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Acid-sensitive sheddable PEGylated, mannose-modified nanoparticles increase the delivery of betamethasone to chronic inflammation sites in a mouse model

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Abstract

Inflammation is implicated in a host of chronic illnesses. Within these inflamed tissues, the pH of the microenvironment is decreased and immune cells, particularly macrophages, infiltrate the area. Additionally, the vascular integrity of these sites is altered with increased fenestrations between endothelial cells. These distinctive properties may be exploited to enhance targeted delivery of anti-inflammatory therapies. Using a mouse model of chronic inflammation, we previously showed that acid-sensitive sheddable PEGylation increases the distribution and retention of nanoparticles in chronic inflammation sites. Here we demonstrated that surface modification of the acid-sensitive sheddable PEGylated nanoparticles with mannose, a ligand to mannose receptors present in chronic inflammation sites, significantly increases the targeted delivery of the nanoparticles to these areas. Furthermore, we showed that the acid-sensitive sheddable PEGylated, mannose-modified nanoparticles are able to significantly increase the delivery of betamethasone-21-acetate (BA), a model anti-inflammatory compound, to chronic inflammation sites as compared to free BA. These results highlight the ability to engineer formulations to target chronic inflammation sites by exploiting the microenvironment of these regions.

Keywords

Lipopolysaccharides; in vitro release; cell uptake; TNF- α release; tissue pharmacokinetics

1. Introduction

Inflammation is a critical part of the immune system that serves to maintain homeostasis in response to a physiological disturbance. This signal-mediated process occurs in reaction to cellular insult resulting from infections, toxins, or physical stresses¹. As a component of innate immunity, inflammation serves to resolve breaches to physiological barriers in the

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human body through the infiltration of immune cells and soluble mediators to the site of tissue injury². Normally, inflammation is a highly regulated process that protects the host through the removal of potentially damaging stimuli and the repair of destroyed tissues. Acute inflammation is characterized by changes in the vasculature including increased vascular flow to the site of injury, increased vascular permeability at the site of tissue damage, and increased leukocyte migration from the vessels into the compromised tissues³. Together, these changes allow for the removal of harmful agents from the tissues and the return of the inflammation sites to homeostatic conditions. As a result of these changes, inflammation sites are initially marked by increased populations of neutrophils, followed by an increased population of macrophages^{1, 3, 4}.

Failure to resolve the antigenic stimulus at the site of tissue injury transforms the beneficial, acute inflammatory process into a chronic, pathological one. Whereas the resolution of inflammation is defined by the presence of anti-inflammatory mediators (e.g. IL-10), chronic inflammation is dominated by the production of pro-inflammatory cytokines, including TNF- α , IL-1 β , and IL-6⁴. Due to the ongoing inflammatory response, cell numbers, particularly for macrophages and T cells, are increased in chronic inflammation sites⁵. Continued activation of the inflammatory response leads to tissue destruction and underlies the implication of chronic inflammation in a host of diseases, including atherosclerosis and chronic obstructive pulmonary disease⁶.

In recent years, it has been found that chronic inflammation sites can preferentially accumulate nanoscale molecules and delivery systems⁷. For example, Quan et al. previously reported a macromolecular prodrug utilizing an acid-labile polymer for conjugation to be administered to rats with adjuvant-induced arthritis⁸. In their study, the researchers found that more of the administered drug was delivered to the inflamed ankles of rats with adjuvant-induced arthritis, as compared with healthy, non-arthritic rats⁸. They described this effect as “extravasation through leaky vasculature and subsequent inflammatory cell-mediated sequestration”, or “ELVIS”⁸. As described by the ELVIS phenomenon, chronic inflammation may be characterized by increased numbers of immune cells. Within the inflamed tissue, these elevated immune cell populations produce a multitude of pro-inflammatory cytokines that contribute to disease progression and tissue destruction^{9–11}. Additionally, the inflammation site is characterized by a decrease in the local pH, with pH decreasing as disease severity increases^{12, 13}. These attributes may be exploited to develop a formulation that improves the targeting of therapies and imaging agents to sites of chronic inflammation.

Previously, our laboratory reported that acid-sensitive sheddable PEGylation of nanoparticles increases the distribution and retention of the nanoparticles in chronic inflammation sites when intravenously (i.v.) administered in a mouse model of chronic inflammation¹⁴. In addition, by taking advantage of the relatively lower pH microenvironment in tumor tissues and the overexpression of mannose receptors by tumor-associated macrophages, we previously demonstrated that surface modification of acid-sensitive sheddable PEGylated nanoparticles with mannose, a ligand to mannose receptors, significantly increased the delivery of nanoparticles into tumor tissues in mouse models¹⁵. As mentioned above, chronic inflammation sites have an increased population of

macrophages, and there are reports of exploiting those macrophages to increase the delivery of drugs and imaging agents to chronic inflammation sites^{16–19}. For example, Put et al. demonstrated the feasibility of targeting the macrophage mannose receptor (MMR) in an inflammatory arthritis model¹⁶. In their work, ⁹⁹Tc-labeled nanobodies directed to MMR were created and evaluated for their potential as a diagnostic aid. The results from this work revealed that, in mice with symptomatic arthritis, the MMR-targeted nanobodies preferentially accumulated in the inflammatory sites¹⁶. We hypothesized, therefore, that surface modification of acid-sensitive sheddable PEGylated nanoparticles with mannose could further increase their distribution and/or retention in chronic inflammation sites. In the present study, we tested this hypothesis in a mouse model of lipopolysaccharide (LPS)-induced chronic inflammation. In addition, we evaluated the feasibility of utilizing the acid-sensitive sheddable PEGylated, mannose-modified nanoparticles to increase the delivery of betamethasone, a common anti-inflammatory agent, into chronic inflammation sites in a mouse model.

2. Materials and Methods

2.1. Materials, cells, and animals

Poly (DL-lactic-co-glycolic) acid (85:15, ester-terminated, 0.55 – 0.75 dL/g) was from Durect Corporation (Birmingham, AL). PEG2000-hydrazone-C18 (PHC), PEG2000-amide-C18 (PAC), and *O*-stearoyl mannose (M-C18) were synthesized using methods previously published by our laboratory²⁰. BA and prednisolone were from TCI America (Portland, OR). Cyanine7.5 NHS ester (Cy7.5) was from Lumiprobe (Hallandale Beach, FL). TNF- α ELISA kit was from Thermo-Fisher Scientific (Waltham, MA). LPS and dimethyl-9,9'-biacridinium dinitrate (lucigenin) were from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC grade and from Sigma Aldrich.

Mouse J774A.1 macrophage cells were from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL), all from Gibco (Grand Island, NY).

All female C57BL/6 mice (18–20 grams) were from Charles River Laboratories (Wilmington, MA). Animal studies were conducted following the U.S. National Research Council Guidelines for Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

2.2. Preparation and characterization of nanoparticles

2.2.1. Preparation of BA-incorporated nanoparticles—Nanoparticles containing BA were prepared according to a method previously reported by our laboratory with slight modifications¹⁵. Briefly, 3 mg of PLGA, 3.6 mg of PHC or PAC, and 1.8 mg of M-C18 were dissolved in 900 μ L of tetrahydrofuran (THF). BA was dissolved in methanol at a concentration of 1 mg/mL, and 200 μ L of this solution was added to the THF solution containing PLGA, PHC or PAC, and M-C18. Nanoparticles were formed by

nanoprecipitation after drop-wise addition of the solvent phase to water under vigorous stirring. Solvent was evaporated by stirring at room temperature for 4 to 6 h. Nanoparticles were collected by centrifugation. In addition to the BA-loaded, acid-sensitive sheddable PEGylated, mannose modified nanoparticles (i.e. BA-AS-M-NPs) and the BA-loaded, acid-insensitive PEGylated, mannose modified nanoparticles (i.e. BA-AI-M-NPs), the following nanoparticles were also prepared similarly: acid-sensitive sheddable PEGylated nanoparticles incorporated with Cy7.5 (i.e. Cy7.5-AS-NPs), acid-sensitive sheddable PEGylated and mannose-modified nanoparticles incorporated with Cy7.5 (i.e. Cy7.5-AS-M-NPs), Cy7.5-labeled BA-AS-M-NPs (Cy7.5-BA-AS-M-NPs) and Cy7.5-labeled BA-AI-M-NPs (Cy7.5-BA-AI-M-NPs). The size and zeta potential of the nanoparticles were determined using a Malvern Zetasizer (Malvern Instruments, Ltd., Westborough, MA).

2.2.2. Determination of entrapment efficiency of BA in the nanoparticles—To quantify the amount of BA within the nanoparticles, supernatant was collected after high-speed centrifugation of the nanoparticles, mixed with an equal volume of methanol, and then analyzed using a reversed phase high performance liquid chromatography (HPLC) method. Briefly, sample was injected into an Agilent 1260 Infinity LC equipped with an Agilent ZORBAX Eclipse Plus C18 column (5 μ m, 4.6 mm $\times$ 150 mm) for chromatographic separation at 30°C and a flow rate of 1.5 mL/min. Measurements were made at 248 nm. Mobile phase consisted of acetonitrile and water at a ratio of 40:60 (v/v). The amount of BA determined to be in the supernatant was subtracted from the total amount of BA added during preparation of the nanoparticles (200 μ g) to calculate the encapsulation efficiency.

2.2.3. Transmission electron microscopy of BA-incorporated nanoparticles—Morphological characterization of the nanoparticles was performed using transmission electron microscopy (TEM). One drop of nanoparticle suspension (5 μ L) was applied to carbon-coated 400 mesh grids after activation and allowed to dry prior to examination. Nanoparticles were visualized using an FEI Tecnai Transmission Electron Microscope (FEI, Hillsboro, OR).

2.3. In vitro stability of BA-incorporated nanoparticles in a simulated biological media

The stability of BA-AS-M-NPs and BA-AI-M-NPs in a simulated biological media was determined by adding aliquots of nanoparticles to 10% FBS in phosphate-buffered saline (PBS, 10 mM, pH 7.4) and incubating at 37°C and 150 rpm in a MaxQ™ 5000 Floor Shaker Incubator (Thermo Fisher Scientific) for 18 h. Particle size was measured at zero and 18 h using the Malvern Zetasizer. The stability of the acid-sensitive sheddable PEGylated nanoparticles (i.e. AS-NPs) was also determined similarly.

2.4. In vitro release of BA from nanoparticles

The release profiles of BA from nanoparticles (i.e. BA-AS-M-NPs and BA-AI-M-NPs) were evaluated by adding a small volume of nanoparticle suspension to a sufficient amount of PBS (pH 6.8 or 7.4, 10 mM) containing 0.05% (v/v) Tween 20 to create sink conditions, which was then placed in a MaxQ™ 5000 Floor Shaker Incubator at 37°C and 150 rpm. Samples were removed at designated time points and centrifuged to collect nanoparticles. The supernatant was collected and lyophilized. Prednisolone was added to samples prior to

lyophilization as an internal control. Lyophilized samples were reconstituted with an equal volume of water and methanol and analyzed using HPLC.

2.5. Cellular uptake study

To confirm the acid-sensitive PEG-shedding from BA-AS-M-NPs, the uptake of BA-AS-M-NPs (and BA-AI-M-NPs as a control) by J774A.1 macrophages in culture was evaluated after the nanoparticles were incubated in a relatively lower pH (i.e. pH 6.8 vs. 7.4) to facilitate PEG-shedding. The nanoparticles were labeled with Cy7.5 or prepared by including PLGA that was conjugated to fluorescein isothiocyanate (i.e. 15% of total PLGA), and incubated in PBS buffer at pH 6.8 or 7.4 for 6 h¹⁵. J774A.1 mouse macrophages were seeded into 24-well plates at a concentration of 2×10^5 cells per 0.5 mL one night prior to the addition of BA-AS-M-NPs or BA-AI-M-NPs. Nanoparticles were collected from PBS buffer (pH 6.8 or 7.4) using centrifugation, re-suspended in serum-free DMEM, and added to macrophages. Following incubation, cells were lysed and the lysate was analyzed for fluorescence intensity using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT). To confirm the surface modification of BA-AS-M-NPs with mannose, the uptake of nanoparticles by J774A.1 cells cultured in the presence or absence of 2 mg/mL of mannose was evaluated. The BA-AS-M-NPs were incubated at pH 6.8 for 6 h before adding to the cell culture, and the extent of uptake of the nanoparticles was determined as mentioned above. Finally, confocal microscopy was used to confirm the uptake or internalization of BA-AS-M-NPs by J774A.1 macrophages. Briefly, Cy7.5-labeled BA-AS-M-NPs were incubated for 4 h in a pH 6.8 buffer to facilitate the shedding of PEG. The nanoparticles were then centrifuged and re-suspended in serum-free DMEM, added to J774A.1 cells grown on glass coverslips in a 6-well plate, and incubated at 37°C. After 4 h of incubation, the nanoparticle-containing media was removed and cells were washed three times with cold PBS. Cells were fixed and permeated using cold ethanol for 10 min and stained using Hoechst 33342 (0.01 mM) for 10 min. After staining, cells were rinsed with PBS and examined using a Leica TCS-SP5 confocal microscope with an oil immersion objective (63×1.4 NA) (Leica Microsystems GmbH, Mannheim, Germany).

2.6. Evaluation of the activity of the BA in BA-AS-M-NPs in macrophages in culture

The activity of BA in BA-AS-M-NPs after uptake by J774A.1 macrophages was analyzed using an enzyme linked immunosorbent assay (ELISA) to measure the inhibition of TNF- α release from cells. Briefly, J774A.1 macrophages were seeded in 12-well plates at 2×10^5 cells per well in 0.75 mL of DMEM. Cells were then incubated with BA-AS-M-NPs, free BA, BA-free AS-M-NPs, or DMEM alone for 12 h at 37°C, 5% CO₂. LPS was added at a concentration of 100 ng/mL to stimulate the production of pro-inflammatory cytokines for 12 additional hours. Samples of media from each well were transferred to an antibody-coated 96-well plate for analysis of cytokine production by ELISA following the manufacturer's instructions. Cells from all groups had a final viability of greater than 80% and were not significantly different from healthy untreated cells, as confirmed by an MTT assay (data not shown).

2.7. In vivo characterization of BA-AS-M-NPs

2.7.1. LPS-induced chronic inflammation model—Chronic inflammation was induced in female C57BL/6 mice using LPS according to a previously reported method²¹. Briefly, 50 μ L of 1 mg/mL LPS solution was injected into the right hind footpad of the mice. Mice were periodically injected intraperitoneally with lucigenin (200 μ L of 3 mg/mL) to monitor the development of chronic inflammation in the right foot over the course of 10 to 14 days. Mice with localized chronic inflammation were randomized into groups for all *in vivo* studies.

2.7.2. In vivo distribution of BA-AS-M-NPs, BA-AI-M-NPs, BA-AS-NPs, and BA in the chronic inflammation site—To evaluate the effect of surface modification of nanoparticles with mannose on the distribution and retention of nanoparticles in the inflamed mouse foot, mice were i.v. injected with Cy7.5-AS-M-NPs or Cy7.5-AS-NPs and periodically imaged for 28 days using the IVIS® Spectrum in vivo imaging system (PerkinElmer, Waltham, MA). To evaluate the effect of acid-sensitive PEG-shedding on the distribution of BA in the inflamed foot, mice were i.v. injected with Cy7.5-BA-AS-M-NPs, Cy7.5-BA-AI-M-NPs, or left untreated. The inflamed foot was collected 48 h after the injection. BA was extracted from homogenized tissue samples using ethyl acetate, which was dried and re-suspended in methanol and water (1:1). The concentration of BA in the tissue homogenate was determined using HPLC. In both experiments, the fluorescence intensities of nanoparticles were measured prior to dosing to confirm that nanoparticles with equal fluorescence intensity values were injected. Finally, to determine if BA-AS-M-NPs were able to enhance the delivery of BA to the inflamed foot, mice were i.v. injected with either BA-AS-M-NPs or free BA or left untreated and were sacrificed 48 h later to collect the inflamed foot for determination of BA content as mentioned above.

2.8. Statistical analysis

For comparing the biodistribution of Cy7.5-AS-M-NPs and Cy7.5-AS-NPs, statistical significance was evaluated using area under the curve (AUC) calculations based on a non-compartmental pharmacokinetic model (PKSolver). The 95% confidence intervals were calculated (GraphPad Prism, La Jolla, CA) and also used for statistical comparison. For all other experiments, a student's two-sided t-test or one-way ANOVA was used and a p value of ≤ 0.05 was considered significant.

3. Results and Discussion

3.1. Surface modification of acid-sensitive sheddable PEGylated nanoparticles with mannose increases their distribution and retention in chronic inflammation sites

Previously, we showed that acid-sensitive sheddable PEGylation of nanoparticles prepared with PLGA increases the distribution and retention of the nanoparticles in chronic inflammation sites when intravenously injected in an LPS-induced chronic inflammation mouse model¹⁴. To test whether surface-modifying the acid-sensitive sheddable PEGylated nanoparticles with mannose, a ligand to mannose receptors (which have increased expression in chronic inflammation sites), can help further increase the distribution and/or retention of the nanoparticles in chronic inflammation sites, mice positive for localized,

LPS-induced chronic inflammation (as confirmed by lucigenin injection and IVIS imaging) in the right footpad were used. Mice were i.v. injected with Cy7.5-labeled AS-M-NPs (size, 168 nm; polydispersity index (PDI), 0.185; zeta potential, -28.6 mV) or Cy7.5-labeled AS-NPs (size, 161 nm; PDI, 0.232; zeta potential, -23.1 mV). The distribution of nanoparticles in the inflamed foot was then monitored using the IVIS Spectrum for 28 days (Fig. 1A). Shown in Fig. 1B are the fluorescence intensity values in the inflamed foot in mice i.v. injected with Cy7.5-AS-M-NPs or Cy7.5-AS-NPs as a function of time. The mean fluorescence intensity value in the inflamed foot of mice i.v. injected with the Cy7.5-AS-M-NPs was higher than that in mice injected with the Cy7.5-AS-NPs (Fig. 1B), and the AUC of the Cy7.5-AS-M-NPs group was significantly larger than that of the Cy7.5-AS-NPs (Table 1). These findings demonstrate that surface-modification of the acid-sensitive sheddable PEGylated nanoparticles with mannose significantly increases their distribution and retention in chronic inflammation sites.

This finding is in agreement with what was reported by Put et al., who showed that the macrophage mannose-receptor (MMR) in chronic inflammation sites can be utilized as a target for ^{99}Tc -labeled anti-MMR nanobodies ¹⁶. Results from their study showed that the MMR found in the synovia of mice with collagen-induced arthritis (CIA) can be used to image or track chronic inflammation in arthritic joints ¹⁶. Therefore, it is clear that the relatively lower pH and the presence of mannose receptors are characteristics of chronic inflammation sites that can be exploited for site-specific delivery of nanoparticles. However, it is important to note that the surface receptor profile of macrophages differs with respect to macrophage subtype (i.e. M1 vs. M2 macrophages); therefore, it is critical to assess the role of the particular subtype(s) in the inflammatory process when designing new formulations for chronic inflammation therapies, as it may be favorable to enhance the activities of some subtypes while suppressing that of others ²².

3.2. Preparation and in vitro characterization of BA-loaded AS-M-NPs

Data in Fig. 1 and our previous study demonstrated the feasibility of increasing the distribution and retention of nanoparticles into chronic inflammation sites by exploiting the relatively lower pH and the presence of mannose receptors in chronic inflammation sites ¹⁴. To test whether our acid-sensitive sheddable PEGylated, mannose-modified nanoparticles can increase the delivery of an anti-inflammatory agent into chronic inflammation sites, we chose betamethasone-21-acetate as a model drug. Betamethasone is a potent glucocorticoid that reduces pro-inflammatory cytokines, upregulates anti-inflammatory cytokines, and is currently used for the symptomatic management of inflammatory conditions ^{23, 24}. BA-loaded AS-M-NPs were prepared using a nanoprecipitation method. As a control, BA-loaded AI-M-NPs were also prepared. Both nanoparticles have a mean particle size of less than 200 nm (Fig. 2A) and a PDI value of less than 0.2. Their zeta potential values were approximately -30 mV. The encapsulation efficiencies of BA in both BA-AS-M-NPs and BA-AI-M-NPs were greater than 75%. A representative TEM micrograph of BA-AS-M-NPs (Fig. 2B) confirmed that nanoparticles are spherical and have a particle size of less than 200 nm.

Shown in Fig. 2C are the size of BA-AS-M-NPs and BA-AI-M-NPs before and after 18 h of incubation in a simulated biological media (i.e. 10% FBS in PBS) at 37°C. No significant difference in size was observed after incubation for both nanoparticles (Fig. 2B), suggesting that the nanoparticles will not likely aggregate when i.v. injected in mice.

The release profiles of BA from BA-AS-M-NPs and BA-AI-M-NPs in different pH values (i.e. pH 6.8 or 7.4) are shown in Fig. 2C. Less than 3% of BA was released from the nanoparticles in 10 days (~40–45% after a month, data not shown), and the rate of release of BA from the nanoparticles at pH 6.8 is not significantly different from that at pH 7.4 (Fig. 2D). The slow release of BA from the nanoparticles is likely related to the large molecular weight and the relatively lipophilic nature of the PLGA (i.e. 0.55 to 0.75 dL/g; PLA to PGA, 85:15; ester-terminated) used to prepare the nanoparticles. The slow release of BA from the nanoparticles in both physiological pH and a relatively lower pH (i.e. 6.8, similar to that in chronic inflammation sites) is expected to be beneficial. Because of their immunosuppressive effects, glucocorticoids such as BA, along with many other anti-inflammatory and anti-rheumatic agents, have severe systemic consequences that can limit their dose and long-term use²⁵. Therefore, a nanoparticle formulation with limited release before the nanoparticles are taken up by immune cells, such as macrophages, in chronic inflammation sites will likely reduce the systemic adverse effects associated with anti-inflammatory treatment. The slow release of BA from the nanoparticles indicates that they should be stable during circulation without significant drug release. Furthermore, the slow release of BA from the nanoparticles at pH 6.8 indicates that burst release of BA from the nanoparticles once distributed into chronic inflammation sites is unlikely, potentially enabling the maximum amount of BA to be taken into macrophages and avoiding clearance of released BA from the chronic inflammation sites.

3.3. In vitro cellular uptake of BA-AS-M-NPs

Acid-sensitive sheddable PEGylation and mannose-modification of the nanoparticles was confirmed by evaluating the uptake of the nanoparticles by J774A.1 macrophages¹⁵. Cells incubated with BA-AS-M-NPs pre-treated in pH 6.8 for 6 h to facilitate the shedding of the PEG chains prior to adding them to cells had significantly higher fluorescence intensity than cells incubated with the same volume of BA-AS-M-NPs pretreated in pH 7.4 for 6 h, or BA-AI-M-NPs pretreated at either pH value (Fig. 3A). Moreover, pre-treatment of BA-AI-M-NPs in pH 6.8 or pH 7.4 for 6 h did not result in a significant difference in the resultant fluorescence intensity (Fig. 3A). Taken together, data in Fig. 3A demonstrate the acid-sensitive sheddable PEGylation of BA-AS-M-NPs. To confirm the surface modification of the nanoparticles with mannose, BA-AS-M-NPs were pretreated at pH 6.8 to facilitate the shedding of the PEG chains and expose the mannose on the surface of the nanoparticles, which were then added to J774A.1 cells cultured in the presence or absence of mannose in media. As shown in Fig. 3B, the presence of mannose in the cell culture media significantly decreased the uptake of the nanoparticles, as compared to the absence of mannose in the media. Finally, the confocal microscopic images in Fig. 3C confirmed that BA-AS-M-NPs were taken up (or internalized) by the J774A.1 cells, and not simply bound on the surface of the cells.

3.4. Evaluation of the activity of BA in the BA-AS-M-NPs in macrophages in culture

To confirm the activity of BA incorporated in BA-AS-M-NPs, the nanoparticles' ability to inhibit the release of TNF- α , a pro-inflammatory cytokine, by J774A.1 macrophages was evaluated. As shown in Fig. 4, incubation of J774A.1 cells with LPS stimulated TNF- α production. Treatment with BA-AS-M-NPs reduced the level of TNF- α production by LPS-stimulated J774A.1 cells to a level not different from that of the unstimulated cells (Fig. 4). BA-free AS-M-NPs did not significantly affect the production of TNF- α by J774A.1 cells, demonstrating that the reduced cytokine levels were due to BA incorporated into the BA-AS-M-NPs (Fig. 4).

Conventionally, glucocorticoids are bound by cytosolic receptors, which dimerize and translocate to the nucleus where they bind to glucocorticoid response elements (GRE), down-regulating pro-inflammatory cytokines and up-regulating anti-inflammatory cytokines^{23, 24}. Because nanoparticles are endocytosed, and therefore, BA is contained inside the nanoparticle within the endolysosomes, the nanoparticles must be able to escape the endolysosomes and release the drug to allow it to bind with the cytosolic glucocorticoid receptors²⁶. We found that the zeta potential of BA-nanoparticles changed from negative to positive at low pH values (e.g. pH 4) (data not shown), indicating that nanoparticles can escape the endolysosomes, enabling BA the opportunity to exert its activity. Using ELISA, we found that the mean level of TNF- α in BA-AS-M-NP-treated cells was not significantly different than that of unstimulated cells (Fig. 4), indicating that BA delivered by BA-AS-M-NPs was released in a sufficient amount to translocate to the nucleus and inhibit the transcription of pro-inflammatory genes. As a comparator, cells were also treated with free BA at an equivalent concentration. These cells showed reduced levels of TNF- α that were not significantly different from that of unstimulated cells, nor cells treated with BA-AS-M-NPs (Fig. 4), again indicating that BA was released from the nanoparticles.

3.5. In vivo biodistribution of BA in chronic inflammation sites

To confirm that acid-sensitive sheddable PEGylation and mannose modification of BA-encapsulated nanoparticles increases the distribution or accumulation of BA in chronic inflammation sites, two experiments were completed. In the first experiment, mice with LPS-induced chronic inflammation in a hind footpad were i.v. injected with BA-AS-M-NPs or BA-AI-M-NPs. In the second experiment, mice with LPS-induced chronic inflammation were i.v. injected with BA-AS-M-NPs or free BA. Shown in Fig. 5 is the percent of BA recovered from the inflamed foot normalized to the weight of the foot. The levels of BA in the foot of mice injected with the BA-AS-M-NPs were distinctly and significantly higher than in mice injected with BA-AI-M-NPs or free BA, demonstrating that BA-AS-M-NPs increased the distribution and/or accumulation of BA in the chronic inflammation site.

As a form of symptom management, glucocorticoids, specifically a suspension of betamethasone sodium phosphate and betamethasone acetate, can be administered by intra-articular injection. This combination approach provides immediate and sustained symptom relief based on the physicochemical properties of the individual betamethasone forms. As demonstrated in Fig. 5, mice i.v. injected with BA-AS-M-NPs had significantly more drug within the inflamed foot at 48 h as compared with mice given free BA. Because samples

were analyzed only 48 h after administration, it is difficult to assess whether this indicates that more drug was initially delivered to the inflamed sites with the injection of BA-AS-M-NPs than with free BA, or that our nanoparticles were retained within the chronic inflammation sites longer than the free BA. However, these results do emphasize the ability of BA-AS-M-NPs to increase the delivery of drug to the inflamed sites. As discussed earlier, the systemic consequences of anti-inflammatory agents can limit their use. In addition to reducing exposure to these agents by limiting release prior to delivery to chronic inflammation sites, it is also necessary to limit systemic exposure by improving their retention within the chronic inflammation sites. Clinically, increased distribution and/or retention of anti-inflammatory agents in chronic inflammation sites could translate to reduced doses and/or dosing frequency, provided the delivered agents have equivalent therapeutic efficacies.

4. Conclusion

In the present study, acid-sensitive PEG-shedding and mannose-modification were implemented as means to improve the delivery of nanoparticles to chronically inflamed sites. Characterization of the nanoparticles revealed that acid-sensitive sheddable PEGylated, mannose-modified, BA-loaded nanoparticles are stable and have limited drug release *in vitro*. Reduced pH is sufficient to facilitate the shedding of PEG, which increases the uptake of nanoparticles by macrophages in culture. More importantly, the inclusion of mannose as a receptor-targeting ligand in the nanoparticles significantly enhances the distribution and retention of nanoparticles within inflamed sites. Because this study solely focused on the ability to actively target immune cells, specifically macrophages, within chronic inflammation sites, future explorations into optimal targeting ligands and improved therapeutic efficacy still need to be conducted to evaluate this approach for modulating the immune response in chronic inflammatory diseases, such as rheumatoid arthritis.

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(A)

(B)

Figure 1.

(A) IVIS images of the LPS-induced inflamed feet of mice i.v. injected with Cy7.5-AS-M-NPs or Cy7.5-AS-NPs monitored over 28 days. (B) The kinetics of the fluorescence intensity values of the inflamed feet of mice i.v. injected with Cy7.5-AS-M-NPs or Cy7.5-AS-NPs. Data are mean $\pm$ S.D. (n = 3).

(A)

(B)

Figure 2. (A) Size distribution curves of BA-AS-M-NPs (red) and BA-AI-M-NPs (green); (B) Representative TEM micrograph of BA-AS-M-NPs. BA-AI-M-NPs have similar morphological and size characteristics (data not shown); (C) The sizes of BA-AS-M-NPs and BA-AI-M-NPs incubated in PBS with 10% FBS for 18 h. No significant change in

nanoparticle size was observed. **(D)** *In vitro* release profiles of BA from BA-AS-M-NPs and BA-AI-M-NPs at pH 6.8 and 7.4. No significant difference in release from the nanoparticles was found at either pH. In C and D, data are mean $\pm$ S.D. (n = 3).

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(A)

(B)

Figure 3.

In vitro cellular uptake of BA-AS-M-NP and BA-AI-M-NPs by J774A.1 macrophages. **(A)** Nanoparticles were incubated at pH 6.8 or 7.4 before adding to cells in culture. **(B)** Uptake of BA-AS-M-NPs by J774A.1 cells in the presence or absence of mannose in cell culture media prior to adding nanoparticles. The BA-AS-M-NPs were incubated at pH 6.8 for 6 h before adding to the cell culture. **(C)** Confocal microscopic images showing the internalization of the BA-AS-M-NPs by J774A.1 cells (**red**, BA-AS-M-NPs; **blue**, cell nucleus). Data in A and B are mean $\pm$ S.D. (n = 3).

Figure 4. Levels of TNF- α released from J774A.1 macrophages treated with BA-AS-M-NPs (pink), free BA (blue), or BA-free AS-M-NPs (green). The concentration of BA was 0.5 μ g/mL. The concentration of BA-free AS-M-NPs was equivalent to that of BA-AS-M-NPs in PLGA content. Cells stimulated with LPS, but not treated, were included as a positive control (yellow), whereas cells not stimulated by LPS and not treated were included as a negative control (purple). Data are mean $\pm$ S.D. ($n > 3$).

Figure 5.

Comparison of the amount of BA recovered from the inflamed feet of mice 48 h after i.v. injection of BA-AS-M-NPs or BA-AI-M-NPs (**A**), or free BA or BA-AS-M-NPs (**B**). Data are mean $\pm$ S.D. (n = 3).

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Table 1

A comparison of AUC for the fluorescence intensity values obtained from imaging of the inflamed feet of mice i.v. injected with Cy7.5-BA-AS-M-NPs or Cy7.5-BA-AS-NPs. Control mice were injected with PBS. Values in the table have been normalized to values obtained for mice injected with PBS (n = 3). Units for AUC values are p/s/cm²/sr*h.

	BA-AS-NPs	BA-AS-M-NPs
Total AUC	1125.36	1917.33
95% CI	872.86 to 1377.87	1736.62 to 2098.05

The AUC_{0-t} for BA-AS-M-NPs is significantly higher than that for BA-AS-NPs (p < 0.05).