments of the gastrointestinal tract. As demonstrated in Fig. 1c, the particle sizes of chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), and TTMC/siRNA/TPP(8) nanoparticles increased slightly at pH 1.2, changed negligibly at pH 5.8, and increased noticeably at pH 6.8, 7.4, and 7.8. At pH 1.2, due to the protonation of the free aminos on chitosan, TMC, and TTMC, the mutual repulsion of the

ammonium groups led to a highly stretched structure in these cationic polymers, thus forming larger nanoparticles (29). However, at pH 6.8, 7.4, and 7.8, the solubility and protonation degree of chitosan, TMC, and TTMC were reduced (13,33). When less than 50% of amino groups were positively charged, the electrostatic interaction between oppositely charged molecules weakened, which caused fracture of nanoparticles and increase in particle size (29). As for the TTMC/siRNA/HA(7) nanoparticles, their particle size increased slightly at pH 1.2 and exhibited negligible changes at pH 5.8, 6.8, 7.4, and 7.8. This might be due to the fact that pH alteration does not affect the twisting effect of HA and TTMC. To simulate the pH environment that orally delivered nanoparticles encounter in the gastrointestinal tract, the pH of nanoparticle suspensions were adjusted to 1.2 and then back to 7.4. Among the nanoparticles tested, TTMC/siRNA/TPP(8) nanoparticles retained their initial particle size and ζ potential (Fig. 1c), which further proved their preferred structural stability. As for the TTMC/siRNA/HA(7) nanoparticles, a significant increase in particle size was observed, which confirmed their poor stability in the gastrointestinal tract.

Collectively, among the four kinds of nanoparticles tested, TTMC/siRNA/TPP(8) nanoparticles exhibited preferable structural stability in the physiochemical milieu of gastrointestinal tract.

Enzymatic Stability in Intestinal Fluids and Homogenates

The stability of naked siRNA was investigated using endogenous nucleases present in rat intestinal lumen and tissue, an experimental situation that mimicked the *in vivo* milieu. As shown in Fig. S3, no siRNA bands were detected after incubation with DF, DTH, DMH, and CMH for 5 min, which implied the existence of abundant RNase in the duodenum and colonic mucosa, whereas clear siRNA bands appeared after incubation with IF, ITH, and IMH for 2 h, which suggested that siRNA was degraded mainly in the duodenum and remained comparatively intact in the ileum.

To ensure the efficiency of *in vivo* RNAi via oral delivery, siRNA must be protected from nuclease degradation in the intestinal tract before it reaches the target cells. Chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles were employed to evaluate their protection of siRNA integrity in DF, DTH, DMH, and CMH that can quickly degraded naked siRNA. As illustrated in Fig. 2a, naked siRNA treated with DF, DTH, DMH, and CMH was completely degraded, whereas siRNA loaded into nanoparticles was partially preserved, as indicated by the migration bands on the gel. Judged by the brightness of the siRNA bands, the TTMC/siRNA/TPP(8) nanoparticles provided good protection for their payload. This phenomenon was consistent with the results of the structural stability of the nanoparticles. A compact structure could prevent nucleases from approaching the encapsulated siRNA in the nanoparticles via strong steric hindrance (34). Partial degradation of encapsulated siRNA after incubation with CMH (Fig. 2a) might be due to the presence of polysaccharidase, such as chitosanase, produced by the flora in the colonic mucosa, which can decompose chitosan and its derivatives and expose siRNA to RNase (5). In addition, the poor stability of nanoparticles based on chitosan or its derivatives in the alkaline pH (pH 7.8) of CMH (Fig. 1c) might also result in the approaching of RNase to siRNA.

The protection of siRNA integrity provided by the ternary polymeric nanoparticles was further quantitatively evaluated by monitoring the variation in $OD_{260\ nm}$ upon incubation with DF. Hyperchoromic effect is the increase of UV absorbance of DNA upon denaturation (12). The formation of double-stranded DNA is mainly driven by hydrogen bonds and hydrophobic interactions between complementary bases, which compromise UV absorbance of DNA due to the limited resonance of the aromatic ring. However, when the double helix structure of the DNA is denatured, the base-base interaction will be reduced, so the UV absorbance will be increased (35). Such base-base interaction also exists between the two strands of siRNA, so it is reasonable to hypothesize that siRNA exhibits hyperchoromic effect when it is denatured. As shown in Fig. 2b, the $OD_{260\ nm}$ of naked siRNA was rapidly increased after the addition of DF, while all the tested nanoparticles could suppress the increment of $OD_{260\ nm}$; and among them the TTMC/siRNA/TPP(8) nanoparticle exhibited the least increment. These results indicated that naked siRNA was quickly denatured, whereas TTMC/siRNA/TPP(8) nanoparticles resisted denaturation, consistent with the results of agarose gel electrophoresis (Fig. 2a).

siRNA Permeation Across the Ileum *Ex Vivo*

The ileum is rich in Peyer's patches and seems to be the major absorption site for orally administered polymeric nanoparticles (36). Moreover, it secretes less nucleases (Fig. S3). Therefore, the transport of ternary polymeric nanoparticles was investigated using *ex vivo* rat ileum. As demonstrated in Fig. 3, the transport of siRNA loaded into nanoparticles was significantly enhanced as compared to that of naked siRNA. Among the nanoparticles investigated, TTMC/siRNA/TPP(8) nanoparticles demonstrated the greatest permeation enhancement. The P_{app} values of the TTMC/siRNA/TPP(8) nanoparticles showed 2.48-, 1.54-, and 1.31-fold increments in comparison with those of chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles, respectively. The enhanced transport of TTMC/siRNA/TPP(8) nanoparticles might

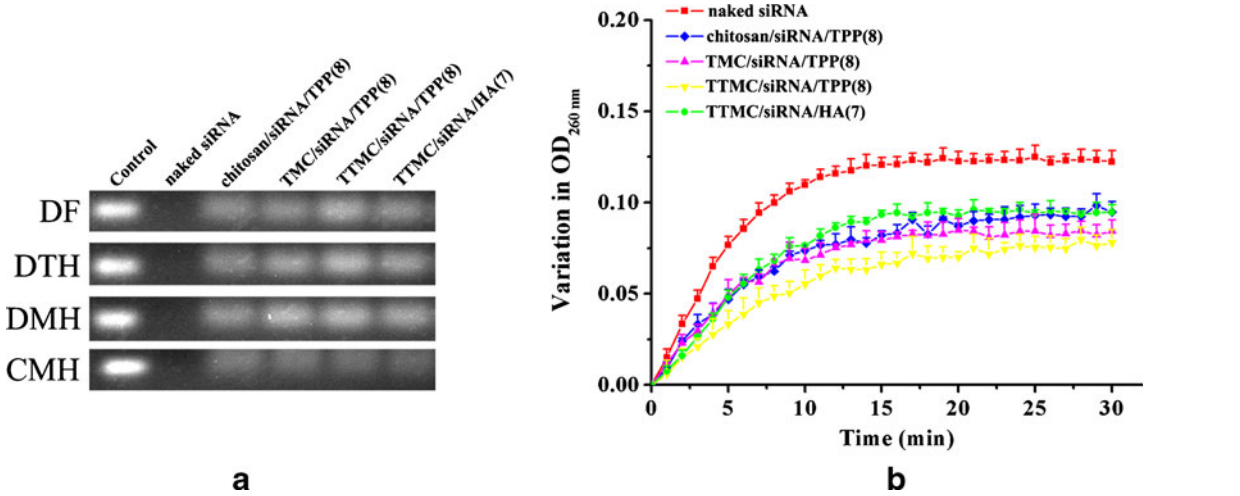

Fig. 2 The enzymatic stability of siRNA encapsulated into chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles. (**a**) Stability of nanoparticles after treatment with duodenal fluids (DF), duodenal tissue homogenates (DTH), duodenal mucosa homogenates (DMH), and colonic mucosa homogenates (CMH) at 37°C for 6 h evaluated by agarose gel electrophoresis. Naked NC siRNA without any treatment served as controls; (**b**) alteration in $OD_{260\ nm}$ of nanoparticles after incubation with equal volume of DF. Naked siRNA incubated with DF served as a control. Indicated values were mean ± SD (n=3).

be due to their stability in the ileum. Besides, thiolation could prolong the residence time on the intestinal mucosa and promote transcellular transport across the intestinal epithelium via forming disulfide bonds (18), which also contributed to the enhanced permeation of TTMC/siRNA/TPP(8) nanoparticles.

Cellular Uptake

Peyer's patches being rich in macrophages are major sites for rapid and substantial transport of nanoparticles following oral administration (36). Orally delivered nanoparticles are internalized by macrophages and translocated to the

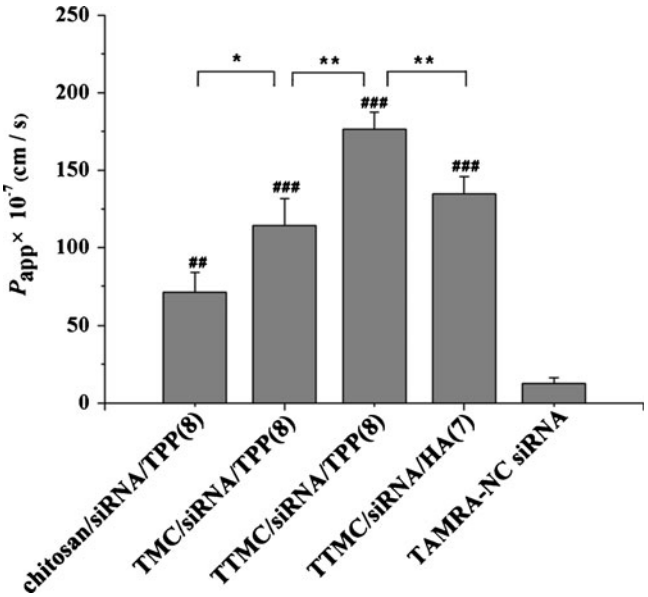

Fig. 3 Apparent permeability coefficients (P_{app}) for TAMRA-NC siRNA loaded chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles across *ex vivo* rat ileum. Naked TAMRA-NC siRNA solution served as a control. Indicated values were mean ± SD (n=3). *P < 0.05 and **P < 0.01. # Statistically significant differences observed from the values of naked TAMRA-NC siRNA (##P < 0.01 and ###P < 0.001).

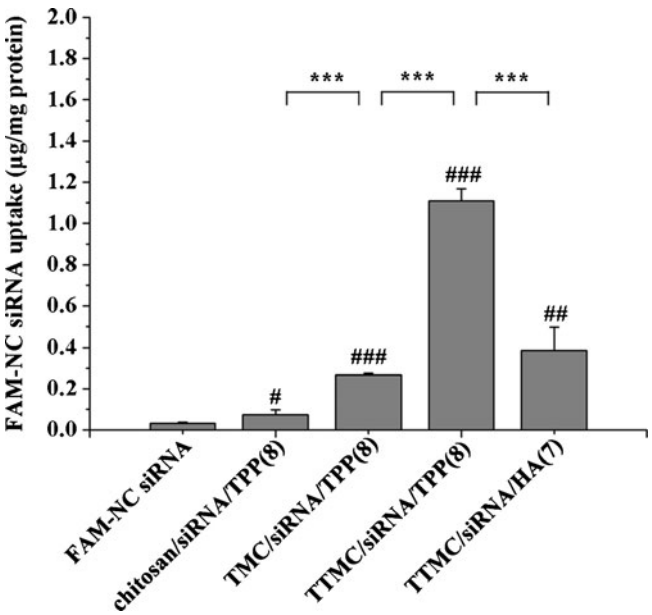

Fig. 4 Cellular uptake of FAM-NC siRNA loaded chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles in Raw 264.7 cells. Naked FAM-NC siRNA solution served as a control. Indicated values were mean ± SD (n=3). ***P < 0.001. # Statistically significant differences observed from the values of naked FAM-NC siRNA (#P < 0.05, ##P < 0.01, and ###P < 0.001).

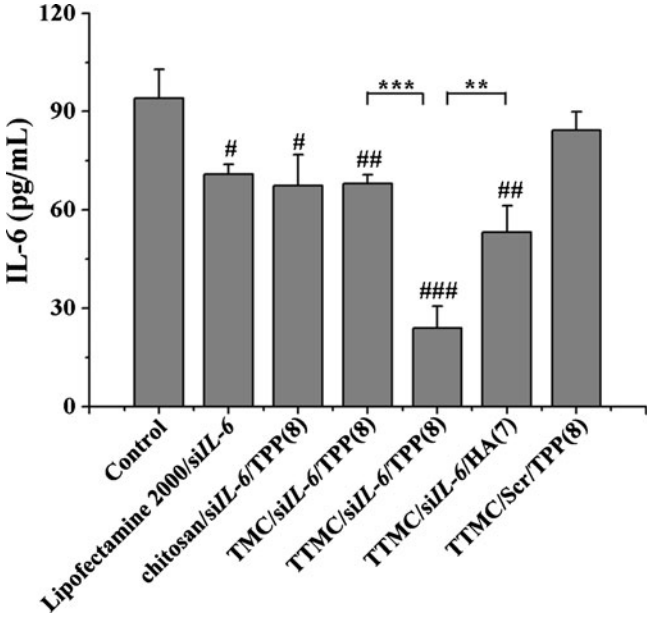

Fig. 5 IL-6 secretion from Raw 264.7 cells following treatment with Lipofectamine 2000/si*IL-6* complexes and nanoparticles loaded with si*IL-6* or Scr in absence of serum, incubation in fresh media for 20 h, and subsequent LPS stimulation for 6 h at 1 μg/mL. Indicated values were mean ± SD ($n=3$). $^{**}P<0.01$ and $^{***}P<0.001$. # Statistically significant differences observed from the values of control ($\#P<0.05$, $\#\#P<0.01$, and $\#\#\#P<0.001$).

systemic circulation (8). Hence, as an important depot for orally administered nanoparticles, the murine macrophage Raw 264.7 cells were adopted as *in vitro* cell model to investigate the cellular uptake and the gene silencing efficiency of the ternary polymeric nanoparticles. As demonstrated in Fig. 4, the TTMC/siRNA/TPP(8) nanoparticles outperformed other nanoparticles tested in cellular uptake, which was consistent with their desired structural stability after dilution with cell culture medium (high ionic strength 0.166 mol/L and pH 7.4). Additionally, TTMC could form disulfide bonds with the cysteine-rich mucin glycoproteins on cell membranes, which enhanced cellular uptake (28).

IL-6 Knockdown in LPS-Activated Raw 264.7 Cells

LPS-activated macrophages release proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (37,38). IL-6 is a multifunctional cytokine that plays important roles in host defense, acute phase reactions, immune response, nerve cell function, and hematopoiesis (39). The ability of ternary polymeric nanoparticles loaded with si*IL-6* to silence IL-6 production in LPS-activated Raw 264.7 cells was tested. As depicted in Fig. 5, TTMC/siRNA/TPP(8) nanoparticles containing Scr evoked no IL-6 inhibition effect, whereas TTMC/siRNA/TPP(8) nanoparticles containing si*IL-6* decreased the secretion of IL-6 from LPS-activated Raw 264.7 cells by 76.1%, which outperformed other nanoparticles tested. This was consistent with the high cellular uptake of TTMC/siRNA/TPP(8) nanoparticles (Fig. 4). The structural stability of TTMC/siRNA/TPP(8) nanoparticles and the disulfide bond of TTMC could enhance cellular internalization, which were beneficial for *IL-6* silencing in LPS-activated Raw 264.7 cells. As noted, the Lipofectamine

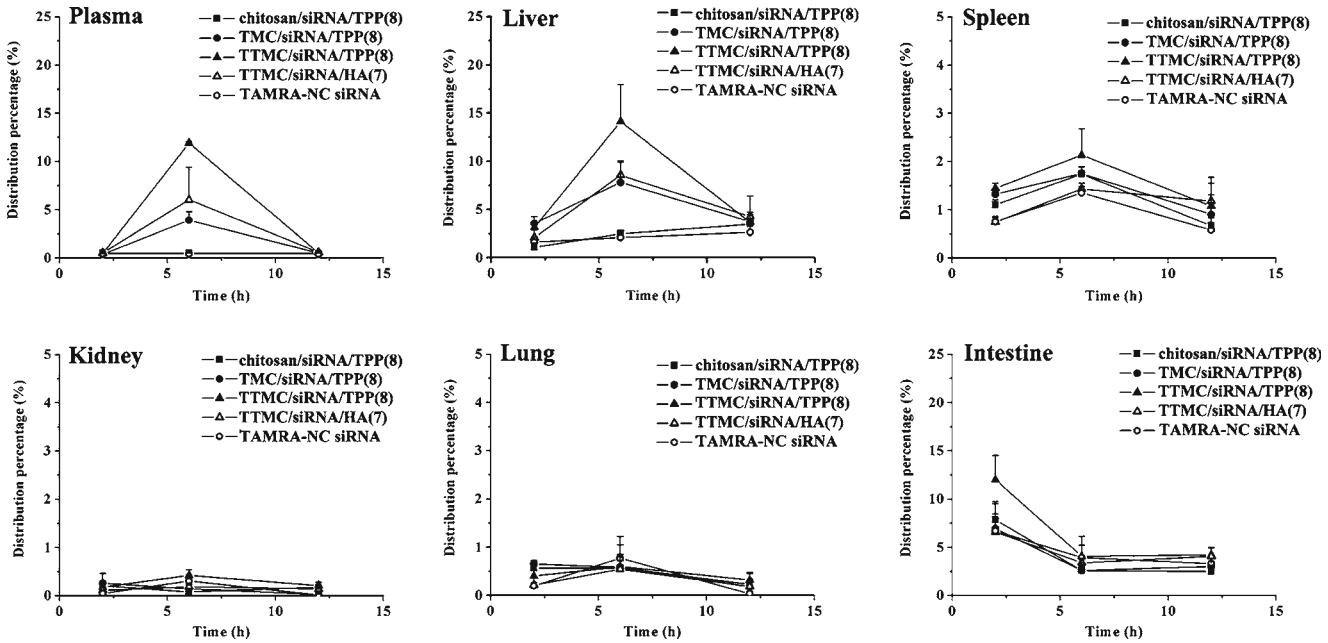

Fig. 6 Biodistribution of TAMRA-NC siRNA in mice after oral administration of TAMRA-NC siRNA loaded chitosan/siRNA/TPP(8), TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles. Indicated values were mean ± SD ($n=4$).

2000/si*IL*-6 complexes induced a 29.1% decrement in IL-6 secretion, significantly lower than the inhibition of the si*IL-6* loaded TTMC/siRNA/TPP(8) nanoparticles.

To investigate the impact of serum on gene silencing efficiency, *IL-6* knockdown assay was carried out in the transfection medium containing 10% FCS. After treatment with the si*IL-6* loaded TTMC/siRNA/TPP(8) nanoparticles and the Lipofectamine 2000/si*IL-6* complexes, the relative IL-6 expression levels of Raw 264.7 cells were 43.2±1.2% and 117.1±2.4%, respectively. These results indicated that Lipofectamine 2000/si*IL-6* complexes failed to exert a gene silencing effect in the presence of serum. By contrast, the si*IL-6* loaded TTMC/siRNA/TPP(8) nanoparticles significantly inhibited the LPS-induced IL-6 expression, which might be due to their desirable stability in cell culture medium.

Biodistribution

To exert systemic effects, orally delivered nanoparticles must enter the systemic circulation via intestinal absorption, and subsequently reach the targeted tissues. Fig. 6 depicted the biodistribution of TAMRA-NC siRNA in mice at 2, 6, and 12 h post oral administration. Following oral administration, the TMC/siRNA/TPP(8), TTMC/siRNA/TPP(8), and TTMC/siRNA/HA(7) nanoparticles remarkably promoted the intestinal absorption of siRNA as compared to the naked TAMRA-NC siRNA solution, which was evidenced by 8–26 folds enhancement in the TAMRA-NC siRNA distribution level in plasma. Notably, the intestinal distribution of the TAMRA-NC siRNA encapsulated into these nanoparticles was decreased with time, while the distribution of TAMRA-NC siRNA was increased in plasma, liver, and spleen within 6 h post oral administration. These results confirmed an efficient absorption and subsequent entry into the systemic circulation and other reticuloendothelial tissues (liver and spleen). Specifically, the TTMC/siRNA/TPP(8) nanoparticles, rather than other nanoparticles examined, showed the highest distribution percentages in most organs and plasma in mice. This might be again attributed to their desired stability in the intestinal tract (Fig. 1 and Fig. 2) and their enhanced intestinal permeation (Fig. 3).

CONCLUSIONS

In the present work, siRNA loaded ternary polymeric nanoparticles were formed with cationic polymers and anionic components by ionic gelation. The kinds of cationic polymers and anionic components selected in forming the nanoparticles significantly affected their particle size, ζ potential, structural stability, protection of siRNA integrity against adverse factors in the intestinal environment, permeability across *ex vivo* rat ileum, macrophage uptake, *in vitro* gene silencing, and *in vivo* biodistribution. More elaborate formulation strategies for the ternary polymeric nanoparticles included modifying chitosan with proper functional groups (trimethyl groups and thiol groups) and involving the TPP rather than the HA. TTMC/siRNA/TPP(8) nanoparticles had been proved to possess superior structural stability and siRNA protection in the harsh intestinal environment, good permeability across the intestinal epithelium, enhanced gene silencing efficiency in Raw 264.7 cells, and high systemic biodistribution after oral administration, which were expected to be a potential delivery vehicle for oral siRNA administration. These results may serve as guidelines for the development of orally available siRNA therapeutics.

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