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RESEARCH PAPER

Trimethyl Chitosan-Cysteine Nanoparticles for Systemic Delivery of TNF-α siRNA *via* Oral and Intraperitoneal Routes

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ABSTRACT

Purpose The lack of effective delivery vehicles impedes *in vivo* applications of siRNA. The trimethyl chitosan-cysteine (TC) nanoparticles (NPs) were developed for *in vivo* delivery of tumor necrosis factor α (TNF-α) siRNA *via* oral gavage and intraperitoneal injection.

Methods The nanoparticles formulated from TC conjugate of 100, 200, and 500 kDa were prepared through ionic gelation with sodium tripolyphosphate, termed as TC100 NPs, TC200 NPs, and TC500 NPs, respectively. They were evaluated in terms of stability, siRNA protection, cellular uptake and TNF-α knockdown in peritoneal exudates macrophage cells (PECs), and *in vivo* TNF-α silencing in acute hepatic injury mice.

Results TC100 NPs exhibited poor stability in simulated physiological environment compared to TC200 NPs and TC500 NPs. Compared to TC500 NPs, TC200 NPs could significantly enhance *in vitro* and *in vivo* cellular uptake by PECs and facilitate cytoplasmic siRNA release, resulting in high *in vitro* and *in vivo* TNF-α knockdown. Superior TNF-α suppressing level was obtained with TC200 NPs *via* oral gavage rather than intraperitoneal injection.

Conclusions The efficacies of *in vivo* TNF-α silencing were related to the molecular weight of TC conjugate and the administration route, which would assist in the rational design of siRNA vehicles.

KEY WORDS administration route · gene delivery · molecular weight · polymeric nanoparticles · TNF-α siRNA

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INTRODUCTION

Small interfering RNA (siRNA) has become an interesting class of developmental nucleic acid-based therapeutics (1–3). siRNA-mediated knockdown of tumor necrosis factor α (TNF-α) as a major proinflammatory cytokine offers an appealing strategy to treat systemic inflammation (4). Despite the considerable progress in the field with respect to the robust efficacy of RNA interference (RNAi) when applied to cultured cells, systemic delivery of siRNA, especially *via* oral route with a high rate of patient compliance, still remains a challenging task in achieving the improved *in vivo* RNAi efficiency (5,6).

Recently emerged RNAi strategy takes benefit from existing expertise in pDNA transfer (5). The ability of polymeric nanoparticles (NPs) to overcome rapid nuclease degradation and restrictive cellular uptake has been used to facilitate the successful delivery of pDNA and siRNA (7–10). However, despite similar properties with pDNA, delivery of siRNA poses distinct demands for polymeric NPs because of its small size (21–23 bp), low charge density, and action site in the cytosol (5). A previous report has demonstrated that the efficient gene transfection mediated by pDNA and siRNA require their polymeric vehicles with different binding affinities (9). The linear polyethylenimine is known to be a successful agent for pDNA delivery, however, it shows limited feasibility in siRNA delivery since it has low binding affinity for siRNA molecule (11). Due to the low charge density contributed by short siRNA molecules, it was assumed that polymer with high molecular weight (MW) as its vehicle might offer better siRNA complexation and extracellular stability, which however would compromise efficient intracellular release (5,7,12). The MW of polymer would also exert considerable influences on the physicochemical properties of the NPs, which might affect their cell entry and subsequent gene silencing (7). In addition, since siRNA is small and does not bear adequate negative charge, it cannot form stable NPs with cationic polymer through simple complexation. Therefore,

the ionic gelation technique has been applied to prepare siRNA loaded polymeric NPs in the presence of ionic crosslinker (13).

In our previous study, trimethyl chitosan-cysteine (TC) NPs have been demonstrated high efficacies in pDNA transfer by preventing nuclease degradation, promoting cellular uptake, and improving intracellular release (14). In view of these advantages for pDNA transfer, the efficacy of TC NPs for siRNA transfer needs further evaluation to demonstrate their versatilities in nucleic acid delivery. In the present investigation, TC conjugate with different MW (100, 200, and 500 kDa) were synthesized and siRNA loaded TC NPs were prepared through ionic gelation with sodium tripolyphosphate (TPP). The siRNA loaded TC NPs were characterized in terms of particle size, ζ potential, stability, siRNA integrity in physiological fluids, and glutathione (GSH)-responsive release. *In vitro* and *in vivo* cellular uptake and gene silencing efficacy against lipopolysaccharide (LPS)-induced TNF-α production were monitored in murine peritoneal exudate cell macrophages (PECs). Finally, the *in vivo* TNF-α silencing was determined in acute hepatic injury mice *via* oral gavage and intraperitoneal injection.

MATERIALS AND METHODS

Materials, Animals, and Cell Culture

Chitosan (deacetylation degree of 85% and MW of 100, 200, and 500 kDa) was purchased from Golden-shell Biochemical Co., Ltd. (Zhejiang, China). TPP, LPS, D-galactosamine (D-GalN), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), *L*-cysteine hydrochloride (Cys), and ethidium bromide (EB) were bought from Sigma (St. Louis, MO, USA). The siRNA duplexes were supplied by GenePharma (Shanghai, China) and dissolved in DEPC-treated water before use. 2′-*O*-methyl modified TNF-α-specific siRNA contained the sequences of sense 5′-GUCUCAGCCUCUUCUCAUUCCUGCT-3′ and antisense 5′-AGCAGGAAmUGmAGmAAmGAmGGmCUmG AmGAmCmAmU-3′, wherein the (m) pattern mN represented a 2′-*O*-methyl base. The negative control siRNA (Scr siRNA) contained the scrambled sequences of sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′-ACGUGACACGUUCGGAGAATT-3′. 5′-fluorescein phosphoramidite (FAM)-labeled negative control siRNA (FAM-siRNA) was used for *in vitro* siRNA quantification.

Male C57BL/6 mice (6–8 wk) were supplied by Shanghai Slaccas Experimental Animal Co., Ltd. Animal experiments were performed according to the approved protocol by the Institutional Animal Care and Use Committee, Fudan University, China.

Caco-2 cells (human colon adenocarcinoma) were 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). To obtain murine peritoneal exudate cell macrophages (PECs), C57BL/6 mice were sacrificed and intraperitoneally injected 1 mL of DMEM containing 10% FCS. The abdominal fluids were extracted and centrifugated at 1,500 rpm for 5 min. The collected PECs were cultured in DMEM supplemented with 10% FCS.

Preparation and Characterization of TC Conjugate

TC conjugate was prepared as previously described (14). Briefly, chitosan was reacted with methyl iodide in the methyl-2-pyrrolidone/NaOH solution for 2 h at 65°C to yield trimethyl chitosan with quaternization degree of 30% (12), followed by the purification and lyophilization. To obtain TC conjugate, 100 mg of trimethyl chitosan and 200 mg of Cys were dissolved in 10 mL of water, into which EDAC and NHS were added at a final concentration of 200 mM, respectively. The pH of the solution was adjusted to 5.0, and the reaction was carried out at ambient temperature for 5 h in dark. The obtained TC conjugate was dialyzed against pH 5.0 HCl solution (MWCO 3500 Da) at 4°C and lyophilized. The amount of immobilized sulphydryl on TC conjugate was determined with Ellman's reagent (15). TC100, TC200, and TC500 were adopted, where the numbers denote the MW (kDa) of chitosan.

Preparation and Characterization of TC NPs

TC NPs were prepared through ionic crosslinking. In brief, siRNA solution (0.2 mg/mL) was mixed with TPP solution (1 mg/mL) at 1:17 (w/w), into which TC solution (1 mg/mL) was drop-wisely added at the TC/TPP weight ratio of 8:1. The resultant mixture was incubated at 37°C for 30 min to allow the formation of TC NPs.

Particle size and Zeta potential of TC NPs were monitored with a Zetasizer Nano-ZS (Malvern, UK). The association of siRNA with the NPs was determined with gel retardation assay on 4% (w/v) agarose gel electrophoresis containing 0.25 μg/mL of EB. Stability of TC NPs in 0.2 M phosphate buffered saline (PBS, pH 7.4) was evaluated in terms of particle size and ζ potential. TC NPs were diluted with isotonic PBS (0.2 M, pH 7.4) upon 100 and 250 folds.

siRNAIntegrity in Physiological Fluids

Blood was collected from C57BL/6 mice and centrifugated at 12,000 rpm for 4 min to collect the serum. To obtain the gastric and intestinal fluids, 1 mL of pH 1.2 HCl solution and 2 mL of cold PBS (0.2 M, pH 7.4) was used to rinse the

gastric and intestinal lumen, respectively. The solution was centrifugated at 7,500 rpm and 4°C for 20 min to collect the supernatant as the gastric and intestinal fluids, respectively. As for the intestinal homogenates, the fresh intestinal tissue was washed with PBS and homogenized with 2 mL of cold PBS, followed by centrifugation at 7,500 rpm and 4°C for 20 min, and the supernatant was collected. As for the preparation of peritoneal fluids, 1 mL of PBS was intraperitoneally injected. The peritoneal fluids were drawn off 5 min later and centrifugated at 7,500 rpm and 4°C for 5 min, and the supernatant was collected.

The integrity of siRNA loaded into TC NPs in physiological fluids was determined both quantitatively and qualitatively. TC NPs containing 1 μg of siRNA were mixed with equal volume of physiological fluids. After incubation for determined time at 37°C, the mixture was heated at 80°C for 5 min to inactivate the nucleases. The siRNA was dissociated from TC NPs by the addition of heparin sodium (1 mg/mL), and its integrity was subsequently evaluated on 4% (w/v) agarose gel electrophoresis.

The hyperchromic effect was adopted to quantitatively evaluate siRNA degradation (16). Briefly, TC200 NPs and TC500 NPs containing 1 μg of siRNA were mixed with equal volume of mouse serum, gastric fluids, intestinal fluids, intestinal homogenates, and peritoneal fluids, respectively. The alteration in absorbance at 260 nm was constantly monitored at 37°C for determined time (20 min for the gastric fluids while 1 h for other physiological fluids). Naked siRNA treated with corresponding physiological fluids served as controls.

Cell Binding

The cell binding affinity was determined according to the method previously reported (14,17,18). Caco-2 cells and PECs were resuspended in isotonic buffer (44.4 g/L glucose, 200 mg/L KCl, 2.90 g/L $Na_2HPO_4 \cdot 12H_2O$, 200 mg/L KH_2PO_4, pH 7.4) at a concentration of 1×10^6 cells/mL, and 1 mL of the cell suspension was incubated with 100 μL of TC NPs at 37°C for 2 h. Following centrifugation at 1,500 rpm for 5 min, the cell pellets were resuspended in 0.2 M PBS (pH 7.4), which was subjected to ζ potential analysis. The cells incubated with 100 μL of 0.2 M PBS (pH 7.4) served as controls.

In Vitro Cellular Uptake

Caco-2 cells were seeded on 24-well plate at 1×10^5 cells per well and cultured for 24 h. PECs were seeded on 24-well plates at 2×10^5 cells per well and cultured for 2 h to allow the adherence of cells and the removal of non-adherent cells. TC200 NPs and TC500 NPs loaded with FAM-siRNA and naked FAM-siRNA solution were added to the cells at a siRNA dose of 0.4 μg per well. Following incubation for 4 h, cells were washed three times with 0.2 M PBS (pH 7.4) and lysed with 0.5% (w/v) SDS (pH 8.0). The lysate was quantified for FAM-siRNA by spectrofluorimetry (λ_{ex}=480 nm, λ_{em}=520 nm) and protein content by the Lowry method. Uptake level was expressed as the amount of FAM-siRNA associated with 1 mg of cellular protein.

GSH-Responsive siRNA Release

TC200 NPs and TC500 NPs containing 1 μg of FAM-siRNA were incubated with 1 mL of DMEM containing 0 mM, 4.5 μM, and 10 mM GSH at 37°C and 100 rpm, respectively. At each time interval, the suspension was centrifugated at 12,000 rpm for 10 min and 0.5 mL of the supernatant was withdrawn for FAM-siRNA quantification by spectrofluorimetry. An equal volume of the corresponding release medium was added, and the precipitate was resuspended before further incubation.

In Vitro TNF-α Knockdown in PECs

PECs were seeded at 1×10^6 cells per well and further cultured for 2 h. The culture media were replaced by 1 mL of serum-free DMEM prior to the experiment. TC200 NPs and TC500 NPs containing TNF-α siRNA or Scr siRNA and naked siRNA solution were added to the cells at a siRNA dose of 0.4 μg per well. Following incubation for 4 h, the culture media were changed to serum-containing media and a further 20-h incubation was allowed before LPS (100 ng/mL) stimulation for 5 h. The supernatant of the culture media was collected for the determination of extracellular TNF-α production by ELISA (R&D Systems, USA). Additionally, the intracellular RNA was isolated from the transfected cells based on the Trizol reagent protocol (Invitrogen, USA). cDNA was synthesized from the total RNA with PrimeScript®RT reagent kit (Takara Biotechnology Co. Ltd, China) based on the manufacturer's instructions. Synthesized cDNA, the forward and reverse primers, and the SYBR Premix Ex Taq™ (Takara Biotechnology Co. Ltd, China) were run on the ABI PRISM 7900HT Real-Time PCR system (Applied Biosystems, USA) for the quantification of *TNF-α* mRNA level. Sequences of the primers in this study were designed with Primer Bank (Supplementary Material Table S1). The ribosomal mRNA *36B4* was used as an internal control (19).

In Vivo Cellular Uptake in PECs

TC200 NPs, TC500 NPs, and naked siRNA solution containing 1 μg of FAM-siRNA were intraperitoneally injected to C57BL/6 mice, and PECs were isolated and seeded on 24-well plate 2 h later. Following further incubation for 2 h, the cells were washed with PBS and thereafter lysed

with 0.5% (w/v) SDS (pH 8.0). The lysate was quantified for FAM-siRNA by spectrofluorimetry and protein content by the Lowry method. Uptake level was expressed as the amount of FAM-siRNA (μg) associated with 1 mg of cellular protein.

In Vivo TNF-α Knockdown in PECs

TC200 NPs and TC500 NPs containing TNF-α siRNA or Scr siRNA and naked siRNA solution were intraperitoneally injected to C57BL/6 mice at a siRNA dose of 200 μg/kg, and murine PECs were isolated and seeded on 24-well plate 2 h later. The cells were allowed to adhere for 2 h before the media containing non-adherent cells were removed, and the serum-containing media were then added. After 20 h the cells were stimulated with LPS (100 ng/mL). The supernatant was harvested 5 h later and extracellular TNF-α production was detected by ELISA (R&D Systems, USA). Intracellular *TNF-α* mRNA levels were determined by realtime-PCR as described above.

In Vivo RNAi *via* Oral Gavage and Intraperitoneal Injection

TC200 NPs and TC500 NPs containing TNF-α siRNA or Scr siRNA and naked TNF-α siRNA solution were orally or intraperitoneally administered to C57BL/6 mice at a siRNA dose of 200 μg/kg. After 24 h, LPS (12.5 μg/kg)/D-GalN (1.25 g/kg) was intraperitoneally injected. Blood was collected 2 h post LPS/D-GalN stimulation to quantify serum TNF-α production by ELISA (R&D Systems, USA), and liver, spleen, and lung of mice were taken, cut into pieces, washed with saline, immersed in the RNAlater solution (Qiagen, USA) for 24 h. The tissues were then homogenized in liquid nitrogen, followed by cell lysis and RNA extraction with the Trizol reagent. Intracellular *TNF-α* mRNA levels were monitored by realtime-PCR as described above.

Male C57BL/6 mice were orally administered with TC200 NPs containing TNF-α siRNA or Scr siRNA at a siRNA dose of 200 μg/kg and PBS, and 24 h post administration, LPS/D-GalN (12.5 μg/kg and 1.25 g/kg) were intraperitoneally injected. Liver was harvested 6 h post administration, fixed in paraffin, cross-sectioned, and stained with haematoxylin/eosin (H&E) for histological examination.

Statistical Analysis

Data were presented as mean ± SD. Statistical analysis was evaluated with Student's *t*-test (two-tailed) and statistical significance was considered at $P<0.05$.

RESULTS

Preparation and Characterization of TC NPs

The free sulphydryl contents of TC100, TC200, and TC500 conjugate were 108.5±8.1, 122.0±0.5, and 121.5±1.7 μmol/g ($n=3$), respectively. During the preparation of TC conjugate, part of the free sulphydryl groups could be oxidized to be disulfide bonds. The disulfide contents of TC100, TC200, and TC500 conjugate were 209.9±6.5, 199.0±0.2, and 198.2±0.4 μmol/g ($n=3$), respectively.

TC NPs were prepared through ionic crosslinking of cationic TC by a polyanion (TPP) and simultaneous encapsulation of siRNA. To obtain TC NPs with optimal particle size, ζ potential, and siRNA encapsulation efficiency, TC/TPP/siRNA weight ratio of 136:17:1 was identified (20). Such relatively low siRNA/TC conjugate weight ratio adopted might be due to the addition of negative charges contributed by TPP. The resultant TC100 NPs, TC200 NPs, and TC500 NPs possessed particle sizes of 118±4, 153±0, and 153±2 nm ($n=3$), respectively, ζ potentials of 25±3, 29±2, and 32±1 mV ($n=3$), respectively, and PDI values of 0.109±0.003, 0.121±0.011, and 0.115±0.025, respectively. Scanning electron microscopy (SEM) image suggested that the morphology of TC NPs were sub-spherical, monodispersed, and well-defined (Supplementary Material Fig. S1). The siRNA encapsulation efficiencies of TC100 NPs, TC200 NPs, and TC500 NPs were (68.1±5.6)%, (72.5±4.3)%, and (84.7±6.1)%, respectively. The association of siRNA with TC NPs was evaluated by agarose gel electrophoresis (Fig. 1a). In comparison with the motility of the naked siRNA, the movement of siRNA encapsulated into TC100 NPs, TC200 NPs, and TC500 NPs was completely retarded, which demonstrated the strong encapsulation ability of NPs towards siRNA.

When TC200 NPs and TC500 NPs were diluted up to 250 folds with 0.2 M PBS (pH 7.4), their particle sizes and ζ potentials were slightly decreased (Fig. 1b), suggesting their abilities to maintain stability in 0.2 M PBS (pH 7.4). However, upon a 100-fold dilution with 0.2 M PBS (pH 7.4), the particle size of TC100 NPs was dramatically increased and a considerable decrease in ζ potential was noted, which indicated their poor stability.

siRNA Integrity in Physiological Fluids

To evaluate the integrity of orally or intraperitoneally delivered siRNA, TC200 NPs and TC500 NPs loaded with TNF-α siRNA and naked siRNA were incubated with various biological fluids including mouse serum, gastric fluids, intestinal fluids, intestinal homogenates, and peritoneal fluids before gel electrophoresis. As shown in Fig. 2a, no bands were observed for naked siRNA following incubation with gastric fluids for 5 min and other physiological fluids for 2 h, implying

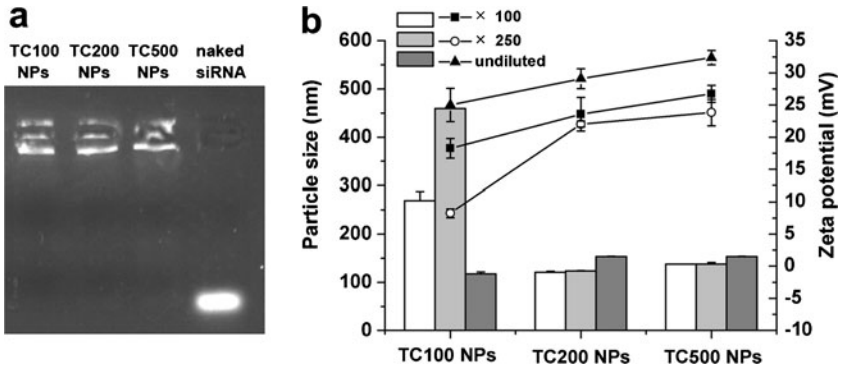

Fig. 1 The siRNA encapsulation and stability of TC NPs. (**a**) Agarose gel electrophoresis of TC NPs containing TNF-α siRNA and naked siRNA. (**b**) Stability of TC NPs in terms of particle size (column) and ζ potential (scatter) upon dilution with 0.2 M PBS (pH 7.4) ($n = 3$).

that abundant RNase in the gastrointestinal tract, peritoneal cavity, and blood could rapidly degrade siRNA. After incubation with intestinal fluids and homogenates for 1 h and gastric fluids for 10 min, ambiguous siRNA bands were noted for TC100 NPs, suggesting the degradation of siRNA. In comparison, clear migration bands for siRNA encapsulated into TC200 NPs and TC500 NPs suggested that they effectively protected siRNA from degradation in physiological environment. Additionally, by comparing the brightness of the siRNA bands in various physiological fluids, gastric fluids and intestinal homogenates were assumed to be more readily to damage the siRNA integrity. In contrast, incubation with peritoneal fluids and serum led to negligible degradation of siRNA loaded into TC200 NPs and TC500 NPs. Therefore, it was reasonable to hypothesize that siRNA loaded into TC NPs would be more stable *via* intraperitoneal rather than peroral delivery.

Since TC100 NPs exhibited poor stability and protection of siRNA in physiological environment, TC200 NPs and TC500 NPs were used in the following investigations. The ability of TC200 NPs and TC500 NPs to preserve siRNA integrity was further confirmed by quantitatively monitoring the hyperchromic effect of nucleic acids. Following treatment with various physiological fluids, inappreciable increment of OD_{260} value was noted for siRNA encapsulated into TC NPs compared to the rapidly increased OD_{260} value of naked siRNA (Fig. 2b). Such results accorded well with the qualitative assessments using gel electrophoresis.

In Vitro Cell Binding and Cellular Uptake

Cell binding served as the initial step for cellular uptake. Surface negative charges of PECs were counteracted following nanoparticle treatment, verifying binding of NPs onto cell membranes (Fig. 3a). TC200 NPs-treated cells exhibited more obvious increment in ζ potentials than TC500 NPs-treated cells, demonstrating preferable binding affinity towards cell surfaces. As indicated in Fig. 3b, TC NPs effectively delivered their payload to PECs as evidenced by the significantly elevated cellular uptake amount compared to naked siRNA. Moreover, the cellular uptake amount of TC200 NPs was significantly higher than that of TC500 NPs, which was consistent with the higher cellular binding affinity of TC200 NPs.

GSH-Responsive siRNA Release

As shown in Fig. 4, the release of siRNA from TC200 NPs and TC500 NPs was significantly promoted under high intracellular GSH concentration (10 mM) while partly inhibited under low extracellular concentration (4.5 μM). TC200 NPs exhibited higher release amount, with approximately 100% of the encapsulated siRNA being released from TC200 NPs in comparison to the cumulative release of 50% for TC500 NPs within 8 h in the presence of 10 mM GSH.

In Vitro TNF-α Knockdown in PECs

To determine the ability of TC NPs to mediate gene silencing *in vitro*, the efficacy of TNF-α siRNA loaded TC NPs to silence TNF-α expression in PECs was evaluated. As shown in Fig. 5, naked TNF-α siRNA and TC NPs containing Scr siRNA evoked no gene knockdown. TC200 NPs encapsulated with TNF-α siRNA mediated effective gene silencing in PECs, of which the relative TNF-α production was suppressed to 70% (Fig. 5a). Accordingly, TC200 NPs containing TNF-α siRNA significantly decreased the *TNF-α* mRNA expression levels to 66% (Fig. 5b). TC200 NPs outperformed TC500 NPs in suppressing TNF-α production in PECs *in vitro* (Fig. 5).

In Vivo Cellular Uptake and TNF-α Knockdown in PECs

The ability of intraperitoneally administered TC NPs to deliver siRNA into PECs was carried out in C57BL/6 mice. As illustrated in Fig. 6a, TC200 NPs and TC500 NPs significantly promoted the cellular uptake of siRNA by PECs *in vivo*, as evidenced by the 19- and 15-fold enhancement in uptake amount compared to siRNA in free form, respectively. TC200 NPs again outperformed TC500 NPs, which accorded well with the *in vitro* cellular uptake assessments.

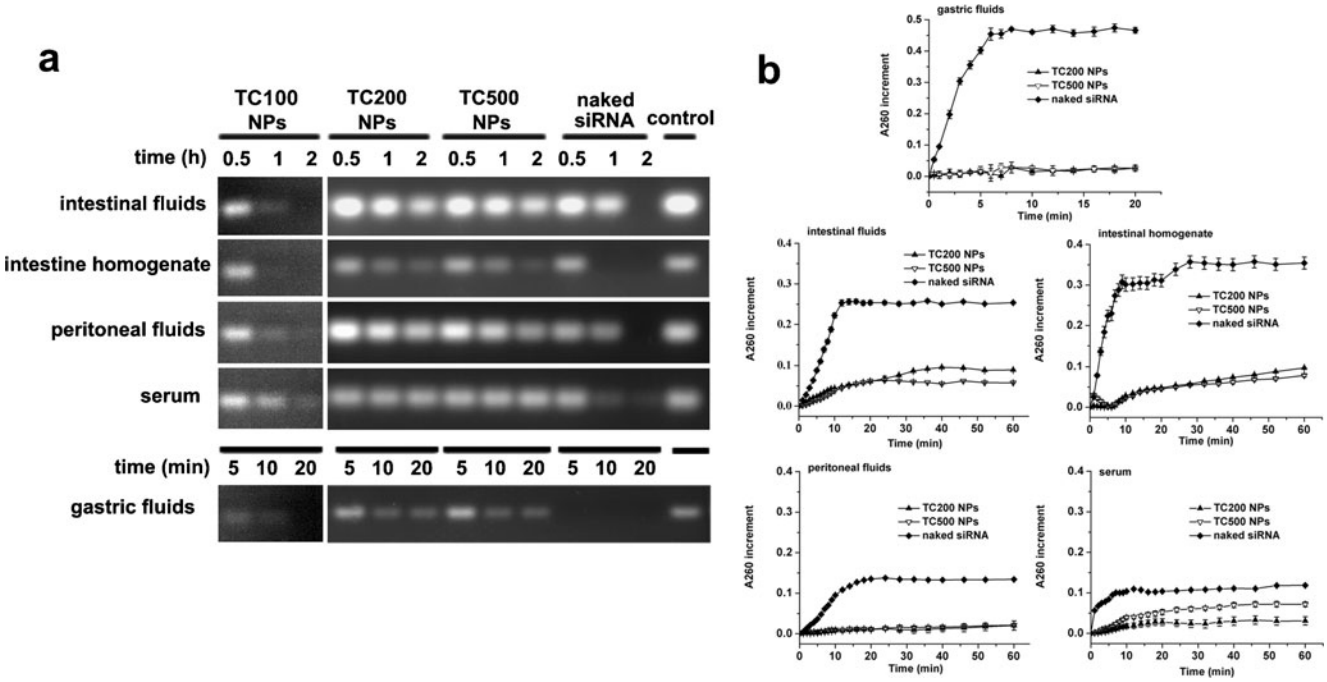

Fig. 2 (**a**) The siRNA integrity following treatment with physiological fluids as evaluated by agarose gel electrophoresis. (**b**) Variation in the OD_{260} value of siRNA encapsulated into TC200 NPs and TC500 NPs after incubation with various physiological fluids as a function of incubation time ($n = 3$). Naked siRNA incubated with corresponding physiological fluids was used as a control.

The *in vivo* gene transfection *via* intraperitoneal injection was shown in Fig. 6b and Supplementary Material Fig. S2, naked siRNA and TC NPs loaded with Scr siRNA demonstrated no gene silencing effect. Significant inhibition of TNF-α and *TNF-α* mRNA production in PECs was observed for TC NPs. In addition, TC200 NPs worked better than TC500 NPs in the depletion of TNF-α production.

In Vivo RNAi *via* Oral Gavage and Intraperitoneal Injection

TNF-α is a proinflammatory cytokine and plays a central role in mediating the inflammatory responses of LPS/D-GalN-induced acute hepatic injury. Therefore. LPS/D-GalN-induced acute hepatic injury characterized by the overexpression of TNF-α was adopted as mouse models to evaluate the RNAi efficiency mediated by TC NPs *via* single oral gavage or intraperitoneal injection. As shown in Fig. 7a, orally delivered TC200 NPs and TC500 NPs at a siRNA dose of 200 μg/kg notably blocked LPS/D-GalN-triggered serum TNF-α production by 74% and 46%, respectively. Intraperitoneally delivered TC200 NPs and TC500 NPs also effectively suppressed serum TNF-α production by 39% and 32%, respectively. These results indicated that TC NPs mediated significantly higher RNAi efficiency *in vivo via* peroral than intraperitoneal administration. Neither the naked TNF-α siRNA nor TC NPs containing Scr siRNA evoked any RNAi effect. Accordingly, orally delivered TC200 NPs and TC500 NPs as well as intraperitoneally delivered TC200 NPs significantly reduced *TNF-α* mRNA levels in macrophage-enriched organs (liver, spleen, and lung, Fig. 7b), among which orally delivered TC200 NPs exhibited the highest *TNF-α* mRNA knockdown

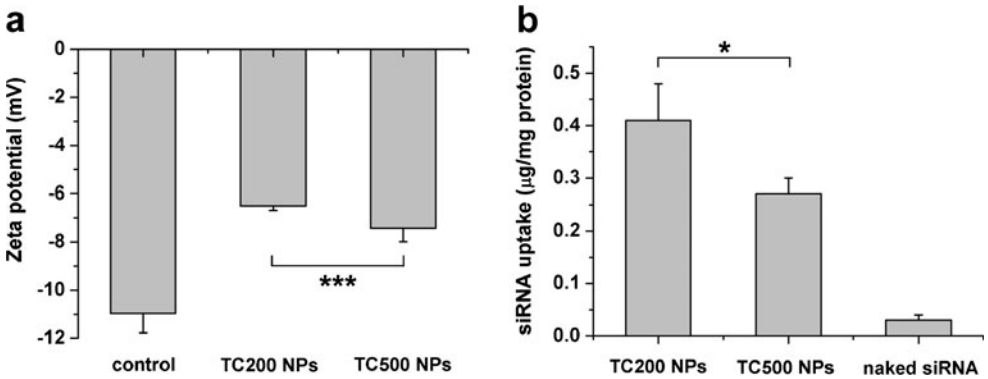

Fig. 3 Cell binding (**a**) and cellular uptake (**b**) of FAM-siRNA encapsulated into TC200 NPs and TC500 NPs in PECs ($n = 3$). * $P < 0.05$ and *** $P < 0.001$.

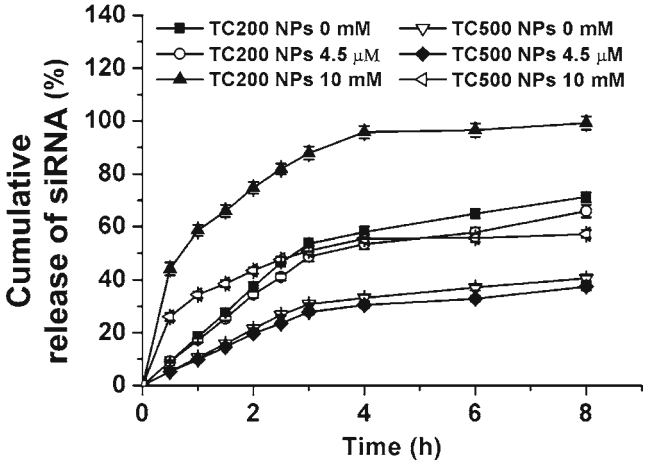

Fig. 4 Release profiles of FAM-siRNA from TC200 NPs and TC500 NPs in GSH-supplemented DMEM ($n = 3$).

efficiencies. Histology assessments suggested obvious blockage of liver damage including piecemeal necrosis, disarranged hepatocytes, and congestion after oral delivery of TNF-α siRNA loaded TC200 NPs (Fig. 7c).

DISCUSSION

In our previous report, TC/pDNA nanocomplexes were prepared through simple complexation, which exhibited desired binding affinity and protection of pDNA (14). However, since siRNA is a small (21–23 bp), linear, and anionic biomacromolecule, its limited electrostatic interaction with TC conjugate made it difficult to achieve sufficient complex stability *via* simple complexation (13). To improve the ability of TC conjugate to encapsulate siRNA, TPP as a regional crosslinker to direct cationic polymer into compact configurations was used to physically crosslink TC conjugate *via* ionic gelation (13). In addition, our previous research demonstrated that the introduction of disulfide crosslinking could also contribute to the improved stability of TC NPs (20). Thus, siRNA could be encapsulated tightly as evidenced by gel retardation assessment (Fig. 1a). The higher ζ potential of TC500 NPs as compared to TC100 NPs and TC200 NPs might be attributed to the longer polymeric chain of TC500 conjugate which carried more positive charges. The stability of TC200 NPs and TC500 NPs in simulated biological environment was remarkably improved compared to TC100 NPs (Fig. 1b). The shorter polymer chains and less positive charges of TC100 conjugate could lead to the insufficient entanglement and electrostatic interaction with TPP and siRNA, which yielded too loosened structures to resist the massive dilution by solutions with high ionic strenght, resulting in the poor stability of TC100 NPs (14). The stability of NPs in biological environment was the prerequisite for effective protection of their payload from nucleases degradation *in vivo* (5,21). Therefore, TC100 NPs with poor stability had the inability of preventing encapsulated siRNA from rapid degradation in murine physiological fluids (Fig. 2a). In the following *in vitro* and *in vivo* investigations, TC200 NPs and TC500 NPs with preferable stability and siRNA protection were chosen as potential siRNA carriers.

With the above efforts in the nanoparticle formulation, TC NPs were anticipated to possess exceptional capabilities to conquer various extracellular and intracellular barriers to *in vitro* gene transfection and mediate potent TNF-α gene knockdown in macrophages. The first barrier to gene transfer was the siRNA degradation by the prevailing nucleases in physiological environment (22). TC200 NPs and TC500 NPs could effectively encapsulated siRNA (Fig. 1), thus protecting siRNA from degradation *via* steric shielding (Fig. 2) (13). The second barrier to gene transfer might be the restrictive cellular uptake (12). TC200 NPs and TC500 NPs enhanced the siRNA uptake in PECs *in vitro* (Fig. 3b). Trimethylation provided sufficient positive charges for TC NPs to be directed onto negatively charged cell membranes, and cysteine conjugation offered free sulphydryl to form disulfide bonds with the cysteine-rich mucin glycoproteins, which collectively facilitated the internalization of TC NPs (14,18). TC200 NPs exhibited higher cell binding affinity and uptake amount in PECs than TC500 NPs (Fig. 3), which might be ascribed to their shorter polymeric chains that favored the adsorption and penetration of the cell membrane (23). The higher cellular uptake amount

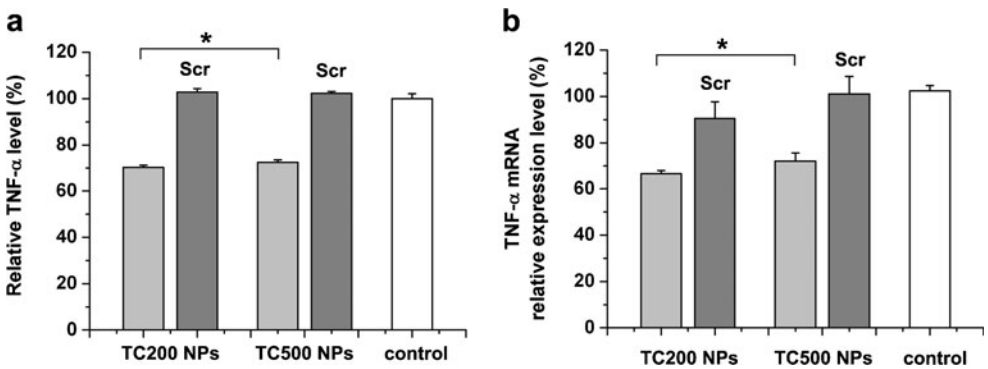

Fig. 5 TNF-α secretion from PECs (**a**) and *TNF-α* mRNA expression in PECs (**b**) following treatment with TC200 NPs and TC500 NPs loaded with TNF-α siRNA or Scr siRNA at a siRNA dose of 0.4 μg/well, incubation in fresh media for 20 h, and subsequent LPS stimulation for 5 h at 100 ng/mL ($n = 3$). * $P < 0.05$.

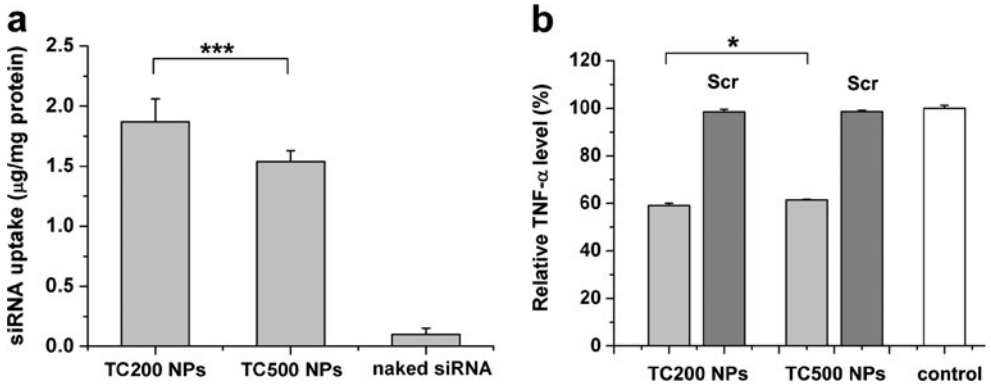

Fig. 6 *In vivo* cellular uptake and TNF-α knockdown in PECs. (**a**) *In vivo* uptake of TC200 NPs and TC500 NPs containing FAM-siRNA and naked FAM-siRNA by PECs following intraperitoneal injection to mice at a siRNA dose of 200 μg/kg ($n = 3$). (**b**) TNF-α production in PECs which were extracted 2 h after intraperitoeanl injection of TC200 NPs and TC500 NPs containing TNF-α siRNA at a siRNA dose of 200 μg/kg, incubated for 22 h, and stimulated with 100 ng/mL LPS for 5 h ($n = 3$). * $P < 0.05$ and *** $P < 0.001$.

of TC200 NPs could contribute to their superior silencing efficiency as compared to TC500 NPs. The third barrier to gene transfer was the endolysosomal entrapment and degradation. Treatment with Bafilomycin A1 and chloroquine exerted negligible effects on the cellular uptake and transfection efficiency of TC NPs in macrophages, which demonstrated that endolysosomal pathway might be not involved in their cellular internalization as well as transfection processes

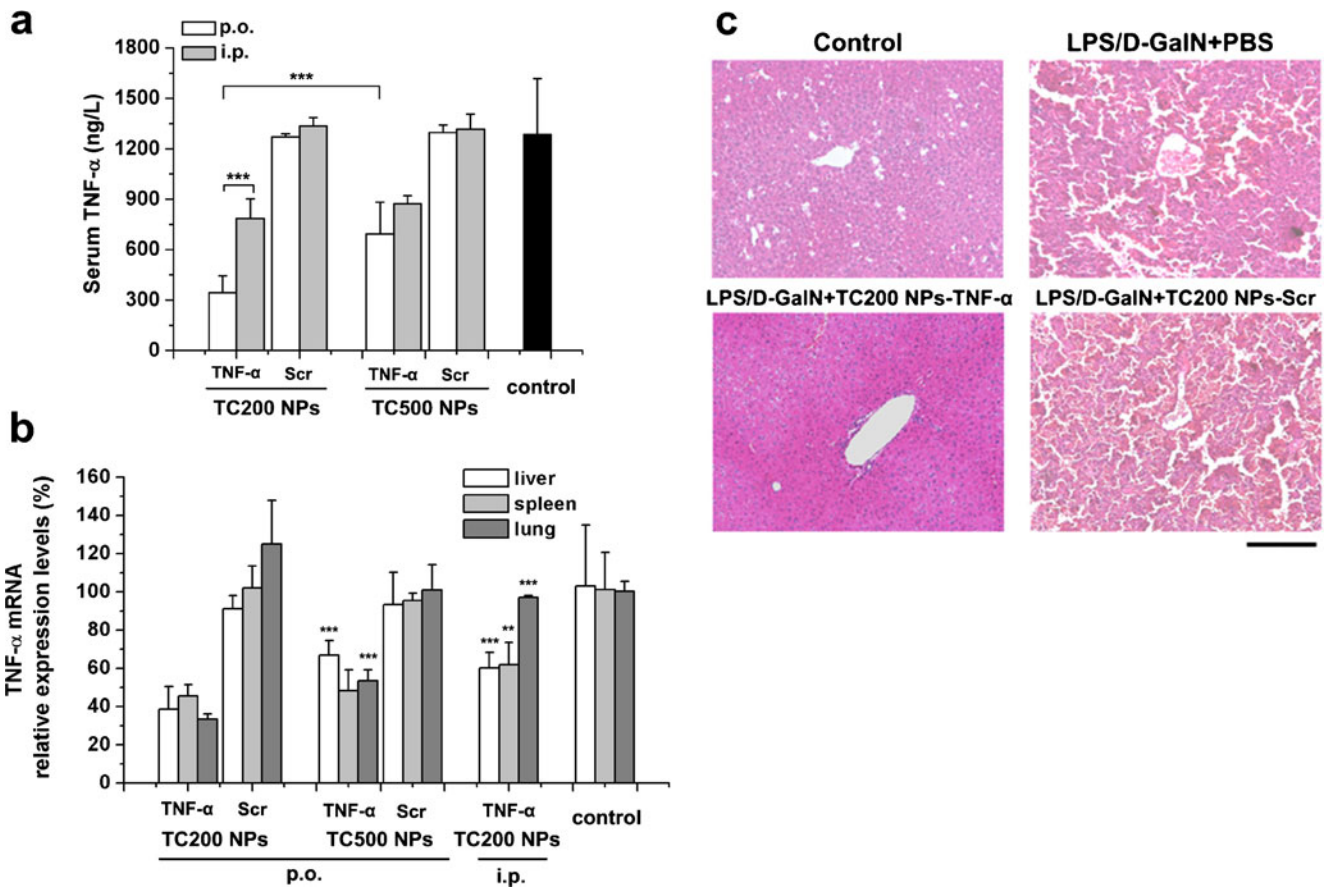

Fig. 7 The potent capacity of TC NPs in attenuating LPS/D-GalN-induced systemic TNF-α production *in vivo* after peroral (p.o.) and intraperitoneal (i.p.) administration. (**a**) Serum TNF-α levels in mice delivered with TC200 NPs and TC500 NPs containing TNF-α siRNA or Scr siRNA at a siRNA dose of 200 μg/kg ($n = 4$–10). (**b**) *TNF-α* mRNA levels in mouse liver, spleen, and lung 24 h post administration of TC200 NPs and TC500 NPs ($n = 3$). Statistical significance observed from *TNF-α* mRNA relative expression level of TNF-α siRNA loaded TC200 NPs *via* oral gavage. (**c**) H&E stained liver sections from mice 6-h post LPS/D-GalN stimulation. *Bar* represented 1 mm. ** $P < 0.01$ and *** $P < 0.001$.

(Supplementary Material Fig. S3 and Fig. S4). Such results implied that TC NPs might directly deliver the encapsulated siRNA to the cytoplasm instead of experiencing endolysosomal entrapment and degradation (24,25). The fourth barrier to gene transfer seemed to be insufficient cytoplasmic release of siRNA. The encapsulation of siRNA into NPs was required for its extracellular protection, however, the siRNA release in the cytoplasm was demanded to allow the incorporation of siRNA into the mRNA target (12). The disulfide bonds of TC conjugate could be presented in the structure of TC NPs. The rapid release of siRNA at high GSH concentration (Fig. 4) could be attributed to the promoted nanoparticle dissociation caused by the cleavage of disulfide bonds within TC NPs (26). TC500 NPs exhibited lower release rate and cumulative release amount compared to TC200 NPs, which might be due to the stronger binding affinity to siRNA resulting from its longer polymeric chain and more postive charges (23). Considering that the efficient RNAi required sufficient siRNA amount in the cytoplasm, the slow and incomplete siRNA release from TC500 NPs might lead to their inferior *in vitro* gene silencing efficiency. Consequently, upon successfully overcoming the above four major barriers to gene transfer, TC200 NPs mediated efficient *in vitro* RNAi in PECs which are difficult to transfect (Fig. 5).

The administration route was a crucial determinant for the success of siRNA-based therapeutics because with the same formulation, administration route would influence the drug efficacy (12,27–29). Intraperitoneally administered TC NPs could directly deliver siRNA to peritoneal cells, which can be as a depot for subsequent systemic distribution (12,30). The following two issues might represent the prerequisites for achieving high *in vivo* RNAi efficiency *via* intraperitoneal delivery: (1) siRNA integrity in peritoneal fluids and blood stream where TC NPs could be translocated; (2) effective *in vivo* uptake and gene knockdown mediated by TC NPs in murine PECs. As demonstrated in Fig. 2, TC200 NPs and TC500 NPs effectively protected siRNA from degradation in both peritoneal fludis and serum. Moreover, they delivered their payload to PECs after intraperitoneal injection (Fig. 6a). Thus, TC200 NPs and TC500 NPs demonstrated preferable capability to mediate TNF-α gene knockdown in PECs *in vivo* (Fig. 6b and Supplementary Material Fig. S2). Compared to TC500 NPs, the improved silencing efficiency mediated by TC200 NPs following intraperitoneal injection might be attributed to their higher *in vivo* cellular uptake levels and cytoplasmic siRNA release.

Considering the patient compliance and cost-effectiveness in the clinics, the peroral administration seemed to be the desired alternative. However, as for oral delivery, the siRNA integrity in the gastrointestinal tract and the siRNA transport across the intestinal epithelial barriers would determine its *in vivo* RNAi efficacy (31,32). TC200 NPs and TC500 NPs effectively improved the siRNA integrity in gastrointestinal environment (Fig. 2). The trimethyl group could interact with negatively charged components in the mucosa and epithelial cell membranes *via* electrostatic interactions and the cysteine residues would form disulfide bonding with mucin in the mucosa and epithelial cell membranes, which synergistically improved mucoadhesion as well as cell binding and cellular uptake by Caco-2 cells to facilitate the siRNA transport across the intestinal epithelial barriers (Supplementary Material Fig. S5 and Fig. S6) (18). The lower P_{app} values for TC500 NPs might be resulted from the following two aspects: (1) the longer polymeric chains reduced the extent of interpenetration with epithelial membrane associated components, thus decreasing the permeation (23); (2) the lower cell binding and cellular uptake amount in Caco-2 cells led to their inferior transcytosis in enterocytes. Compared to TC200 NPs, the inferior gene silencing efficacies of TC500 NPs following oral gavage might be ascribed to their lower permeation across the intestinal epithelium.

Since TC200 NPs and TC500 NPs could overcome the extracellular and intracellular barriers to *in vivo* RNAi, they efficiently mediated TNF-α knockdown in LPS/D-GalN induced acute hepatic injury mice *via* both peroral and intraperitoneal administration (Fig. 7). The extracellular environment for *in vitro* and *in vivo* gene silencing efficacy assessment was dramatically different, which made it possible to achieve better transfection efficiency *in vivo* than *in vitro*. Such results were in accordance with those of a previous report (19). That report demonstrated that macrophage-targeting β1,3-D-glucan particles successfully deliver Map4k4 siRNA for attenuating TNF-α related systemic inflammation *via* both peroral and intraperitoneal administration, however, the disparity in the RNAi efficiency *via* the two administration routes was unknown (19). Considering that overcoming the barriers to peroral delivery was more challenging compared to intraperitoneal delivery, intraperitoneally administered TC NPs were assumed to mediate more potent gene silencing *in vivo*. Interestingly, suppression levels of serum TNF-α production mediated by TC200 NPs *via* peroral administration was significantly higher than intraperitoneal administration (Fig. 7a). Such result might be attributed to the disparities in the absorption mechanisms and macrophage trafficking pathways following peroral and intraperitoneal administration. Orally delivered TC200 NPs could be efficiently absorbed through normal intestinal enterocytes as well as M cells (Supplementary Material Fig. S6) while intraperitoneally delivered TC200 NPs might be directly absorbed *via* the peritoneum. Moreover, TC200 NPs delivered *via* oral gavage could transfect gut-associated macrophages (GAMs) residing in the Peyer's patches to induce efficient RNAi in macrophage-enriched organs (Fig. 7b) (19). Intraperitoneally delivered TC200 NPs might be translocated with the migration of PECs to induce relatively low RNAi efficiency in macrophage-enriched organs (Fig. 7b). As for TC500 NPs, orally delivery did not exhibit more potent gene

silencing than intraperitoneally injection (Fig. 7a), suggesting that optimizing the delivery carriers might play the crucial roles in determining the suitable administration route.

In addition, the cytotoxicity of TC NPs as siRNA carriers was evaluated to address their safety concern. Supplementary Material Fig. S7 suggested that TC NPs with or without TNF-α siRNA induced no cytotoxicity in Raw 264.7 cells after a 6-h incubation. Considering the demonstrated biodegradability and biocompatibility of chitosan (MW 780 kDa)-based drug carriers (33), TC NPs were expected to be degraded and eliminated from the body after oral administration. Thus, TC NPs as oral delivery vehicles should be relatively safe.

CONCLUSIONS

In the present study, TC NPs were demonstrated to effectively mediate gene silencing *in vivo via* both peroral and intraperitoneal administration by conquering various extracellular and intracellular barriers. TC200 NPs and TC500 NPs could protect siRNA from nuclease degradation in physiological environment, deliver siRNA to macrophages *in vitro* and *in vivo* without experiencing the endolysosomal pathway, and sufficiently release siRNA in the cytoplasm to trigger subsequent gene silencing. The superior efficacy of TC200 NPs in inducing RNAi *in vitro* and *in vivo* suggested the important role of polymer MW in the optimization of polymeric NPs-based vehicles. Moreover, RNAi efficiency of TC200 NPs in acute hepatic injury mice was correlated to their administration route. The polymer MW- and administration route-dependent *in vivo* RNAi would provide guidelines for the rational design of siRNA vehicles.

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