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Mannosylated bio reducible nanoparticle-mediated macrophage-specific TNF- α RNA interference for IBD therapy

Bo Xiao^{1,*}, Hamed Laroui¹, Saravanan Ayyadurai¹, Emilie Viennois^{1,2}, Moiz A. Charania¹, Yuchen Zhang¹, and Didier Merlin^{1,2}

¹Department of Biology and Chemistry, Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, 30302, USA

²Atlanta Veterans Affairs Medical Center, Decatur, 30033, USA

Abstract

The application of RNA interference (RNAi) for inflammatory bowel disease (IBD) therapy has been limited by the lack of non-cytotoxic, efficient and targetable small interfering RNA (siRNA) carriers. TNF- α is the major pro-inflammatory cytokine mainly secreted by macrophages during IBD. Here, a mannosylated bio reducible cationic polymer (PPM) was synthesized and further spontaneously assembled nanoparticles (NPs) assisted by sodium triphosphate (TPP). The TPP-PPM/siRNA NPs exhibited high uniformity (polydispersity index = 0.004), a small particle size (211–275 nm), excellent bio reducibility, and enhanced cellular uptake. Additionally, the generated NPs had negative cytotoxicity compared to control NPs fabricated by branched polyethylenimine (bPEI, 25 kDa) or Oligofectamine (OF) and siRNA. *In vitro* gene silencing experiments revealed that TPP-PPM/TNF- α siRNA NPs with a weight ratio of 40:1 showed the most efficient inhibition of the expression and secretion of TNF- α (approximately 69.9%, which was comparable to the 71.4% obtained using OF/siRNA NPs), and its RNAi efficiency was highly inhibited in the presence of mannose (20 mM). Finally, TPP-PPM/siRNA NPs showed potential therapeutic effects on colitis tissues, remarkably reducing TNF- α level. Collectively, these results suggest that non-toxic TPP-PPM/siRNA NPs can be exploited as efficient, macrophage-targeted carriers for IBD therapy.

Keywords

Mannosylation; Bio reducible polymer; Macrophage-targeted delivery; RNA interference; IBD therapy

1. Introduction

Inflammatory bowel disease (IBD), which mainly consists of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing disorder associated with uncontrolled inflammation in the gastrointestinal tract [1]. The main goal of IBD therapy is to induce and maintain remission, achieve mucosal healing, and reduce surgeries and hospitalizations [2, 3]. The conventional treatments are limited to anti-inflammatory drugs and immune-suppressive medications. Although some of these treatments are effective in controlling

* Author for correspondence: Tel.: +1 (404) 413 3597, Fax: +1 (404) 413 3580, bxiao@gsu.edu.

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inflammation, their usefulness has been restricted by problems with long-term efficacy and safety issues [4, 5]. Accumulating evidence has shown that macrophage plays an important role in the pathogenesis of chronic inflammation, contributing to disease progression and/or maintenance by producing pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) [6]. Recently, TNF- α has become an important target for IBD therapy, as its inhibition dramatically reduces inflammatory markers and relieves structural damage to the mucosa [5]. Biological therapeutic strategies against TNF- α have been shown to effectively treat IBD in multiple clinical trials using the monoclonal antibody, infliximab [7, 8]. However, serious infections and side effects have been reported, including infusion reactions and antibody formation against TNF- α [9, 10].

RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) of 19–23 base pairs is a powerful tool for post-transcriptionally silencing gene expression [11], and has been recognized as an efficient approach for downregulating TNF- α expression in macrophages [12]. The success of RNAi is largely dependent on the utilized carrier and delivery method. For example, siRNA can be condensed into nanoparticles (NPs), which protect it against premature degradation, transport it across cell membranes and facilitate its escape from the lysosome/endosome, leading to the effective release of siRNA to the cytoplasm of target cells, where it can enter the RNAi pathway [13, 14]. In terms of delivering the NPs to the proper target cells, receptor-mediated gene delivery is a promising approach for achieving target specificity and avoiding non-specific interactions [15]. The mannose receptor (MR), a 175-kDa transmembrane protein of the C-type lectin family, is exclusively expressed on the surface of macrophages [16]. MRs can undergo high-affinity binding to infectious agents containing terminal mannose residues, triggering their transport into endocytic pathways, and resulting in MHC presentation and subsequent T cell activation [17]. This receptor system can bind to NPs coated with mannose residues, leading to their rapid internalization within membrane-bound vesicles [18]. The introduction of mannose to various non-viral gene delivery carriers, such as polyethyleneimine [19], lipid [20], gelatin [21] and chitosan [22], have been shown to provide selective macrophage targeting, improved cellular uptake and yielding a high transfection efficiency.

Recently, bio-reducible poly(amido amine)-based NPs have been proposed as promising gene carriers [23–27]. Their structures meet the fundamental design criteria of good siRNA carriers, and they have a number of unique beneficial features, including: (1) well-defined structures composed of disulfide bonds, primary amine groups and tertiary amine groups; (2) a high siRNA-condensing ability, wherein the abundant positively charged groups facilitate the efficient condensation of siRNA into NPs [28]; (3) good biostability, wherein the structural integrity of the disulfide backbone-based polymers has a low susceptibility to humidity and is well maintained in the extracellular environment [29]; (4) a “proton-sponge” effect, in which the polymer’s abundant primary and tertiary amine groups can promote endosomal/lysosomal escape [26, 29, 30]; and (5) good bio-reducibility and limited cytotoxicity. In contrast to the mildly oxidizing extracellular environment, the high concentration of glutathione (0.5–10 mM) and other small redox molecules with free thiol groups (5–10 mM) mean that the intracellular compartment has a reducing environment [23, 30]. In addition, abundant intracellular protein thiols represent an even larger redox pool [31]. In the reducing environment, an efficient disulfide-thiol exchange reaction disassembles the bio-reducible NPs, either alone or in cooperation with redox enzymes. Meanwhile, cytotoxicity is relatively low due to polymer degradation. Therefore, a strategy that combines the unique properties of bio-reducible polymers with the advantages of macrophage-specific mannose residues would be expected to show low cytotoxicity and high macrophage-specific TNF- α siRNA (siTNF) transfection efficiency for IBD therapy. Finally, sodium triphosphate (TPP) is a non-toxic negatively charged molecule that can form a crosslink between polycation and siRNA to yield condensed stable NPs [32].

In the present study, we report the first attempt to synthesize a mannosylated bioreducible polymer and fabricate polycation/siRNA NPs with or without TPP, as depicted in Scheme 1. We evaluated the physicochemical properties (*e.g.*, hydrodynamic diameters, siRNA complexation capability and bioreducible ability) of our NPs in relation to their capacity as siRNA delivery carriers. Additionally, we examined the cytotoxicity and cellular uptake profile of these NPs and demonstrate their negative toxicity and high RNAi efficiency.

2. Materials and Methods

2.1. Materials

Cystamine bisacrylamide (CBA) was obtained from Polyscience (Warrington, PA, USA). Branched polyethylenimine (bPEI₈₀₀, 800 Da), branched polyethylenimine (bPEI, 25 kDa), D-(+)-mannose, sodium triphosphate pentabasic (TPP), 2-(*N*-morpholino) ethanesulfonic acid sodium salt (MES), 1-ethyl-3-(diethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), lipopolysaccharides (LPS) from *Salmonella enteric* serotype typhimurium were purchased from Sigma-Aldrich (St. Louis, USA). Heterobifunctional PEG derivatives (NH₂-PEG-COOH and OH-PEG-COOH, MW 2000) were obtained from Jenkem Technology Company (Beijing, China). OligofectamineTM (OF) transfection reagent, Alexa Fluor 568 phalloidin (M_w = 1590), 4',6-diamidino-2-phenyl-indole dihydrochloride (DAPI), Stealth RNAiTM siRNA negative control, FITC fluorescently tagged siRNA and Vybrant[®] MTT cell proliferation assay kit were obtained from Invitrogen (Eugene, USA). GelRed was from Biotium (Hayward, USA). TNF- α siRNA (siTNF) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Synthesis of p(CBA-bPEI)-PEG-Man

The reducible cationic polymer p(CBA-bPEI) was synthesized by Michael addition reaction between CBA and bPEI₈₀₀ as previously described [24, 33]. Briefly, CBA and bPEI₈₀₀ was dissolved in 10% aqueous methanol and stirred in tightly-closed flask for 24 h at 60 °C under nitrogen atmosphere. Subsequently, 10 mol% excess of bPEI in methanol was added to consume unreacted acrylamide groups and the reaction was further maintained for 6 hours under the same condition. The reaction mixture was diluted with 1 M HCl to pH 5.0, and dialyzed (MWCO = 3500 Da) for 5 days against deionized water. The polymeric product was concentrated on a rotary evaporator and lyophilized.

The coupling of mannose to the amine groups of polymer was carried out by a modified method described in previous reports [20, 21]. Initially, D-(+)-mannose (10 μ M) was dissolved in 0.1 M sodium acetate buffer at pH 4 and 60 °C for 2 h, resulting in the ring opening of mannose molecules. In the second step, NH₂-PEG-COOH solution was mixed thoroughly with mannose solution and reacted for 24 h. The aldehyde group of the ring-opened mannose reacts with the amino group of PEG, yielding mannosylated PEG (Man-PEG-COOH). The product was purified by ultrafiltration using a filtration membrane (MWCO = 1000 Da). In the third step, p(CBA-bPEI) was dissolved in MES buffer (25 mM, pH 5.0) and the carboxyl group of Man-PEG-COOH was activated for 2 h by EDC/NHS in MES buffer. The molar ratio of NHS to EDC was 1:1. Subsequently, the activated Man-PEG-COOH solution was added to the p(CBA-bPEI) solution, and the resulting mixture was allowed to react at ambient temperature with stirring for 2 days. The reaction was quenched by adding hydroxylamine, and adjusting the pH of the reaction system to 8.0 with addition of a NaOH solution. The polymer was dialyzed (MWCO = 3500 Da) against deionized water for 5 days, concentrated on a rotary evaporator and lyophilized. The final product was named as PPM. OH-PEG-COOH was used to synthesize p(CBA-bPEI)-PEG (PPP) as a control to PPM.

2.3. Polymer Characterization

¹H NMR spectrum was recorded on a Bruker Avance Spectrometer (INOVA-400 NMR). Polymers were dissolved in D₂O to prepare a 2 wt % solution for NMR measurement. To test the molecular weight (Mw) of the p(CBA-bPEI), it was dissolved in 0.5 M sodium nitrate to concentrations ranging from 0.001 to 0.005 g/mL, and the viscosity-average Mw of the polymer was determined approximately 20.25 kDa using an Ubbelohde capillary viscometer based on a reported method [34]. The buffering capacity of the polymers was determined by acid-base titration method over the pH ranging from 2 to 10.5, as described in previous research [35]. Briefly, polymers (10 mg) were dissolved in 0.15 M NaCl (aq., 10 mL) and 1 M HCl (aq., 0.1 mL). The polymer solutions were titrated with 0.1 N NaOH to pH 10.5 and the changes in the pH of the polymers solutions were monitored by a pH meter. Titration of 0.15 M NaCl and bPEI (25 kDa) solutions were also performed in the same manner as controls. The buffering capacities of the polymers were compared at a pH of 5.1–7.4 to determine the behavior of the polymers at the endolysosomal pH. The buffering capacity can be calculated from the following equation [36]:

$$\text{Buffering capacity}(\%) = \frac{\Delta V_{\text{NaOH}} \times C_{\text{NaOH}}}{m_{\text{polymer}}} \times 100(\%)$$

Here, ΔV_{NaOH} is the volume of NaOH solution (0.1 M) which brought the pH values of polymer solutions from 5.1 to 7.4, C_{NaOH} is the concentration of NaOH solution (0.1 N), and m_{polymer} is the mass (10 mg) of the polymer.

2.4. Preparation of siTNF/polymer NPs

All the NPs were fabricated based on different polymers to siRNA weight ratios. The weight ratio of polymer to TPP was set to 4:1. Polymers and TPP were dissolved in RNase-free water to obtain solutions with the concentration of 4 mg/mL and 1 mg/mL, respectively. The siRNA stock solution was prepared in RNase-free water with the concentration of 0.1 mg/mL. NPs were prepared by a complex coacervation technique. Equivalent volumes of siRNA solutions were mixed with or without different amount of TPP solution, followed by the addition of different amount of polymers solutions, and vortexed for 10 s. The resulting NPs were allowed to incubate for 30 min at room temperature for complete NPs formation. NPs were prepared immediately prior to the experiments.

2.5. Agarose gel electrophoresis assay

The siRNA condensing capacities of PPM/siRNA and TPP-PPM/siRNA NPs were evaluated by agarose gel electrophoresis. The NPs were fabricated at various weight ratios ranging from 3 to 20. Agarose gel (4%, W/V) containing GelRed solution (0.5 µg/mL) was prepared in Tris-Acetate-EDTA buffer. After 30 min of incubation at room temperature, the samples were electrophoresed at 100 V for 20 min. The identical TPP-PPM/siRNA NPs were incubated in the presence of DTT (25 mM) for 1 h or 3 h at 37 °C and electrophoresed. Non-reducible bPEI (25 kDa)/siRNA NPs were used as a control. The resulting siRNA migration patterns were viewed under UV transilluminator.

2.6. Particle size and morphology measurement

Particle size (nm) and polydispersity index (PDI) of NPs were measured by photon correlation spectroscopy (PCS) employing Brookhaven equipment (Model 90Plus Nanoparticle Size Analyzer; Brookhaven Instruments Corporation, Holtsville, NY, USA) in 0.15 M NaCl at 25 °C. The average diameters (nm) were calculated using 3 runs. Each run is an average of 10 measurements. For morphology test, a drop of suspension of TPP-PPM/

siRNA with the weight ratio of 40:1 was mounted on a freshly cleaved glass slide using carbon adhesive tape and sputter-coated with a mixture of gold and palladium (60:40) in an argon atmosphere under low pressure. The image was measured by scanning electron microscopy (SEM, LEO 1450VP, Zeiss, Germany).

2.7. In vitro experiments

2.7.1. Cytotoxicity assay—For 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide assay (MTT), RAW 264.7 macrophages and Caco2-BBE cells were seeded at a respective density of 8×10^3 and 2×10^4 cells/well in 96-well plates and incubated overnight. The cells were incubated with freshly prepared NPs suspensions for 6 h, and the siRNA concentration in the medium is set as 100 nM. Cells were then incubated with MTT (0.5 mg/mL in supplemented 100 μ L of serum free DMEM) at 37 °C for 4 h. Thereafter, the media were discarded and 50 μ L dimethyl sulfoxide (DMSO) was added to each well prior to spectrophotometric measurements at 490 nm. Untreated cells were used as a negative reference, whereas NPs fabricated by bPEI (25 kDa) or OF and siRNA, which are the most frequently used gene carriers, were selected as positive controls.

Cell-attachment assays were performed to investigate the real-time cytotoxicity of NPs using electrical impedance sensing (ECIS) technology (Applied BioPhysics, Troy, NY). The ECIS model 1600R was used for these experiments. The measurement system consists of an 8-well culture dish (ECIS 8W1E plate), the surface of which is seeded with Caco2-BBE cells at a density of 1×10^6 /well. Once cells reached confluence, NPs were added to the wells and the siRNA concentration in the medium is set as 100 nM. Control cell cultures were used for each experiment, and the toxicity of bPEI (25 kDa)/siRNA NPs (weight ratio = 40:1) was also tested. Basal resistance measurements were performed using the ideal frequency for Caco2-BBE cells, 500 Hz, and a voltage of 1 V.

2.7.2. Intracellular NPs uptake visualization—Raw 264.7 macrophages were seeded in four-chamber tissue culture glass slide (BD Falcon, Bedford, MA, USA) at a density of 1.0×10^4 cells/well and incubated overnight. The culture medium was exchanged to serum-free DMEM containing TPP-PPM/FITC-siRNA NPs (weight ratio, 40:1). The FITC-siRNA concentration in the medium is set as 100 nM. After co-culture, the cells were fixed in 4% paraformaldehyde for 20 min. To observe cellular uptake of NPs, DAPI was diluted 10,000 times and added to the wells for staining cells for 5 min. Images were acquired using an Olympus equipped with a Hamamatsu Digital Camera ORCA-03G.

2.7.3. Quantification of intracellular uptake—Raw 264.7 macrophages were seeded in 24-well plates at a density of 5.0×10^4 /well. After overnight incubation, medium was replaced with serum- and antibiotics-free medium and cells were treated with different NPs, at a final concentration of 100 nM FITC-siRNA. After 6 h of co-incubation, cells were washed three times with PBS containing Ca^{2+} and Mg^{2+} and lysed in 150 μ L lysis buffer (1% Triton X-100, 2% SDS in PBS) on ice for 30 min. Lysates were transferred to Eppendorf tubes and centrifuge (20 min, 12,000 g, 4 °C) to remove cell debris. Then 100 μ L of the supernatant was transferred to a black 96-well plate for measuring the fluorescence on a Perkin-Elmer EnSpire multimode plate reader (Perkin Elmer, Boston, MA, USA). The protein content was determined by the Bio-Rad protein assay with bovine serum albumin as a standard. For determination of the mean fluorescence intensity (MFI), fluorescent signals were corrected for the amount of protein.

2.7.4. Anti-inflammation effect of siTNF-loaded NPs—Raw 264.7 macrophages were seeded in 24-well plates at a density of 4.0×10^5 cells and incubated overnight. NPs fabricated by polymers and siRNA with or without TPP were added for 6 h. The siRNA

concentration in the medium is set as 100 nM. As controls, cells were transfected with scramble siRNA-loaded TPP-PPM/siRNA NPs (weight ratio, 40:1) or siTNF using OF reagent according to the manufacturer's instructions. Cells were then incubated in complete medium. After 18 h, cells were stimulated with LPS (100 ng/mL) for 3 h. Finally, TNF- α secretion in the supernatant was measured with an eBioscience kit (mouse TNF- α ELISA kit, eBioscience, San Diego, CA) and concentration of TNF- α was normalized to the amount of protein.

2.8. Ex vivo experiments

2.8.1. Experimental animals—FVB male mice (7–8 weeks) were used in all animal experiments. Mice were group housed (25 °C), photoperiod (12:12-h light-dark cycle), and allowed unrestricted access to potables and standard mouse chow. All the animal experiments were approved by Georgia State University Institutional Animal Care and Use Committee.

2.8.2. DSS-induced colitis—Colitis was induced by replacing their drinking water with a 3% (wt/vol) DSS (35 kDa). For each of the animal experiments, groups of mice were treated with DSS or normal water for eight days. Mice were observed daily and evaluated for changes in body weight and development of clinical symptoms of colitis. After eight days DSS treatment, mice were sacrificed and colon was removed for further study. Tissue samples were evaluated for mucosal architecture change, cellular infiltration, inflammation, goblet cell depletion, surface epithelial cell hyperplasia, and signs of epithelial regeneration. Tissue sections with a thickness of 6 μ m were fixed with 4% paraformaldehyde for 30 min and stained with hematoxylin and eosin. Images were acquired using an Olympus equipped with a Hamamatsu Digital Camera ORCA-03G.

2.8.3. Anti-inflammation effect of siTNF-loaded NPs—Colonic tissues were cultured in RPMI-1640 medium (Cellgro, Manassas, VA, USA) without FBS as our previous study [37]. Briefly, colitis tissues (8–10 mm) of DSS-treated mice were placed in 12-well culture plates with the mucosal surface facing upwards. The wells were flooded in serum-free RPMI-1640 medium supplemented with penicillin and streptomycin and then different amount of TPP-PPM/siRNA NPs were added. The final TNF- α siRNA concentration in the medium is set as 100 nM, 200 nM and 300 nM, respectively. After 6 h, tissues were incubated in RPMI-1640 medium containing 10% FBS overnight (12 h). Supernatant was collected and centrifuged at 12,000 g for 10 min at 4 °C and stored at –80 °C until analyzed. Samples were processed as described in the *in vitro* part by an ELISA protocol.

2.8.4. NPs uptake visualization—Colitis tissues (6–8 mm) of DSS-treated mice were placed in 24-well culture plates with the mucosal surface facing upwards. The wells were flooded in serum-free RPMI-1640 medium supplemented with penicillin and streptomycin and then TPP-PPM/FITC-siRNA NPs suspensions were added. The final FITC-siRNA concentration in the medium is set as 200 nM. After 6 h, tissues were washed with PBS for 3 times, fixed in 10% buffered formalin (EMD Millipore, MA, USA) and then embedded in paraffin. 3 μ m sections were stained with Alexa Fluor 568 phalloidin and DAPI. Images were acquired using an Olympus equipped with a Hamamatsu Digital Camera ORCA-03G.

2.8.5. Flow cytometry analysis—Colonic tissues (10–15 mm) were placed in 6-well culture plates with the mucosal surface facing upwards. The wells were flooded in serum-free RPMI-1640 medium supplemented with penicillin and streptomycin, and then TPP-PPM/FITC-siRNA NPs (weight ratio, 40:1) were added. The final FITC-siRNA concentration in the medium is set as 200 nM. After 6 h, tissues were further incubated in serum-free medium overnight (12 h). Intestines were then cut into pieces 5 mm in length,

which were transferred into 50 mL conical tubes and shaken at 220 rpm for 20 min at 37 °C in Hank's Buffered Salt Solution (HBSS; Life Technologies) with 5% FBS containing 2 mM EDTA. This process was repeated two additional times. Cells suspensions were passed through a strainer and the remaining intestinal tissue was washed and then minced, transferred to a 50 mL conical tube and shaken for 20 min at 37 °C in HBSS with 5% FBS and type VIII collagenase (1.5 mg/mL; Sigma). Cell suspensions were collected and passed through respective 100 μ m and 40 μ m strainers and were pelleted by centrifugation at 1500 rpm for 5 min.

For splenocytes, spleens were dissected and cut into small fragments. The tissue was deposited on the 100 μ m cell strainer and squeezed by the syringe handle. The tissue residues were rinsed using HBSS with 5% FBS. The cells were collected by centrifugation at 1500 rpm for 5 min. Red Blood Cell lysis buffer (3 mL) was added to the spleen cells and incubated for 3 min at room temperature. After that, 37 mL of HBSS with 5% FBS was added to the cell suspension and the splenocytes were collected by centrifugation at 1500 rpm for 5 min.

Isolated splenocytes or colon cells were resuspended in HBSS containing 5% FBS. After incubation with the blocking Ab 2.4G2 anti-Fc γ RIII/I for 10 min, the cells were stained by labeled antibodies (Abs) for 45 min. Samples then were washed twice in HBSS containing 5% FBS. The samples were fixed in flow cytometry buffer containing 1% paraformaldehyde and stored at 4 °C. Abs used for analysis were from eBioscience unless otherwise noted: anti-Mouse CD11c APC, anti-Mouse MHC Class II Alexa Fluor[®] 700, anti-mouse CD11b eFluor[®] 450, anti-mouse F4/80 antigen PE-Cy7, PerCP rat anti-mouse CD45 (BD pharmingen), anti-mouse CD4 APC, anti-mouse CD4 Alexa Fluor[®] 700, anti-mouse CD4 eFluor[®] 450, anti-mouse CD4 PE-Cy7, anti-mouse CD4 FITC and PerCP rat anti-mouse CD4 (BD pharmingen).

2.9. Statistical analysis

Statistical analysis was performed using Student's *t*-test. Data were expressed as mean $\pm$ standard error of mean (S.E.M.). Statistical significance was represented by **P*<0.05 and ***P*<0.01.

3. Results and discussion

3.1. Synthesis and characterization of PPM

A bioreducible bPEI derivative was selected as the backbone polymer on the basis of previous reports showing that such biopolymers have limited cytotoxicity and high plasmid/siRNA transfection efficiency [24, 36, 38, 39]. The synthesis route is illustrated in Scheme S1 (Supplementary data).

The proper synthesis of the polymers was confirmed by ¹H NMR. As seen in Fig. 1a, the peaks between δ 2.5 and 3.0 ppm in the spectrum of p(CBA-bPEI) were specific to bPEI₈₀₀. Additionally, the characteristic acrylamide peaks between δ 5 and 7 ppm disappeared, which was consistent with a previous report [24] and confirmed that the p(CBA-bPEI) had been successfully synthesized. As shown in Fig. 1b, the main peak of PEG (corresponding to the methylene group) registered at 3.6 ppm. As expected, a very strong peak at 3.6 ppm was also observed in Fig. 1c, indicating the successful conjugation of PEG to p(CBA-bPEI). The ¹H NMR spectrum (Fig. 1d) of the mannosylated polymer showed extra signals compared to the non-glycosylated polymer (*e.g.* δ 4.5); these originated from incorporated mannose [40], suggesting that mannose had been successfully grafted to the polymer. The ratio between the integration of PEG proton peaks and the integration of poly(CBA-bPEI) proton peaks was $\sim$ 1.6 PEG per bPEI₈₀₀ unit. Degree of the mannosylation of PEG was

found to be almost 100% from the calculation the integration of mannose proton peaks and the integration of PEG proton peaks.

Although a good proton buffer capacity does not necessarily guarantee a high transfection efficiency for polymers, it is often studied as a property for siRNA carriers [25]. Here, we titrated the polymers and evaluated their buffering capacities between pH 5.1 and pH 7.4, compared to that of bPEI (25 kDa). As shown in Fig. 2, the buffering capacities of p(CBA-bPEI), PPP and PPM were approximately 74.4%, 56.5% and 48.1%, respectively, of that of bPEI (25 kDa). As the buffering capability of polymers was reported to mainly depend on the presence of primary, secondary and tertiary amine groups [41], the reduced buffering capacity of the synthesized polymers may be attributed to the lower density of their amino groups following the introduction of disulfide and PEG groups to the side chains of bPEI₈₀₀. The decreased buffering capacity may also reflect that the mass fraction of bPEI₈₀₀ was decreased by the introduction of disulfide, PEG and mannose groups.

3.2. Agarose gel electrophoresis

In comparison to plasmid DNA, siRNA has an extremely low charge density and high stiffness. In addition, a double-stranded siRNA of 21 bp is only around 5.67 nm in length [42]. These intrinsic properties of siRNA largely limit their NP-forming ability. To overcome this major obstacle, various covalent crosslinkers, such as glutaraldehyde and genipin, have been employed to assist in the formation of NPs. In the present study, we used the ionic crosslinker, TPP, because the residues of covalent crosslinkers can induce cytotoxicity to the cells. In contrast, TPP has been FDA approved for use in the food industry, based on its non-toxicity [43]. TPP has a linear structure that carries five negative charges, and can thus crosslink protonated PPM in an aqueous medium. Most importantly, TPP has been reported to enhance the siRNA condensation capacity of polycations and increase the efficiency of NPs-mediated gene silencing [44].

To examine the optimum PPM-to-TPP ratio for fabricating our NPs, we studied the effect of different PPM/TPP ratios on the size profile of NPs. As depicted in Fig. S1 (Supplementary data), the PPM/TPP weight ratio was clearly critical and controlled the size of the NPs. As the PPM/TPP weight ratio increased from 0.5 to 20, the particle size first gradually decreased from 1193 nm to 569 nm and then dramatically increased to 1704 nm. The optimum PPM/TPP ratio (corresponding to the smallest NP particle size) was 4:1. This was in good agreement with a previous report in which chitosan was used for polycation [45].

As cationic polymers are known to restrict negatively charged siRNA by forming NPs via electrostatic interactions, we used agarose gel electrophoresis to examine the siRNA-condensing ability of the NPs in the absence or presence of DTT. As shown in Fig. 3a and Fig. 3b, PPM/siRNA NPs were found to retard siRNA beginning at a weight ratio of 20:1, whereas TPP-PPM/siRNA NPs condensed siRNA completely at a weight ratio of 10:1 in the absence of DTT. Consistent with a previous report [44], our results showed that TPP can strengthen the association between polycations and siRNA.

The release of siRNA from the NPs was investigated by gel electrophoresis in the presence of 25 mM DTT, which mimics the intracellular reducing environment. As shown in Fig. 3b–d, the TPP-PPM/siRNA NPs generated at a weight ratio of 3:1 successfully released all of their siRNAs into the gel. When NPs were generated in the presence of DTT for 1 h and 3 h, almost all of the siRNAs were released from NPs representing weight ratios of 5:1 and 7:1. As expected, the non-bioreducible bPEI (25 kDa) retarded siRNA irrespective of DTT (data not shown). These findings suggest that PPM can be efficiently degraded in a reducing environment, and support the notion that our NPs will undergo controlled release of siRNA

via bio-reduction in cytoplasm after cellular uptake, potentially leading to efficient long-term RNA silencing.

3.3. Particle size and morphology of NPs

TPP-PPM/siRNA NPs were fabricated at a fixed PPM/TPP weight ratio of 4:1 by adjusting the weight ratio of PPM to siRNA. As shown in Fig. 4a, TPP had a strong impact on NP size. The average sizes of TPP-free PPM/siRNA NPs with different weight ratios were in the range of 302 nm to 363 nm. With the introduction of TPP to the NPs, the average sizes of TPP-containing TPP-PPM/siRNA NPs were markedly decreased, ranging from 211 nm to 275 nm. Fig. 4b and 4c illustrate the size distributions of PPM/siRNA (40:1) NPs with or without TPP. The TPP-free NPs had a wider size distribution than the TPP-containing NPs (PDI value < 0.01), which was in agreement with a previous report [46]. These data collectively indicate that TPP is highly effective in facilitating siRNA condensation and uniform NP formation in our system.

Fig. 4d shows a representative SEM image of TPP-PPM/siRNA (weight ratio, 40:1) NPs, which were largely spherical, with mean diameters ranging from 150 to 200 nm. The particle size measured by SEM was smaller than that determined by PCS, which is consistent with previous finding [47]. This may reflect that fully hydrated (swollen) particles are measured during PCS, whereas SEM measurements are performed in a dry state. In addition, PCS calculates an intensity average size, whereas SEM evaluates a number average size, which is generally lower [48].

3.4. Cytotoxicity of the NPs

Cytotoxicity is a primary concern in the development of gene transfection agents. To evaluate the cytotoxicity of our developed NPs, we treated RAW 264.7 and Caco2-BBE cells with various weight ratios of TPP-PPM/siRNA NPs (siRNA, 100 nM) and examined cell viability using MTT assays. As shown in Fig. 5a, the TPP-PPM/siRNA NPs did not cause obvious cytotoxicities among RAW 264.7 or Caco2-BBE cells. In contrast, OF/siRNA complexes applied at the recommended transfection condition showed significantly higher cytotoxicity compared to our NPs, and bPEI (25 kDa)/siRNA (weight ratio, 40:1) inhibited the proliferation of both cell lines cell by over 85%. This might explain why OF and PEI (25 kDa) are recommended for transfection of highly confluent cells, enabling the survival of sufficient cells for subsequent experiments.

The cytotoxicity of cationic polymers is known to depend on their surface charge, and excess positive charges on the surfaces of NPs can interact with cellular components (*e.g.*, cell membranes) and impair membrane integration [49, 50]. Cationic polymers may also interfere with critical intracellular processes, such as by disrupting protein kinase C activity [51]. In the present study, we designed our NPs such that the PEG groups of PPM should be exposed at the NP surface, decreasing the interaction between the positively charged groups of the NPs and the negatively charged cellular components. In addition, the bioreducible polymers should be degraded in the reducing intracellular environment, further decreasing their toxicity. Consistent with this, our NPs exhibited much better compatibility than bPEI (25 kDa)/siRNA or OF/siRNA complexes.

It has been reported that the MTT assay is not suitable for real-time analysis of NP cytotoxicities [48], so we also used ECIS for *in vitro* toxicity tests. As shown in Fig. 5b, Caco2-BBE cells attached to the electrode surface form a confluent layer with a resistance around 40,000 Ohms. Such cells were then co-incubated with different NPs containing the same siRNA concentration. At 20 hours post-transfection, TPP-PPM/siRNA NPs-transfected Caco2-BBE monolayers showed a slight increase in resistance, while those co-

cultured with bPEI (25 kDa)/siRNA (40:1) experienced sharp decreases in resistance. Together, these results indicated that our NPs did not show obvious cytotoxicity or deleterious effects on monolayer resistance (representing intestinal barrier function).

During the above-described experiments, changes in cell morphology were investigated by direct optical microscopy at 20 h post-treatment. As shown in Fig. 5c, untreated control Caco2-BBE cells contacted each other and exhibited normal shapes with clear outlines, and cells treated with TPP-PPM/siRNA (weight ratios 20:1 and 40:1) had similar morphologies. In contrast, cells cultured with bPEI (25 kDa)/siRNA NPs showed significant morphological changes and small irregular cell bodies.

3.5. Cellular uptake of NPs by macrophages

Efficient cellular uptake is a major requirement for the therapeutic use of siRNAs [52]. To confirm the efficient cellular uptake of TPP-PPM/siRNA NPs in Raw 264.7 cells, we used fluorescence microscopy at different time points (0 h, 3 h and 6 h). As shown in Fig. 6, the FITC fluorescence signals of TPP-PPM/FITC-siRNA NPs in the cells increased with time. After 3 h of incubation, numerous NPs had been internalized. After 6 h, green fluorescence was mainly detected in the cytoplasm of almost all cells. The TPP-PPM/FITC-siRNA NPs and OF/FITC-siRNA complexes yielded comparable FITC fluorescence intensities. Together, these results demonstrate that TPP-PPM/siRNA NPs can be efficiently internalized into Raw 264.7 cells.

To quantitatively compare the cellular uptakes of siRNA carried in NPs of various weight ratios, we performed cell lysis and NP dissociation in a lysis buffer containing 1% Triton X-100 and 2% SDS (to avoid quenching the fluorescent signal) [53], and examined fluorescence. As shown in Fig. 7a, free FITC-siRNA exhibited negligible cellular uptake, whereas the NPs significantly enhanced the cellular uptake of siRNA. For TPP-PPP/siRNA NPs, the cellular uptake of siRNA increased sharply with increasing weight ratios, peaking at the weight ratio of 40:1. The same trend was observed for TPP-PPM/siRNA NPs, which showed dramatically higher siRNA internalization compared to TPP-PPP/siRNA NPs at the same weight ratios, except for the weight ratio of 10:1. As noted above, the enhanced cellular uptake mediated by TPP-PPM/siRNA NPs can be explained by the introduction of mannose at the surfaces of the NPs. As shown in Fig. 7b, a pronounced increase in cellular uptake was observed for TPP-PPM/siRNA NPs compared to PPM/siRNA NPs with the same weight ratio (40:1). These results indicate that the introduction of TPP to our NPs improved the cellular uptake efficiency of siRNA. To confirm the mannose receptor-mediated cellular uptake of TPP-PPM/siRNA NPs, we also examined the cellular uptake of NPs (weight ratio, 40:1) by Raw 264.7 cells in the presence of mannose (as a competitor for the mannose groups in the PPM). As shown in Fig. 7c, the cellular uptake of TPP-PPM/siRNA NPs decreased substantially in the presence of 20 mM free mannose, suggesting that the uptake of TPP-PPM/siRNA NPs is mediated by mannose receptor-mediated endocytosis.

3.6. NP-mediated TNF- α silencing *in vitro*

To investigate the effectiveness of RNAi in our system, we next tested whether siTNF-loaded NPs could block TNF- α expression/secretion in LPS (100 ng/mL)-stimulated Raw 264.7 cells *in vitro*. As shown in Fig. 8a, LPS treatment increased the concentration of TNF- α in the supernatant by 12.9-fold compared with that in the negative control (121.6 pg/mg protein). In contrast, significantly less TNF- α secretion was observed from cells that were pre-treated with TPP-PPM/siRNA NPs prior to LPS stimulation, and this effect was weight-ratio-dependent. Furthermore, the RNAi efficiency of the NPs increased with the weight ratio, reaching a maximum value (471.6 pg/mg protein) at a weight ratio of 40:1. The trend was similar to that seen for the quantification of cellular uptake (Fig. 7a). The NP (40:1)-

mediated decrease in TNF- α expression did not significantly differ from that induced by the leading commercial product (OF; 447.0 pg/mg protein), indicating that our NPs had an efficient anti-inflammatory effect on LPS-treated macrophages.

Notably, the concentration of siTNF contained in our NPs was 2-fold less than that used in the OF/siRNA complexes. The improved gene-silencing effects of our NPs could be attributed to their good siRNA condensation ability, targeted delivery capacity, and increased intracellular internalization (see above). Appropriate controls were performed, and showed that scrambled siRNA-loaded NPs (weight ratio, 40:1) exhibited negligible anti-inflammatory effects on LPS-stimulated macrophages (Fig. 8a). The biological effects observed herein showed that the NPs taken up into the cells effectively released siTNF. Thus, we indirectly demonstrated that siTNF was released from the endocytotic vesicles. Most importantly, the TPP-containing NPs were more efficient for RNAi compared to TPP-free NPs (Fig. 8b). This was in agreement with previous studies [44, 54], and further improved our system.

3.7. NP-mediated TNF- α silencing *ex vivo*

Since our TPP-PPM/siTNF NPs showed a considerable gene silencing ability *in vitro*, we next investigated their RNAi effect in an *ex vivo* test. The DSS-induced colitis mouse model is commonly utilized to address the pathogenesis of IBD and determine the therapeutic efficacy of NP-based drug/gene delivery formulations. DSS-treated mice exhibited an obvious weight loss (about 12.5%) compared to the healthy control group (Fig. 9a), and rectal bleeding was observed after 8 days of DSS treatment (data not shown). As shown in Fig. 9b, H&E-stained colon sections from the healthy control group had normal colon histology with no sign of inflammation or disruption of healthy tissue morphology, whereas tissues from DSS-treated mice exhibited clear signs of inflammation, including goblet cell depletion and a high level of infiltration of inflammatory cells into the mucosa. As shown in Fig. 9c, the TNF- α levels in colitis tissues were significantly higher (243.2 pg/mg) than those in healthy colon tissues (6.4 pg/mg protein). Interestingly, when colitis tissues were treated with TPP-PPM/siTNF NPs, the TNF- α level was significantly and siTNF-concentration-dependently reduced, with a 61.0% decrease at 300 nM compared to the untreated colitis control. Furthermore, there was an obvious difference in TNF- α levels between siTNF concentrations of 100 nM and 300 nM, but not between 200 nM and 300 nM. Together, these results demonstrate that TPP-PPM/siTNF NPs could efficiently inhibit TNF- α synthesis/secretion in tissue samples from the DSS-induced mouse model of colitis.

Finally, we evaluated the targeting efficiency of TPP-PPM/siRNA NPs to colonic cells. As shown in Fig. 10a, co-incubation of TPP-PPM/FITC-siRNA NPs with colitis tissues from DSS-treated mice for 6 h revealed that large amounts of NPs efficiently penetrated into colitis tissue and accumulated therein. To further examine whether our NPs could target colon macrophages, we co-incubated mouse colitis tissues with TPP-PPM/FITC-siRNA NPs overnight (12 h) and carried out flow cytometry. Our results demonstrated that about 29.5% of the colon macrophages had taken up NPs (Fig. 10b), whereas relatively few epithelial cells exhibited FITC signals (data not shown). This indicates that our TPP-PPM/siRNA NPs could be preferentially taken up by macrophages. The high penetration ability and macrophage-targeting efficiency of TPP-PPM/siRNA NPs might reflect the combined effect of enhanced epithelial permeability and retention, small particle size, surface PEGylation, and the conjugation of mannose ligands. Given that the use of some potent therapies for IBD has been limited by their profound systemic toxicities, above data suggest that our system may be a highly promising strategy for delivering anti-TNF agents to the site of inflammation while minimizing systemic toxicity.

4. Conclusion

In the present study, a macrophage-targeted bio-reducible PPM conjugate was successfully synthesized and exploited to fabricate nanoparticles (NPs) with sodium triphosphate (TPP) and siRNA by electrostatic interaction. The well-formed NPs showed an enhanced siRNA condensation capacity, a desirable size distribution (around 240 nm), and a good macrophage-targeting ability. Importantly, these NPs were efficiently taken up by macrophages and conferred high-level RNAi, decreasing TNF- α expression and conferring anti-inflammatory effects *in vitro* and *ex vivo*. The generated TPP-PPM/siRNA NPs are expected to be non-cytotoxic and efficient macrophage-targeting carriers for IBD therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
 ^1H NMR spectra (400 MHz, D_2O) of (a) p(CBA-bPEI), (b) PEG, (c) p(CBA-bPEI)-PEG (PPP), and (d) p(CBA-bPEI)-PEG-Man (PPM).

Figure 2.

Acid-base titration curves of polymers. A total of 10 mg of each polymer was dissolved in 10 mL of 0.15 M NaCl solution, which was subsequently set to pH 2 using 1 M HCl. The solutions were then titrated to pH 10.5 with 0.1 N NaOH. For comparison, titration curves of 0.15 M NaCl and bPEI (25 kDa) solutions are presented as controls. Dashed lines indicate the endosomal pH range (5.1 and 7.4).

Figure 3. Agarose gel electrophoresis of NPs with different weight ratios in the presence or absence of DTT (25 mM). (a) PPM/siRNA NPs without TPP or DTT. (b) TPP-PPM/siRNA NPs without DTT. (c and d) TPP-PPM/siRNA NPs incubated with DTT for (c) 1 h and (d) 3 h.

Figure 4.

Particle size (nm), size distribution and morphological characterization of NPs. (a) Particle sizes of NPs generated at weight ratios ranging from 10:1 to 40:1 with or without TPP. Each point represents the mean $\pm$ S.E.M. ($n=3$). Statistical significance was assessed using the Student's *t*-test (* $P<0.05$ and ** $P<0.01$). (b) Size distribution of PPM/siRNA NPs (weight ratio, 40:1), which had a polydispersity index (PDI) of 0.297 ± 0.006 . (c) Size distribution of TPP-PPM/siRNA NPs (weight ratio, 40:1), which had a PDI of 0.004 ± 0.001 . (d) Scanning electron microscopy (SEM) of a suspension of TPP-PPM/siRNA NPs (weight ratio, 40:1).

Figure 5.

Cytotoxicity of NPs toward Raw 264.7 macrophages and Caco2-BBE cells. (a) The MTT test was used to determine the cytotoxicity of NPs compared to bPEI (25 kDa)/siRNA and Oligofectamine (OF)/siRNA complexes. SiRNAs were used at a concentration of 100 nM except for the OF/siRNA complexes, which were generated according to the manufacturer's standard protocols. Toxicity is given as the percentage of viable cells remaining after treatment for 4 h. Each point represents the mean $\pm$ S.E.M. ($n=5$). Statistical significance was assessed using the Student's *t*-test (* $P<0.05$ and ** $P<0.01$). (b) Electrical impedance-sensing (ECIS) of Caco2-BBE cells was used to determine cell viability after a long exposure to an NP suspension (siRNA, 100nM). As controls, ECIS was also performed on untreated cells or cells treated with bPEI (25 kDa)/siRNA NPs (siRNA, 100 nM). (c) Morphology of Caco2-BBE cells grown for 20 h in 8-well ECIS culture dishes with or without NPs.

Figure 6.

Cellular uptake of TPP-PPM/siRNA nanoparticles (NPs) (weight ratio, 40:1) and Oligofectamine (OF)/siRNA complexes in RAW 264.7 macrophages at different time points. Cells were treated with NPs loaded with FITC-siRNA (green) and processed for fluorescence staining. FITC-tagged scrambled siRNA (100 nM) was used for the transfections except for the OF/siRNA complexes, which were generated according to the manufacturer's standard protocols. Fixed cells were stained with DAPI (purple) for visualization of nuclei. Scale bar represents 10 μ m.

Figure 7.

Quantification of cellular uptake of NPs into RAW 264.7 macrophages. To determine the mean fluorescence intensity (MFI), fluorescent signals were corrected for the amount of protein. (a) Cellular uptake of TPP-PPP/FITC-siRNA and TPP-PPM/FITC-siRNA NPs at weight ratios ranging from 10:1 to 40:1. As controls, we tested cell lysates from untreated cells and those treated with FITC-siRNA. (b) Comparing cellular uptake of PPM/FITC-siRNA and TPP-PPM/FITC-siRNA NPs at a weight ratio of 40:1. (c) Competitive cellular uptake of TPP-PPM/FITC-siRNA NPs (weight ratio, 40:1) was assessed by adding mannose (20 mM) as a competitor. Each point represents the mean $\pm$ S.E.M. ($n=3$). Statistical significance was assessed using the Student's *t*-test (* $P<0.05$ and ** $P<0.01$).

Figure 8.

TNF- α RNA interference (RNAi) efficiency in NP-treated RAW 264.7 macrophages. (a) The inhibition of TNF- α secretion by TPP-PPM/TNF- α siRNA (siTNF) NPs at weight ratios ranging from 10:1 to 40:1. As controls, RAW 264.7 macrophages were treated without NPs, with scrambled siRNA-loaded NPs, and with Oligofectamine (OF)/siTNF complexes. SiTNF was used at a concentration of 100 nM except for the OF/siTNF complexes, which were generated according to the manufacturer's standard protocols. (b) Comparison of the TNF- α RNAi efficiency of PPM/siTNF and TPP-PPM/siTNF NPs at a weight ratio of 40:1. Each point represents the mean $\pm$ S.E.M. (n=5). Statistical significance was assessed using the Student's *t*-test (**P*<0.05 and ***P*<0.01).

Figure 9.

NP-mediated TNF- α RNAi efficiency in colitis tissues. (a) Changes of body weight over time in mice treated with 3% DSS in drinking water. Mouse body weight was normalized as a percentage of day-zero body weight and depicted as the mean of each treatment group $\pm$ S.E.M. (n=6). (b) Hematoxylin and eosin-stained colon sections from healthy control and DSS-treated mice sacrificed on day 8. Scale bar represents 50 μ m. (c) Inhibition of TNF- α secretion by TPP-PPM/siTNF NPs (weight ratio, 40:1) at different siTNF concentrations (100 nM, 200 nM and 300 nM). Colon tissues were incubated with TPP-PPM/siTNF NPs for 12 h. For comparison, colon tissues left untreated or treated with TPP-PPM/scrambled siRNA NPs (siRNA: 300 nM) were tested. Each point represents the mean $\pm$ S.E.M. (n=5). Statistical significance was assessed using the Student's *t*-test (* <0.05 and ** <0.01).

Figure 10.

The targeted delivery of TPP-PPM/siRNA NPs to macrophages in colitis tissues. (a) Tissue uptake of NPs after 6 h co-incubation with colitis tissues *ex vivo*. Colitis tissues were treated with FITC-siRNA-loaded NPs (green; 200 nM) and processed for fluorescence staining. Fixed cells were stained with Alexa Fluor 568 phalloidin and DAPI for visualization of actin (red) and nuclei (purple), respectively. Scale bar represents 50 μm . (b) Representative flow cytometric analysis of FITC fluorescence-positive colon macrophages in mice colitis tissue treated with FITC-siRNA-loaded NPs. Cells were isolated from the colon after overnight co-incubation with NPs and measured by flow cytometry. The presented results are representative of two independent experiments. Data are expressed as mean $\pm$ S.E.M. (n=3).

Scheme 1.

Schematic illustration of TPP-PPM/siRNA nanoparticle (NP) formation and macrophage-targeting delivery, and the release of siRNAs to the cytoplasm.