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Pharmacokinetic and Biodistribution Studies of *N*-(2-Hydroxypropyl)methacrylamide (HPMA) Copolymer-Dexamethasone Conjugates in Adjuvant–induced Arthritis (AA) Rat Model

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Abstract

N-(2-Hydroxypropyl)-methacrylamide (HPMA) copolymer has been found to be arthrotropic (joint-targeting) in adjuvant-induced arthritis (AA) rat model using magnetic resonance imaging (MRI). In this manuscript, we report the quantitative pharmacokinetics and biodistribution (PK/BD) of ^{125}I-labeled HPMA copolymer-dexamethasone conjugate (P-Dex) in AA rats. Structural parameters of the prodrug such as the molecular weight (MW) and Dex content were found to have strong impact on the PK/BD profiles of P-Dex. The increase of MW (14,000 24,000 and 42,000 g/mol) and Dex content (0, 151 and 313 μmol/g) enhances the arthrotropism of P-Dex. For the conjugate with highest MW and Dex content (P-H-MW/Dex), the percentage of injected doses per gram (ID/g) of ankle synovial tissue at day 7th post administration is 1 % g^{-1}, which confirms P-Dex as an arthrotropic macromolecular prodrug. For liver and spleen, the ID/g values are 0.51 and 3.64 % g^{-1}, respectively. As an antigen-presenting organ, the sequestration of the prodrug by spleen may be explained by its abnormal enlargement associated with the systemic inflammatory disease model. Gradual reduction of spleen weight due to the inflammation resolution effect of P-Dex may also contribute to the high ID/g values. Increase of Dex content and reduction of MW would increase P-Dex distribution to kidney. The highest ID/g value for kidney at day 7th post administration (0.91 % g^{-1}) was found with P-L-Mw (MW =14,000 g/mol, Dex content = 288 μmol/g), which may suggest kidney tubuli reabsorption of the conjugates. The P-Dex' distribution to heart and lung is minimum.

Keywords

rheumatoid arthritis; pharmacokinetics; biodistribution; dexamethasone; HPMA copolymer; prodrug; arthrotropism

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Supporting Information

A representative SPECT/μ-CT movie depicting the biodistribution of ^{125}I labeled P-H-M_W/Dex in an AA rat at 8 days post treatment is provided as supporting information for this manuscript. This material is available free of charge via the Internet at http://pubs.acs.org.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease affecting approximately 0.5%–1% of the global adult population. It usually involves multiple joints in a symmetrical pattern, with predominant symptoms including pain, stiffness, and swelling of peripheral joints. The prevalence of RA among women almost doubles that in men, and rises with aging. People with RA have a significantly higher rate of mortality compared with age- and sex-matched controls without RA of the same community [1,2]. The etiology of RA is unknown but seems to be multifactorial. The familial nature of RA has long been recognized, suggesting that genetic risk factors are important in the etiology of this disease. Environmental factors (e.g. smoking or infectious agents) are also suggested to play a role in the etiology, though their contribution has yet to be defined [3].

Historically, the treatment of RA was often viewed as a pyramid that consists of the initial use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), followed by the subsequent use of a single disease-modifying anti-rheumatic drugs (DMARDs), which may include drugs such as methotrexate, leflunomide, sulfasalazine, etc. The newly emerged biologic DMARDs (e.g. etanercept, rituximab, certolizumab pegol, etc.) have provided rheumatologists with additional potent disease management tools. Glucocorticoid is the most potent and fast acting anti-rheumatic drug category with proven disease-modifying effects. Due to their notorious systemic side effects, they are only used as a bridging therapy in between of NSAIDs and DMARDs. Based on an expanded knowledge of its pathophysiology, along with the recognition that the functional damage to the joint occurs very early, earlier intervention with DMARDs and short term low dose glucocorticoids has been recommended in recent years [4–6]. Many novel therapeutic interventions for RA have been developed during the last decade with very specific molecular targets. Like glucocorticoids, however, most of them do not have specificity to the organs/tissues affected of RA, which often lead to extra-articular adverse effects.

To address this challenge, we have developed an arthrotropic (joint-targeting) macromolecular dexamethasone (a potent glucocorticoid) prodrug (P-Dex) based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer [7,8]. In these proof of concept studies, Dex was linked to HPMA copolymer side chains via a hydrazone bond, which can be cleaved under acidic environments, such as the acidosis associated with arthritic joint and lysosomal compartment of synoviocytes. A single intravenous (i.v.) administration of P-Dex can lead to effective and sustained resolution of inflammation and joint protection in an adjuvant-induced arthritis (AA) rat model. The arthrotropism of HPMA copolymer has been qualitatively established using MRI [9]. In this manuscript, a pharmacokinetic and biodistribution (PK/BD) study of P-Dex is reported to quantitatively understand the arthrotropism of this prodrug and the impacts of different structural factors on its PK/BD profiles.

Experimental Section

Materials

N-(2-Hydroxypropyl) methacrylamide (HPMA) [10], *N*-methacryloylglycylglycylhydrazinyl dexmathasone (MA-Dex) [8], *N*-methacryloylaminopropyl fluorescein thiourea (MA-FITC) [11], *N*-methacryloyl tyrosinamide (MA-Tyr-NH_2) [12], *S*,*S*'-bis(α,α'-dimethyl-α''-acetic acid)-trithiocarbonate (CTA, purity > 97 %) [13] and *N*,*N*-dioctadecyl-*N*',*N*'-bis(2-hydroxyethyl)-1,3-propanediamine (LA) [14] were prepared as described previously. Sephadex LH-20 resin and PD-10 columns were obtained from GE HealthCare (Piscataway, NJ). The NaI^{125} was purchased from Perkin Elmer (Waltham, MA). All other reagents and solvents were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Acros

Organics (Morris Plains, NJ, USA). All compounds were reagent grade and used without further purification.

Synthesis of P-Dex by RAFT copolymerization

A typical reversible addition–fragmentation chain transfer (RAFT) copolymerization is described as the following. HPMA (1.23 g, 8.6 mmol), MA-Tyr-NH_2 (17.2 mg, 0.08 mmol) and MA-Dex (303 mg, 0.516 mmol) were dissolved in methanol/DMF (8:1, v/v), with 2,2'-azobisisobutyronitrile (AIBN, 6.45 mg, 39.3 μmol) as initiator and CTA (5.66 mg, 21.4 μmol) as the RAFT agent. The solution was purged with Argon and polymerized at 40 °C for 48 h. The resulting polymer conjugates were purified on LH-20 column to remove the unreacted low molecular weight (MW) compounds. The polymer solution was then dialyzed and lyophilized to obtain the tyrosine amide-containing P-Dex (P-Dex-Tyr-NH_2). The weight average molecular weight (M_w) and the polydispersity index (PDI) of the copolymer conjugates were determined by size exclusion chromatography (SEC) using a ÄKTA fast protein liquid chromatography (FPLC) system (GE HealthCare) equipped with UV and RI detectors. HPMA homopolymers with narrow PDI were used as calibration sample. The characterizations of all HPMA copolymer conjugates used in this study are summarized in Table 1.

To quantify Dex content in P-Dex, it was hydrolyzed in 0.1 N HCl (1 mg/mL) overnight. The resulting solution was neutralized and analyzed on an Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Inc., Santa Clara, CA, USA) with a reverse phase C_{18} column (Agilent, 4.6×250 mm, 5 μm). Mobile phase:acetonitrile/water = 2/3; Detection, UV 240 nm; Flow rate: 1 mL/min; Injection Volume: 10 μL. The analyses were performed in triplicate. The mean value and standard deviation were obtained using Excel.

Preparation of ^{125}I-Labeled conjugates

The HPMA copolymer-conjugates were labeled with ^{125}I using a standard chloramine-T assay. Briefly, P-Dex-Tyr-NH_2 (10 mg in 0.2 mL saline and 0.1 mL of 0.5 M PBS) and chloramine T·$3H_2O$ (0.1 mL, 5 mg/mL in 0.05 M PBS) were added to the NaI^{125} (1 mCi) vial and incubated at room temperature for 30 min. Sodium metabisulfite (0.1 mL, 6 mg/mL in 0.05 M PBS) was then added to stop the reaction. Low MW reactants, including free ^{125}I were removed by PD-10 columns. The absence of free ^{125}I in the labeled P-Dex-Tyr-NH_2 was supported by the fact that no radioactivity could be found by a survey meter in the PD-10 columns used in the repeated purifications of the conjugates. P-Dex-Tyr-NH_2-I^{125} was mixed with the unlabeled P-Dex-Tyr-NH_2 and used in the *in vivo* study.

Animal Study

Arthritis was induced in male Lewis rats (180–200 g, Charles River Laboratories, Inc.) as described previously [7]. Briefly, *Mycobacterium tuberculosis* H37Ra (1 mg) and *N*,*N*-dioctadecyl-*N*',*N*'-bis(2-hydroxyethyl) propanediamine (LA, 5 mg) were mixed in paraffin oil (100 μL), sonicated and s.c. injected into the base of the rat's tail. On day 14 post arthritis induction, ^{125}I labeled and unlabeled P-Dex-Tyr-NH_2 conjugates (~ 30 μCi/rat, equivalent free Dex dose is 5 mg/kg) were mixed and administered to AA rats (5–6/group) via tail vein injection. Animals were sacrificed at designated time points (15 min, 30 min, 1 h, 6 h, 48 h and 7 d). Blood and other major organs/tissues including, heart, lungs, kidneys, liver, spleen, and ankle joints were isolated at euthanasia, processed, and analyzed with a gamma scintillation counter (Packard auto-gamma counter, PerkinElmer). All organs and tissues were analyzed without perfusion. These animal experiments were performed according to a University of Nebraska Medical Center IACUC approved protocol.

Pharmacokinetic Analysis

Data were fit to various models, including one and two compartment models with various weighting schemes. The precision (% CV) of estimated parameters, Schwartz criterion (SC), Akaike information criterion (AIC), weighted sum of squared residuals (WSSR), condition number, residual plot analysis and correlation matrix were used to compare the goodness of fit. Using these criteria, the two-compartmental model with 1/y weighting was selected as the model of best fit. The pharmacokinetic parameters, such as total clearance (CL), steady-state volume of distribution (V_{ss}), and biological half-life ($t_{1/2}$) were determined using the bolus intravenous input two-compartmental analysis module of WinNonlin (version 1.5, Pharsight, Mountain View, CA). The area under the curve (AUC) was calculated using the trapezoidal rule. The relative exposure ratio was calculated by dividing AUC_{tissue} with AUC_{blood}.

In vitro stability study of P-Dex in rodent plasma

P-H-Dex (45.0 mg, in Table 1) was dissolved into PBS (pH 7.4, 5 mL). Five milliliters of mouse plasma (Southern Biotech, AL) or rat plasma (collected from male Lewis rats) was added into this solution. The mixture was incubated at 37 °C. The final equivalent free Dex concentration is 508.0 μg/mL. At predetermined time intervals, samples (300 μL) were withdrawn and extracted with tert-butyl methyl ether (3 mL) by vortex for 3 min. After centrifugation at 4,000 rpm for 10 min, the organic phase (2.4 mL) was separated and evaporated. The residue was dissolved into methanol (480 μL) and the Dex content was analyzed using HPLC. The recovery efficiency of the method used was determined as 101.4 ± 5.2%.

In vitro internalization study of HPMA copolymer conjugates in macrophages

Raw 264.7 cells were cultured in 96-well culture plates (150 μL/well) at a density of 5×10^5 cells/mL in DMEM medium (Invitrogen, CA) supplemented with 10 % fetal bovine serum (VWR, PA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, CA). The cells were allowed to adhere for 24 h, and then activated with lipopolysaccharide (LPS, 500 ng/mL) for 18 h. After activation, cells were treated with different FITC-labeled HPMA copolymer conjugates (1 mg/mL) in the presence of LPS (500 ng/mL) for 24 h. The supernatants were then removed, and the cells were rinsed three times with FBS free medium (at 4 °C) and lysed with Triton X-100 (0.1%). The concentration of internalized conjugates in each well was calculated by measuring the fluorescence from FITC-labeled HPMA copolymer conjugates using a Spectra Gemini EM Fluorescence Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA). The results were normalized against protein content of each well as determined by BCA assay (Thermo Scientific, IL).

Statistical Analysis

Differences in cell internalization and organ depositions of different HPMA copolymer conjugates were analyzed using one-way ANOVA. When differences were detected, Tukey's test was used to evaluate the pair-wise differences between the groups.

Results

Synthesis of HPMA copolymer-dexamethasone conjugates and characterization

The general structure of the Dex-containing HPMA copolymer conjugates used in this study is shown in Figure 1. The HPMA copolymer conjugates were prepared by RAFT copolymerization to achieve control of their MW and polydispersity [14,15]. Due to the presence of several comonomers, the PDI values of these conjugates could only be controlled at around 1.3–1.4. The physicochemical characteristics of these conjugates are

summarized in Table 1. For *in vivo* study, two groups of conjugates were prepared: the first group has similar MW of ~ 42 kDa but different dexamethasone content (P-NA-Dex, P-M-Dex, P-H-M_w/Dex); the second group has similar dexamethasone content of ~ 118 mg Dex/g polymer but differs in MW (P-L-M_w, P-M-M_w, P-H-M_w/Dex). All copolymer conjugates contained a small amount of tyrosine amide to allow their ^{125}I labeling and tracking of their distribution after i.v. administration to rats. Similarly, additional Dex-containing HPMA copolymer conjugates were synthesized for cell internalization study, with MA-Tyr-NH_2 being replaced by MA-FITC to allow fluorescent signal detection.

The stability of HPMA copolymer-Dex conjugate in rodent plasma

The release of free Dex from HPMA copolymer-Dex conjugate is minimum in both rat and mouse plasma. No detectable Dex was found during the first 6 h of incubation. At the end of the 72 h, the free Dex released is less than 0.4 % of the total drug loading (Figure 2).

In vitro cell culture study

No significant difference in cell internalization was observed among conjugates with difference MW (Figure 3A). However, the amount of internalized conjugates increased when Dex content was raised (Figure 3B). A significant difference between two Dex-containing conjugates (P-H-M_w/Dex-FITC and P-M-Dex-FITC) and a marginal difference ($p = 0.057$) between P-M-Dex-FITC (medium Dex-containing conjugates) and P-NA-Dex-FITC (without Dex) were observed.

Biodistribution of P-Dex conjugates in major organs and tissues

The injected dose per gram of tissue (ID/g) vs. time profiles of all conjugates in blood and the major organs/tissues are shown in Figure 4. The distribution of P-Dex conjugates in major organs at each time point (15 min, 30 min, 1h, 6h, 48h and 7d) post injection is clearly dependent on MW and Dex content. Among all the tested P-Dex, the one with the highest MW and the highest Dex content (P-H-M_w/Dex) shows the maximum ID/g value in most of the organs examined. The increase of MW and Dex content generally enhances the accumulation of the conjugates to peripheral organs. One organ of exception, however, is the kidney, in which the conjugate with the lowest MW has the highest ID/g values. The amounts of all tested conjugates in heart, lung and kidneys were gradually reduced. By day 7, the ID/g values in heart and lung were less than 0.2 % g^{-1}. The ID/g values of P-L-M_w, P-M-M_w, P-NA-Dex, P-M-Dex in the liver gradually decreased from 15 min to 6 h, but remained constant thereafter. P-H-M_w/Dex had a constant ID/g value of around 0.5 % g^{-1} in the liver throughout the entire experiment duration, with a slight peak at 6 h. The distribution of all conjugates to the spleen decreases initially. After reaching the minimum values, they start to increase continuously until the end of the experiment. Among all conjugate tested, P-H-M_w/Dex shows the highest ID/g value of ~ 3.5 % g^{-1} in spleen by day 7. As shown in Figure 5, however, this increase coincides with the large decrease of the weight of spleen during the experiment. The response of the enlarged spleen to P-Dex treatment is very fast. Within 6 h, the weight of spleen in AA rats treated with all Dex-containing conjugates was reduced by as much as 20 %. On day 2, the spleen weight of the AA rats treated by P-H-M_w/Dex and P-M-Dex reached the range of healthy control rats of matching age (~ 0.5 g/spleen). By day 7, spleens from the P-H-M_w/Dex group weighted ~ 50 % of a normal spleen, while spleens from other treatment groups weighted about the same as the healthy control spleens. Spleens of AA rats treated with P-NA-Dex were also observed with some weight reduction (but still enlarged), which might be attributed to a self-healing process of the AA rat model. However, the joints of these animals still remained severely inflamed. For the other evaluated organs, their weight generally remained constant during the treatment (data not shown).

The arthrotropism of the tested conjugates is clearly evident in Figure 4. All conjugates can be found in the joints within 15 min after administration. Increasing of the MW or Dex content enhances the conjugates accumulation to the ankle joints. P-H-M_w/Dex, which has the highest MW and Dex content continuously accumulate in the ankle joints to an ID/g value of ~ 1 % g^{-1} by 7 d, which is 5 and 14 times higher than that of P-NA-Dex and P-L-M_w, respectively. Similar to the spleen, the soft tissue that can be isolated from the ankle joint also decreased gradually during the experiment, with P-H-M_w/Dex group showing the highest reduction (~ 50 %) by 7 d.

Another site of significant polymer conjugate accumulation is the adjuvant injection site at the base of the AA rats' tail. Comparing to the healthy rats, such preferential distribution is clearly evident in the SPECT/μ-CT movie provided in the supplement materials.

Pharmacokinetic analyses

The PK parameters of the tested conjugates in blood and major organs/tissues were obtained using a two-compartment model (Table 2). The AUC_{blood} values of the tested conjugates are ranked as the following: P-H-M_w/Dex > P-M-Dex > P-M-M_w > P-NA-Dex > P-L-M_w. The organ/tissue relative exposure (AUC_{organ}/AUC_{blood}) data suggest that P-H-M_w/Dex and P-M-Dex are more selective to the organs/tissues of interests such as arthritic ankle and spleen. The $t_{1/2(\beta)}$ of P-H-M_w/Dex and P-M-Dex was 31 h; the $t_{1/2(\beta)}$ of P-NA-Dex and P-M-Dex was 17–18 h; whereas P-L-M_w had the shortest $t_{1/2(\beta)}$ of 13 h. For $t_{1/2(\alpha)}$, P-M-Dex and P-NA-Dex have the highest value of ~ 0.8 h. The value for P-H-M_w/Dex is relatively lower at ~ 0.6 h. For P-L-Mw and P-M-Mw, their $t_{1/2(\alpha)}$ values are ~ 0.5 and 0.3 h, respectively. It is obvious that both the distribution and elimination half-lives of the conjugates are dependent upon the MW and Dex content. The clearance values also demonstrated the similar dependence upon MW and Dex content of the conjugates.

Discussion

The preferential distribution of HPMA copolymers to inflammatory joints has been previously reported using non-invasive magnetic resonance imaging (MRI) [9]. The superior and long lasting anti-rheumatic effect of P-Dex was also demonstrated in the AA rats model when compared to equivalent dose of free dexamethasone treatment [7]. In this study, the *in vivo* biodistribution and pharmacokinetics of P-Dex were investigated. The focus of this work is to quantitatively establish the arthrotropism of the macromolecular prodrug and delineate the significant impact of the MW and dexamethasone content of the HPMA copolymer conjugates on their PK/BD profiles. As the hydrazone bond-containing acid-labile P-Dex was found to be stable in the rodents' plasma in this study (Figure 2), we speculate that the main activation sites of the prodrug are probably the organs/tissues of its major distribution. Therefore, the therapeutic effect and safety profile of the polymer-drug conjugates would be directly related to their PK/BD profiles.

Due to the well-known toxicities of glucocorticoids, long-term systemic presence of P-Dex *in vivo* can be problematic. Therefore, all the conjugates we used in this study were of average MW less than 45,000 g/mol to facilitate their renal glomerular filtration [17]. To evaluate the impact of both MW and dexamethasone content on the PK/BD profiles, one group of polymer conjugates were synthesized with similar molecular weights of ~ 42,000 g/mol but different dexamethasone content; and another group of conjugates were synthesized with similar dexamethasone contents of ~ 118 mg/g but different molecular weight.

The arthrotropism of P-Dex is clearly evident in the biodistribution data (Figure 4) and the SPECT/μ-CT movie (see supplement material). P-H-M_w/Dex and P-M-Dex seem to be the

most favorable conjugates in terms of targeting the arthritic joints by achieving the highest AUC among all the conjugates. The major extra-articular accumulation sites of these two conjugates are spleen, kidneys, base of the tail, and to a lesser extent, the liver. The base of the tail in AA rats is known to be highly inflamed due to the injection of adjuvant. The enhanced vascular permeability associated with inflammation may explain the preferred distribution of P-Dex to this site. The high AUC_{kidney} and AUC_{kidney}/AUC_{blood} values indicate the role of kidney as a major clearance organ for the water-soluble HPMA copolymer-Dex conjugates. MW and Dex content have significant impact on P-Dex' accumulation in kidney. This is clearly demonstrated in the case of P-L-M_w, which has the high Dex content, lowest MW but the highest renal distribution. Conjugates with lower MW such as P-L-M_w would have a faster glomerular filtration, leading to higher renal tubular epithelium exposure of the conjugate for reabsorption. Also important for reabsorption is the drug loading of the conjugate. Higher hydrophobicity of the HPMA copolymer conjugates may enhance the reabsorption process and increase their kidney accumulation [18]. The results we observed in this study agree with the previous data of the doxorubicin-containing HPMA copolymer conjugates, in which the conjugate with low MW showed the highest accumulation in the kidneys [19]. Nevertheless, the impact of MW on HPMA copolymer conjugates' distribution to kidney is not consistent. Other factors may also contribute. For example, Mitra et al showed that for ^{99m}Tc-HPMA copolymer conjugate fraction of 21,000 g/mol, its kidney accumulation at 24 h was higher than both the fractions of 7,000 and 70,000 g/mol [20]. In the study by Lammers et al, renal accumulation of HPMA copolymer at 7 days post administration was increased when MW of the copolymers was increased from 23,000 to 65,000 g/mol [21]. In the study by Wang et al, D-aspartic acid octapeptide-containing HPMA copolymer conjugate (P-D-Asp_8) of 24,000 g/mol showed a higher kidney distribution than P-D-Asp_8 of 46,000 g/mol but lower distribution than P-D-Asp_8 of 96,000 g/mol [22].

For a systemic autoimmune disorder such as RA, spleen plays a critical role in the disease pathogenesis as one of the antigen-presenting organs. Patients with RA are at risk of experiencing spontaneous splenic rupture. Splenomegaly is common in uncomplicated RA and is a feature of Felty's syndrome, in which the patient may be at risk of splenic abscess formation [23]. In AA rats, spleen enlargement (Figure 5) was recorded with visual evidence of inflammation (not shown). We suspect that the presence of sinusoidal blood vessel structures facilitates the continuous extravasation of polymer conjugates in the enlarged spleen. In addition, the accelerated internalization of P-Dex conjugates by the large number of activated macrophages in spleen may be responsible for the demonstrated accumulation of the copolymer conjugates in the organ. Similarly, we also speculate that the conjugates' accumulation in arthritic joints and other inflammatory sites is also due to the internalization by activated inflammatory cells, rather than the impairment of lymphatic drainage as seen in the case of solid tumors [24]. Gradual activation of P-Dex within lysosomes of these cells and the prolonged release of Dex would lead to sustained inflammation resolution, which was observed in previous studies [7,8]. Though no direct evidence is presented, such presumption is supported by the fact that the macrophage-like synoviocytes were able to internalize liposomes after their systemic administration [25].

To understand if MW and Dex content of P-Dex affect endocytosis by macrophage, different conjugates were tested in LPS-activated RAW 264.7 cell culture. As shown in Figure 3A, no significant difference was observed among conjugates with difference molecular weights, which was consistent with two previous studies by others: PVP with peritoneal macrophages and PHEA using explanted rat liver. In both cases, accumulation of low MW fractions was demonstrated and no indication of preferential uptake of any MW fraction was observed in the range from 5,000 g/mol up to about 100,000 g/mol [26]. In contrast (Figure 3B), the amount of conjugates internalized increased when Dex content was

raised. The internalization of P-H-M_w/Dex-FITC by LPS-activated macrophages is significantly higher than that of P-M-Dex-FITC. It should be noted, however, that the present study is still preliminary and more detailed experiments need be done to consider the impacts of other factors such as linearity of conjugate uptake and rate of exocytosis on the cellular uptake kinetics of the P-Dex conjugates.

From the PK data (Table 2), it is obvious that both Dex content and MW have direct impact on $t_{1/2(\alpha)}$, $t_{1/2(\beta)}$ and AUC of the P-Dex conjugates. Reduction of the MW of the conjugates would facilitate their extravasation and tissue distribution; Higher Dex content may help to enhance the tissue sequestration of the conjugates via increased hydrophobicity; Both had led to reduced $t_{1/2(\alpha)}$ values as seen in Table 2. Since the elimination of P-Dex is mainly through kidney, increase in the MW of the polymeric conjugates is expected to impede their glomerular filtration, which in turn will enhance the conjugates' exposure and accumulation to peripheral organs/tissues, such as the arthritis joint. On the other hand, the increase of Dex content in P-Dex conjugates raises their hydrophobicity and may enhance their binding to plasma proteins. This may consequently prolong the conjugates' $t_{1/2(\beta)}$ and facilitate their joint accumulation. In addition, activated synoviocytes in the inflammatory joints may recognize this increase of Dex content and accelerate the endocytosis process of the conjugates.

Besides arthrotropism, the accumulation of P-Dex in spleens of AA rats is also noteworthy. Though such distribution was not highlighted in our previous MRI study, accumulation in spleen is not a surprise because the sequestration of colloidal drug delivery systems by the reticuloendothelial system (RES) is well-recognized [27]. As shown in Figure 5, the response of the enlarged spleen to P-Dex treatment is very fast. Spleens from P-M-M_w and P-M-Dex treated animals at day 7 post injection had similar weight to those obtained from healthy controls. However, spleens from the group treated with P-H-M_w/Dex had about half the weight of spleens obtained from the healthy rats. We believe such significant reduction in spleen weight is the main reason for the gradual increase in spleen ID/g value over time. While there is strong evidence supporting P-Dex' potential therapeutic benefit in reducing the size of enlarged spleen in RA patients with splenomegaly or Felty's syndrome, cautions must be taken to avoid over suppression of spleen functions. Balance needs to be made in selection of the P-Dex structural parameters to achieve superior and long lasting therapeutic effect in the arthritic joints and enlarged spleen, without causing significant adverse events. Liver functions should also be carefully monitors in P-Dex treatment. Clearly, more detailed functional studies are needed to fully characterize the pharmacological and toxicological effects of P-Dex on the RES of the AA model.

Conclusions

The pharmacokinetic and biodistribution data quantitatively confirmed the arthrotropism of HPMA copolymer-dexamethasone conjugates in the AA rat model. Both the increase of MW and Dex content will facilitate P-Dex' arthritic joint-targeting through increasing circulation half-life of the conjugates and presumably enhanced inflammatory cell uptake at the sites of inflammation. The high exposure of spleen to P-Dex is favorable as it helps to ameliorate the spleen enlargement of the AA rat model. Structural parameters, such as MW and drug content must be further optimized to maximize P-Dex arthrotropism and control their distribution to RES in order to balance the efficacy and toxicity. The design of biodegradable multifunctional copolymer carriers [28,29] may be considered in the future optimization process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AA	adjuvant-induced arthritis
AIBN	2,2'-azobisisobutyronitrile
AUC	area under a concentration of analyte vs. time curve
α	macro rate constant associated with the distribution phase
BD	biodistribution
BMD	bone mineral density
β	macro rate constant associated with the elimination phase
CL	total body clearance
CTA	*S*,*S*′-bis(α,α′-dimethyl-α″-acetic acid)-trithiocarbonate
Dex	dexamethasone
DMARD	disease-modifying anti-rheumatic drug
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
HPMA	*N*-(2-hydroxypropyl)-methacrylamide
ID/g	injected dose per gram of tissue
i.v.	intravenous
LA	*N*,*N*-dioctadecyl-*N*’,*N*’-bis(2-hydroxyethyl)-1,3-propanediamine
LPS	lipopolysaccharide
MA-Dex	*N*-methacryloylglycylglycylhydrazinyl dexmathasone
MA-FITC	*N*-methacryloylaminopropyl fluorescein thiourea
MA-Tyr-NH_2	*N*-methacryloyl tyrosinamide
MW	molecular weight
M_w	weight average molecular weight
M_n	number average molecular weight
NSAIDs	nonsteroidal anti-inflammatory drugs
P-Dex	acid-labile HPMA copolymer-dexamethasone conjugates
P-Dex-Tyr-NH_2	tyrosine amide-containing P-Dex
PK	pharmacokinetics
RA	rheumatoid arthritis
RAFT	reversible addition-fragmentation chain transfer
SEC	size exclusion chromatography
$t_{1/2(\alpha)}$	the half life associated with the distribution phase
$t_{1/2(\beta)}$	the half life associated with the elimination phase

V_c	volume of central compartment
V_{ss}	apparent volume of distribution at steady state

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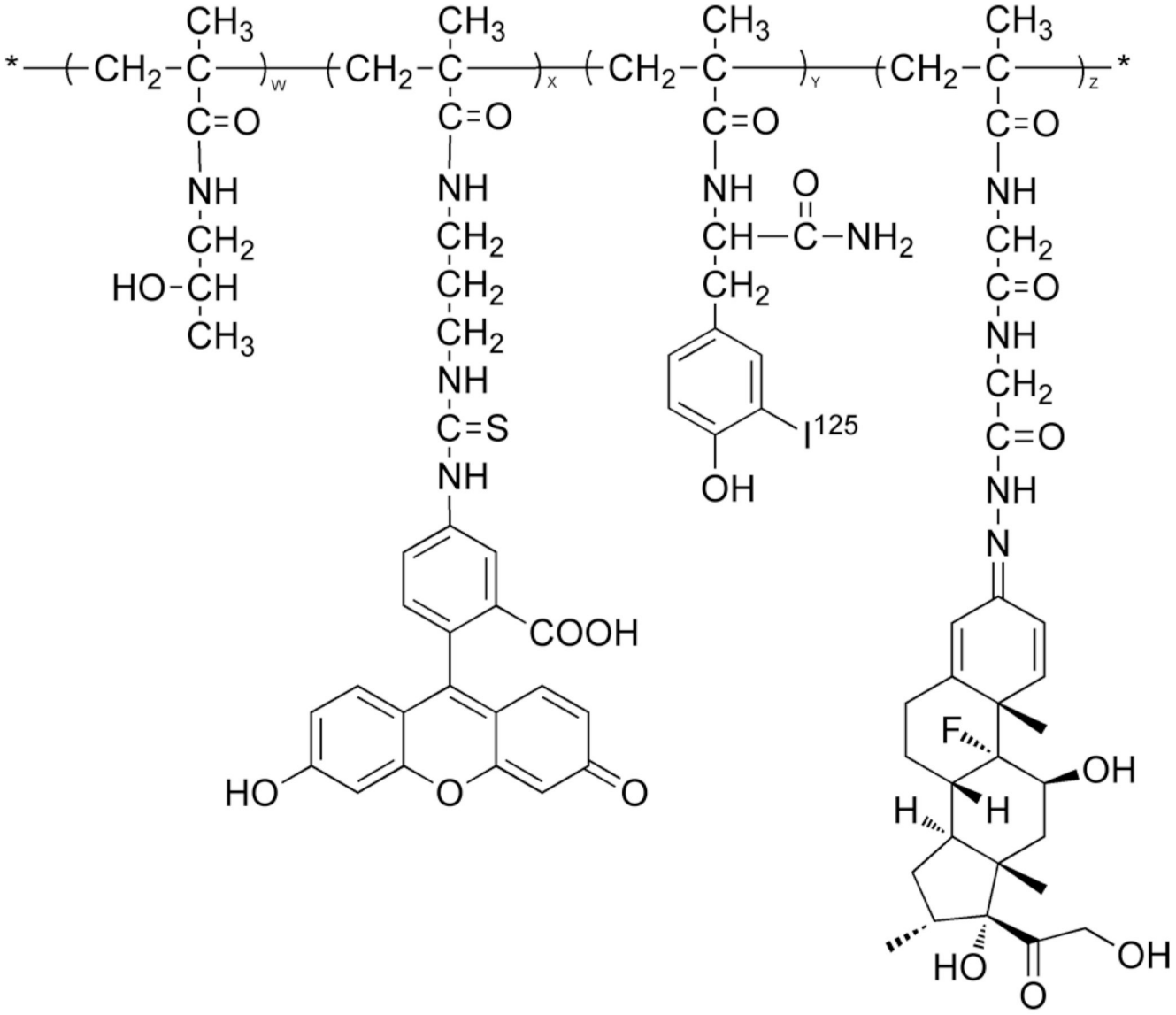

Figure 1.
The general structure of dexamethasone-containing HPMA copolymer conjugates. For all conjugates used in PK/BD and plasma stability studies, x = 0; For all conjugates used in cell culture study, y = 0.

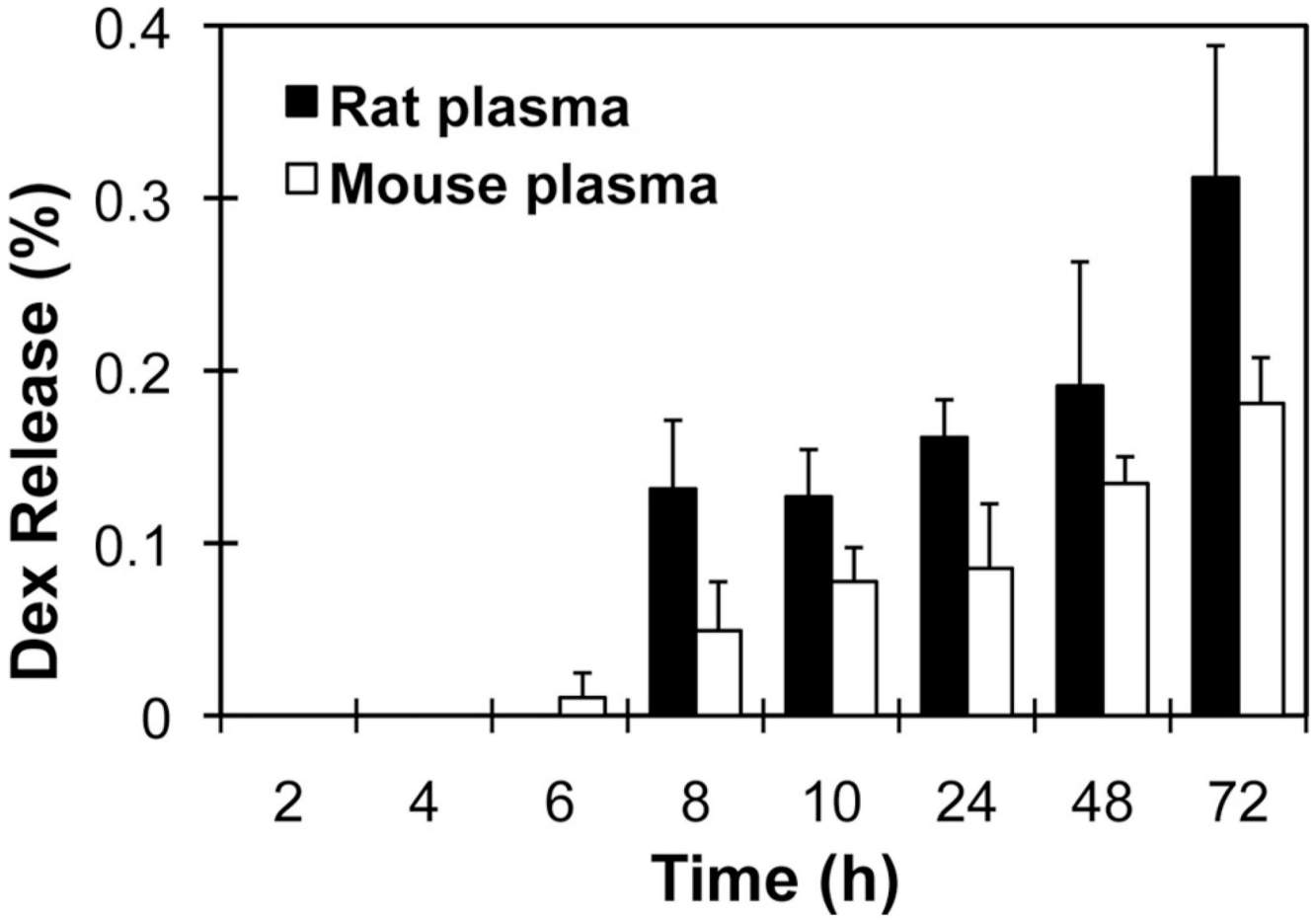

Figure 2.
The release of dexamethasone from P-Dex conjugates in murine plasma.

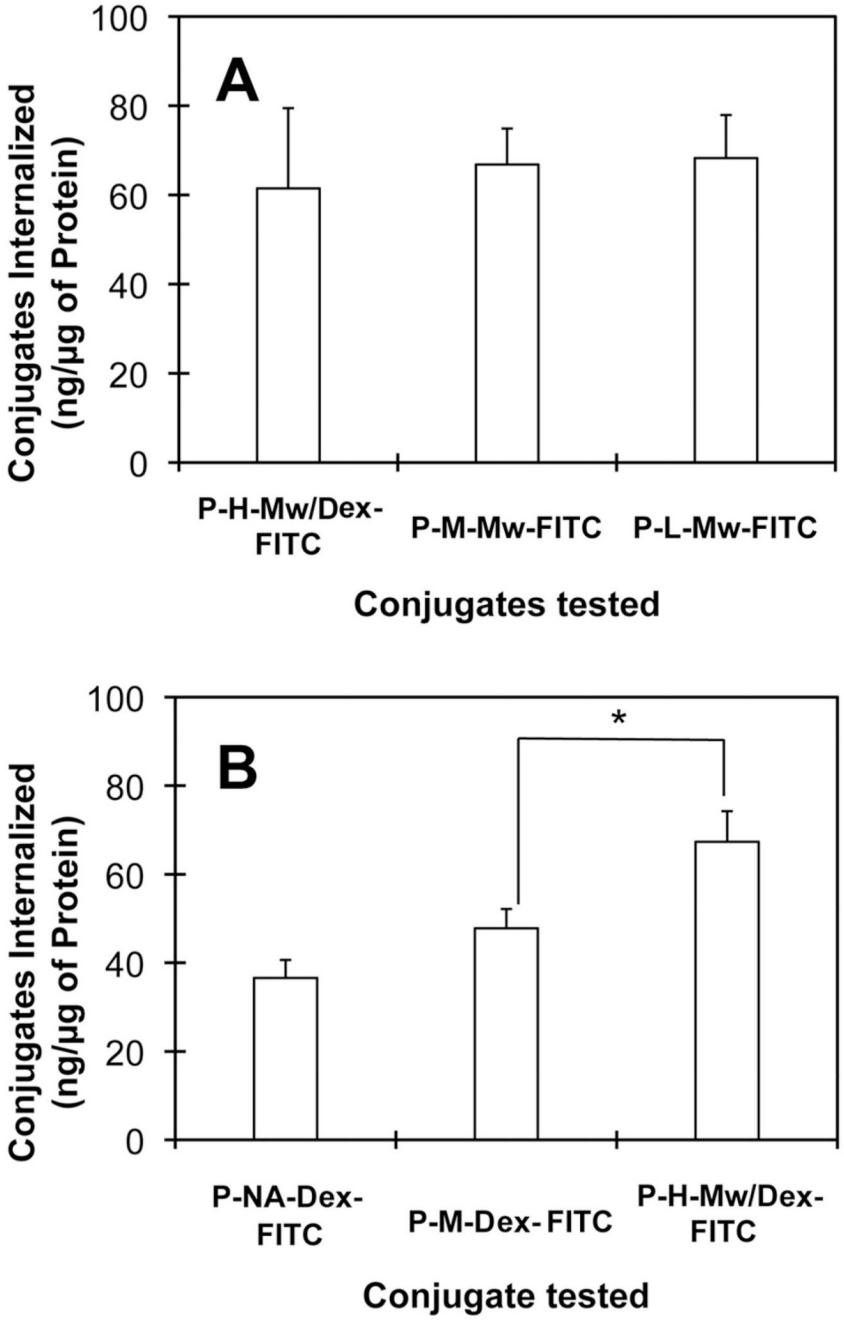

Figure 3.

In vitro internalization of HPMA copolymer conjugates in Raw 264.7 cell cultures (n = 3). (A) Internalization of HPMA copolymer conjugates with different molecular weight; (B) Internalization of HPMA copolymer conjugates with different Dex content. * indicates significant difference ($p < 0.05$, one-way ANOVA).

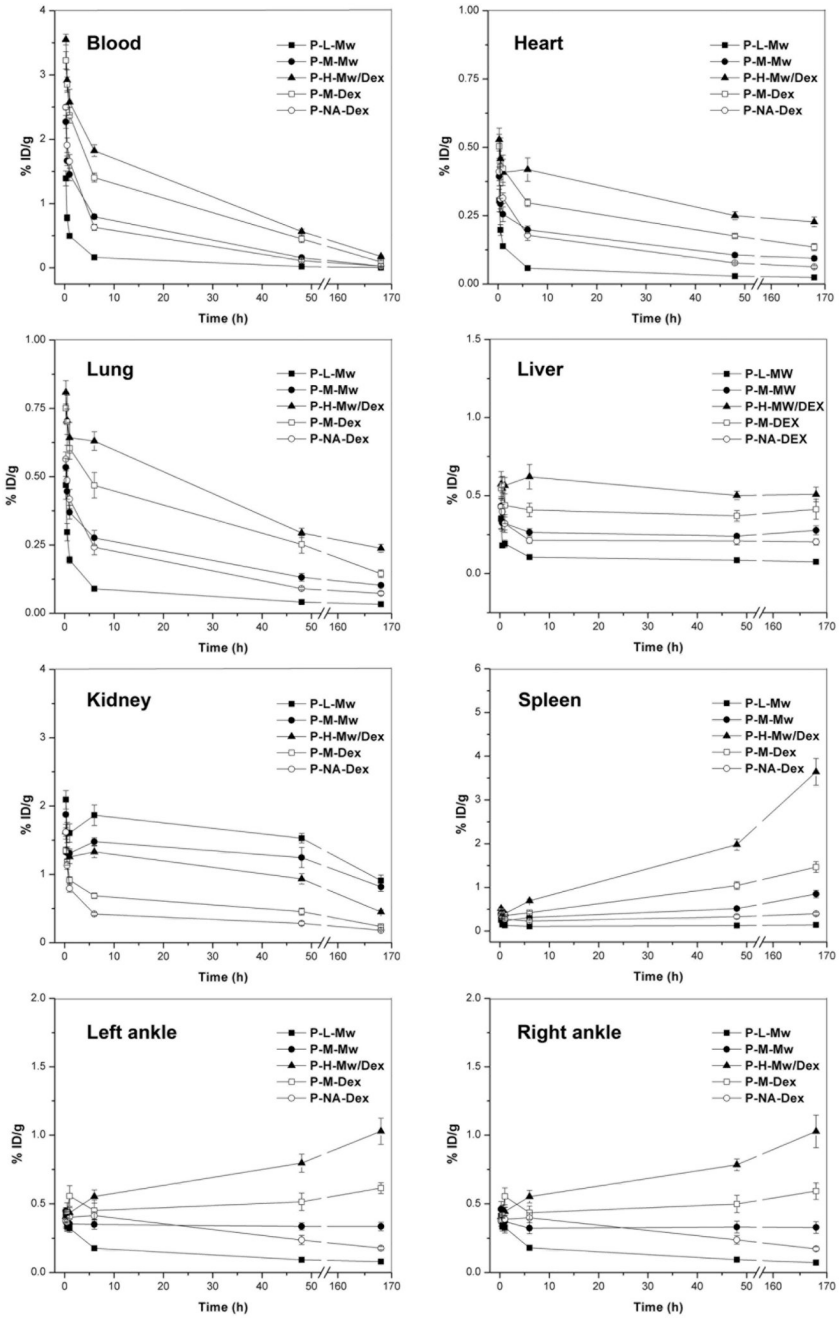

Figure 4.

The pharmacokinetics profiles of HPMA copolymer conjugates with different molecular weights and Dex contents in blood and major organs/tissues over the time course of 7 days post i.v. administration.

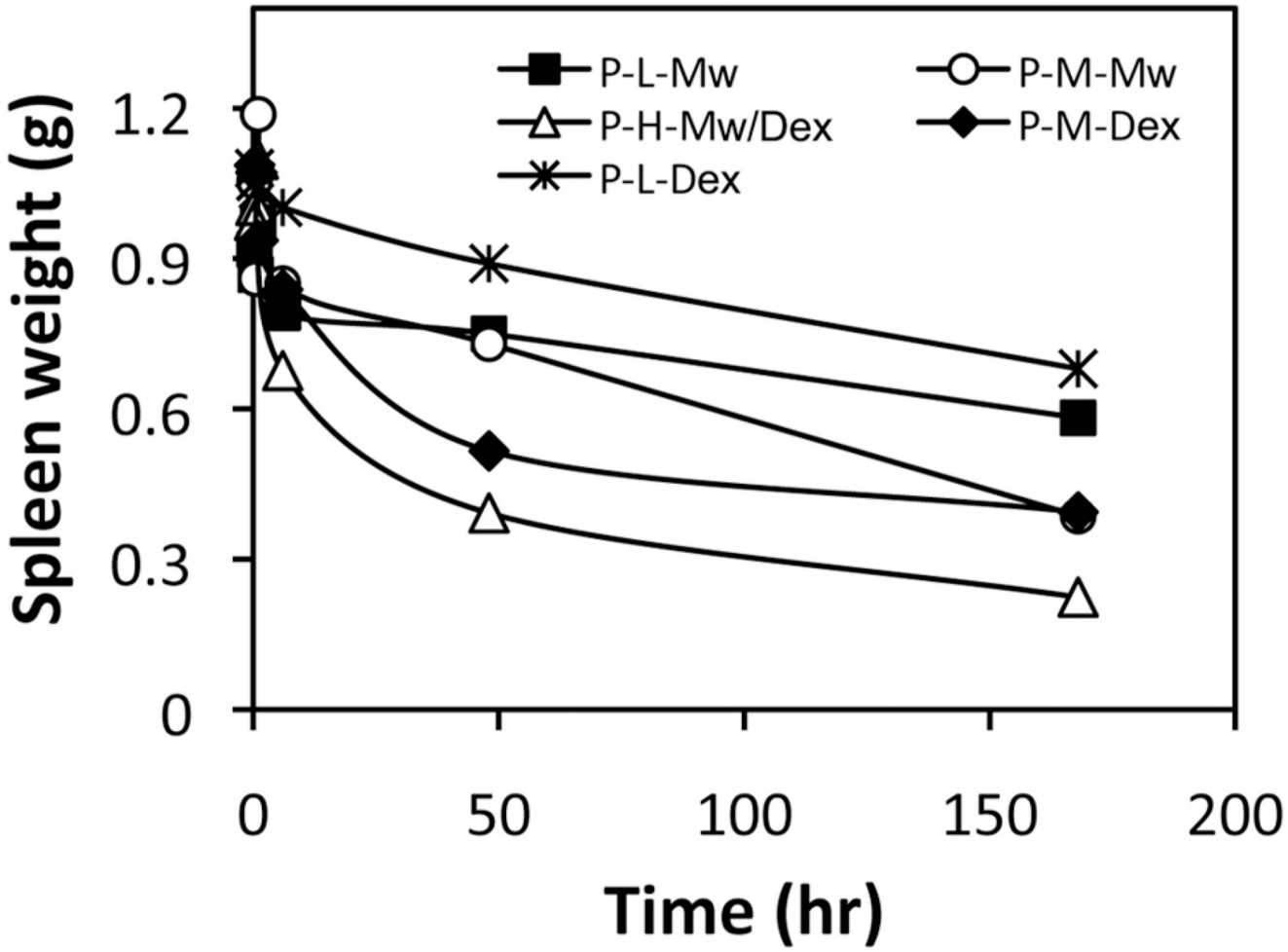

Figure 5.
The change of AA rats' spleen weight after the treatment with different HPMA copolymer conjugates. The average spleen weight of healthy male Lewis rats of matching age is ~ 0.5 g.

Table 1

The characterization of all HPMA copolymer conjugates used in the study

Polymer conjugates*		M_w** (× 10^3 g/mol)	PDI	[MA-Tyr-NH_2] (μmol/g)	[Dex] (μmol/g)	[FITC] (μmol/g)
PK/BD study	P-L-M_w	14	1.3	25.6	288	0
	P-M-M_w	24	1.3	28.2	301	0
	P-H-M_w/Dex	42	1.4	31.3	313	0
	P-M-Dex	44	1.4	29.1	151	0
	P-NA-Dex	40	1.4	31.8	0	0
Cell Culture study	P-L-M_w-FITC	16	1.3	0	321	43.0
	P-M-M_w-FITC	30	1.3	0	334	44.9
	P-H-M_w/Dex-FITC	41	1.4	0	301	42.2
	P-M-Dex-FITC	37	1.4	0	153	49.8
	P-NA-Dex-FITC	37	1.4	0	0	48.9
Plasma stability study	P-H-Dex	37	1.4	0	288	0

*L = low; M = medium; H = high; NA = no.

**The apparent weight average molecular weight (M_W) of the HPMA copolymer-Dex conjugates were calculated according to HPMA homopolymer calibration samples with narrow PDI. Limits of detection for free Dex and free FITC are 0.42 μmol/L and 1.74 nmol/L, respectively. After repeated purifications with LH-20 columns, there is no detectable free Dex and free FITC in final conjugates.

Table 2

The pharmacokinetic (PK) parameters of different HPMA copolymer conjugates after their systemic administration to AA rats. A two-compartmental model with 1/y weighting was used in the calculation.

	P-L-M_w	**P-M-M_w**	**P-H-M_w/Dex**	**P-M-Dex**	**P-L-Dex**
β^*	0.053 ± 0.007	0.038 ± 0.003	0.022 ± 0.002	0.022 ± 0.002	0.040 ± 0.004
α^*	2.17 ± 0.18	1.46 ± 0.24	1.24 ± 0.34	0.85 ± 0.16	0.88 ± 0.13
$t_{1/2(\beta)}{}^*$	13.0 ± 1.7	18.2 ± 1.4	31.6 ± 3.2	31.0 ± 2.4	17.2 ± 1.6
$t_{1/2(\alpha)}{}^*$	0.32 ± 0.03	0.48 ± 0.08	0.56 ± 0.15	0.82 ± 0.15	0.78 ± 0.12
CL^*	18.8 ± 1.6	3.6 ± 0.2	1.1 ± 0.1	1.4 ± 0.1	4.6 ± 0.3
$V_c{}^*$	47.4 ± 2.9	37.0 ± 2.2	24.8 ± 1.9	27.7 ± 1.3	35.8 ± 1.7
$V_{ss}{}^*$	297.6 ± 27.1	91.0 ± 4.9	49.4 ± 3.1	61.8 ± 3.0	102.5 ± 6.4
$AUC_{blood}{}^*$	5.3 ± 0.5	27.6 ± 1.6	90.6 ± 6.6	69.9 ± 3.8	21.8 ± 1.4
$AUC_{left\ ankle}/AUC_{blood}$	3.3	2.1	1.6	1.3	1.9
$AUC_{right\ ankle}/AUC_{blood}$	3.2	2.0	1.5	1.3	1.9
AUC_{liver}/AUC_{blood}	2.8	1.6	1.0	0.9	1.6
AUC_{heart}/AUC_{blood}	1.1	0.7	0.5	0.4	0.7
AUC_{kidney}/AUC_{blood}	43.0	6.9	1.5	1.0	2.1
AUC_{spleen}/AUC_{blood}	4.1	3.7	4.4	2.6	2.6
AUC_{lung}/AUC_{blood}	1.6	0.9	0.6	0.6	0.9

*β, macro rate constant associated with the elimination phase; α, macro rate constant associated with the distribution phase; $t_{1/2(\beta)}$, the half life associated with the elimination phase; $t_{1/2(\alpha)}$, the half life associated with the distribution phase; CL, total body clearance; V_C, volume of distribution of the central compartment; V_{SS}, volume of distribution at steady state; AUC, area under a concentration of analyte vs. time curve.