

Pharmacokinetic and Biodistribution Studies of N-(2-Hydroxypropyl)methacrylamide Copolymer-Dexamethasone Conjugates in Adjuvant-Induced Arthritis Rat Model

Ling-dong Quan, Fang Yuan, Xin-ming Liu, Jian-geng Huang, Yazen Alnouti,
and Dong Wang*

*Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska
Medical Center, Omaha, Nebraska 68198*

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Abstract: N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer has been found to be arthrotropic (joint-targeting) in the 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 $\mu\text{mol/g}$) enhances the arthrotropism of P-Dex. For the conjugate with highest MW and Dex content (P-H- M_w /Dex), the percentage of injected doses per gram (ID/g) of ankle synovial tissue at day seventh postadministration 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 seventh postadministration (0.91% g^{-1}) was found with P-L- M_w (MW = 14,000 g/mol, Dex content = 288 $\mu\text{mol/g}$), which may suggest kidney tubuli reabsorption of the conjugates. The P-Dex's distribution to heart and lung is minimum.

Keywords: Rheumatoid arthritis; pharmacokinetics; biodistribution; dexamethasone; HPMA copolymer; prodrug; arthrotropism

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

* Correspondence should be addressed to Dong Wang, Department of Pharmaceutical Sciences, University of Nebraska Medical Center, 986025 Nebraska Medical Center, COP 3026, Omaha, NE 68198-6025. Phone: 402-559-1995. Fax: 402-559-9543. E-mail: dwang@unmc.edu.

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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 single disease-modifying antirheumatic 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 antirheumatic drug category with proven disease-modifying effects. Due to their notorious systemic side effects, they are only used as a bridging therapy between 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 past 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 (iv) 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 magnetic resonance imaging (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 dexamethasone (MA-Dex),⁸ *N*-methacryloylaminopropyl fluorescein thiourea (MA-FITC),¹¹ *N*-methacryloyl tyrosinamide (MA-Tyr-NH₂),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 Na¹²⁵I was purchased from Perkin-Elmer (Waltham, MA). All other reagents and solvents were purchased from either Sigma-Aldrich (St. Louis, MO) or Acros Organics (Morris Plains, NJ). 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 follows. HPMA (1.23 g, 8.6 mmol), MA-Tyr-NH₂ (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 μ mol) as initiator and CTA (5.66 mg, 21.4 μ mol) 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 an 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-NH₂). The weight average molecular weight (M_w) and the polydispersity index (PDI) of the copolymer conjugates were determined by size exclusion chromatography (SEC) using an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare) equipped with UV and RI detectors. HPMA ho-

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Table 1. The Characterization of All HPMA Copolymer Conjugates Used in the Study

polymer conjugates ^a	M_w^b ($\times 10^3$ g/mol)	PDI	[MA-Tyr-NH ₂] (μ mol/g)	[Dex] (μ mol/g)	[FITC] (μ mol/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

^a L = low; M = medium; H = high; NA = no. ^b The apparent weight average molecular weight (M_w) of the HPMA copolymer–Dex conjugates was calculated according to HPMA homopolymer calibration samples with narrow PDI. Limits of detection for free Dex and free FITC are 0.42 μ mol/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.

mopolymers 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) with a reverse phase C₁₈ column (Agilent, 4.6 \times 250 mm, 5 μ m). Mobile phase, acetonitrile/water = 2/3; detection, UV 240 nm; flow rate, 1 mL/min; injection volume, 10 μ L. The analyses were performed in triplicate. The mean value and standard deviation were obtained using Excel.

Preparation of ¹²⁵I-Labeled Conjugates. The HPMA copolymer conjugates were labeled with ¹²⁵I using a standard chloramine-T assay. Briefly, P–Dex-Tyr-NH₂ (10 mg in 0.2 mL of saline and 0.1 mL of 0.5 M PBS) and chloramine T·3H₂O (0.1 mL, 5 mg/mL in 0.05 M PBS) were added to the Na¹²⁵I (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 ¹²⁵I, were removed by PD-10 columns. The absence of free ¹²⁵I in the labeled P–Dex-Tyr-NH₂ 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-NH₂–¹²⁵I was mixed with the unlabeled P–Dex-Tyr-NH₂ 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

μ L), sonicated and sc injected into the base of the rat's tail. On day 14 post arthritis induction, ¹²⁵I-labeled and unlabeled P–Dex-Tyr-NH₂ conjugates (\sim 30 μ Ci/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 days). 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 counter (Packard autogamma 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 μ g/mL. At predetermined time intervals, samples (300 μ L) were withdrawn and extracted with *tert*-butyl methyl ether (3 mL) by vortexing 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 μ L) and the Dex content was analyzed using HPLC. The recovery efficiency of the method used was determined as 101.4 \pm 5.2%.

In Vitro Internalization Study of HPMA Copolymer Conjugates in Macrophages. RAW 264.7 cells were cultured in 96-well culture plates (150 μ L/well) at a density of 5 \times 10⁵ cells/mL in DMEM medium (Invitrogen, CA) supplemented with 10% fetal bovine serum (VWR, PA), 100 U/mL penicillin, and 100 μ g/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

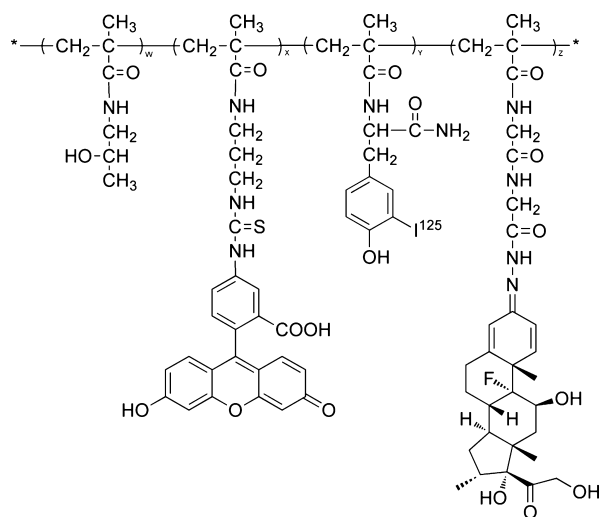


Figure 1. The general structure of dexamethasone-containing HPMA copolymer conjugates. For all conjugates used in PK/BD and plasma stability studies, $x = 0$; for all conjugates used in cell culture study, $y = 0$.

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 pairwise 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.^{15,16} 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–

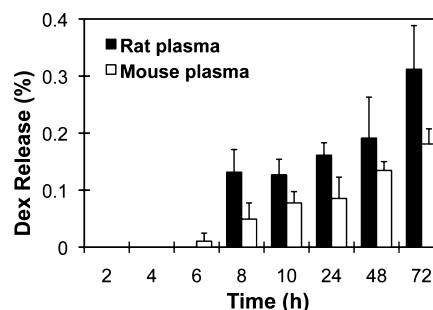


Figure 2. The release of dexamethasone from P–Dex conjugates in murine plasma.

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 ¹²⁵I labeling and tracking of their distribution after iv administration to rats. Similarly, additional Dex-containing HPMA copolymer conjugates were synthesized for cell internalization study, with MA–Tyr–NH₂ 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 different 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, 1 h, 6 h, 48 h and 7 days) postinjection 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

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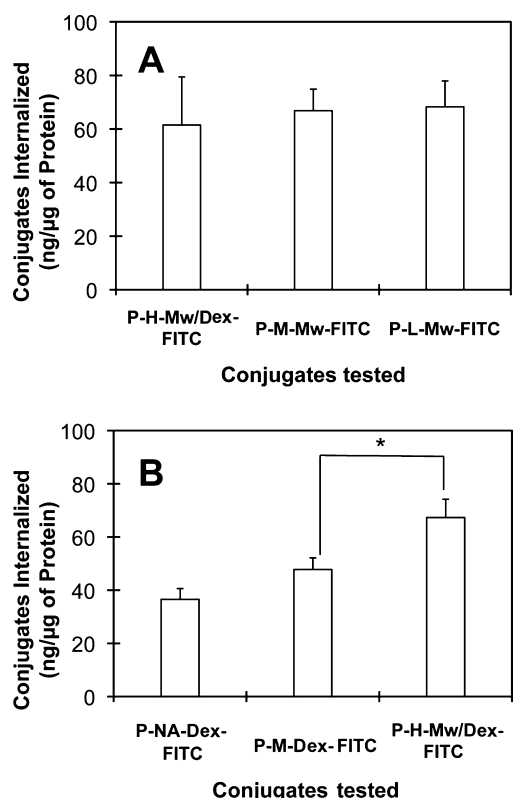


Figure 3. *In vitro* internalization of HPMA copolymer conjugates in RAW 264.7 cell cultures ($n = 3$): (A) internalization of HPMA copolymer conjugates with different molecular weight; (B) internalization of HPMA copolymer conjugates with different Dex content. * indicates significant difference ($p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

a constant ID/g value of around $0.5\% \text{ g}^{-1}$ 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 conjugates tested, P-H- M_w /Dex shows the highest ID/g value of $\sim 3.5\% \text{ g}^{-1}$ 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 ($\sim 0.5 \text{ g/spleen}$). By day 7, spleens from the P-H- M_w /Dex group weighed $\sim 50\%$ 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 accumulated in the ankle joints to an ID/g value of $\sim 1\% \text{ g}^{-1}$ by 7 days, 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 the P-H- M_w /Dex group showing the highest reduction ($\sim 50\%$) by 7 days.

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 Supporting Information.

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 follows: P-H- M_w /Dex > P-M-Dex > P-M- M_w > P-NA-Dex > P-L- M_w . The organ/tissue relative exposure ($AUC_{\text{organ}}/AUC_{\text{blood}}$) 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- M_w 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 $\sim 0.8 \text{ h}$. The value for P-H- M_w /Dex is relatively lower at $\sim 0.6 \text{ h}$. For P-L- M_w and P-M- M_w , 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 noninvasive MRI.⁹ The superior and long lasting antirheumatic effect of P-Dex was also demonstrated in the AA rat 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.

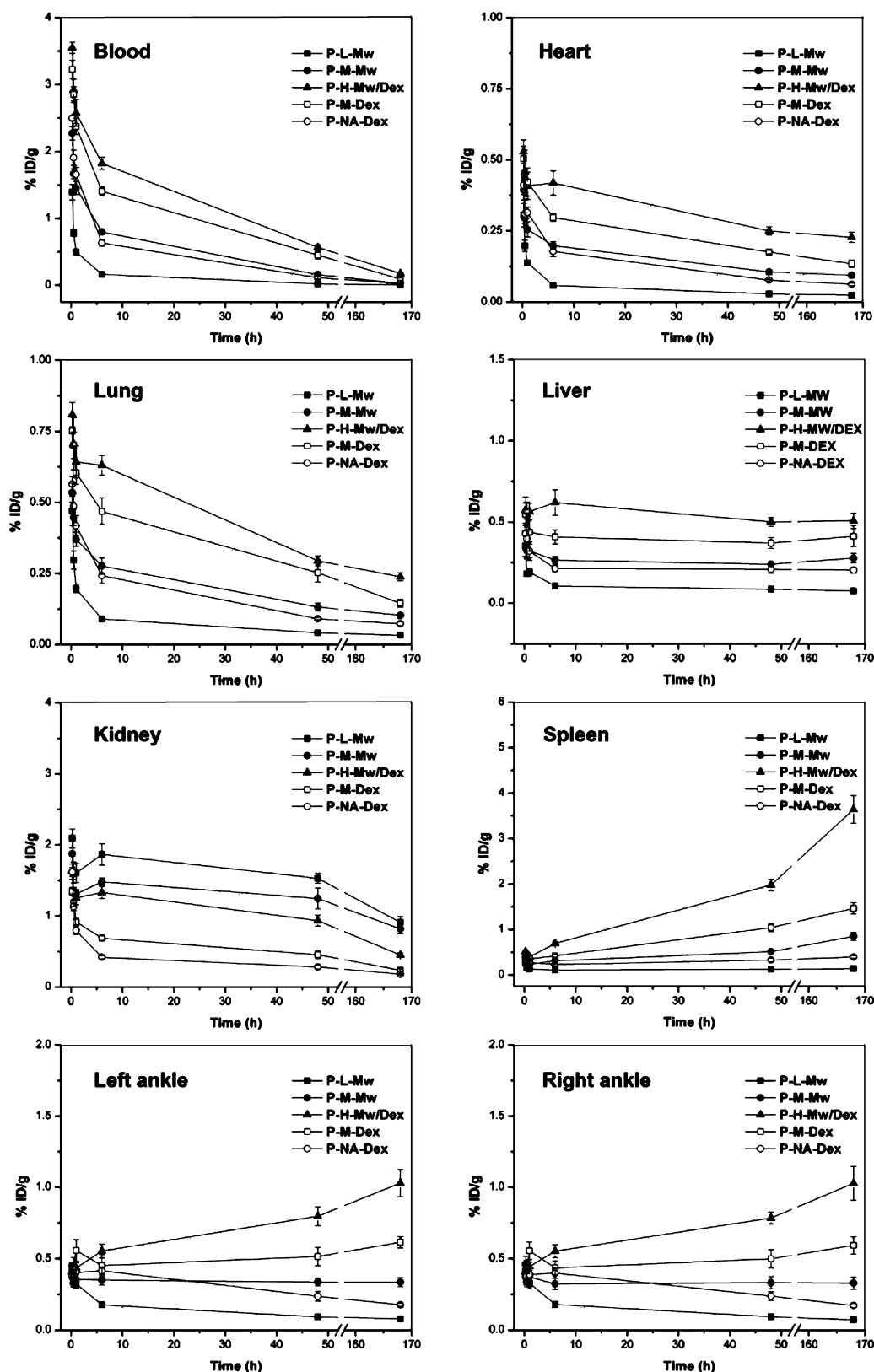


Figure 4. The pharmacokinetics profiles of HPMA copolymer conjugates with different molecular weights and Dex contents in blood and major organs/tissues over the time course of 7 days post iv administration.

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 dexametha-

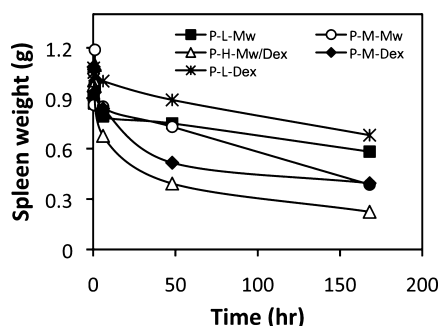


Figure 5. The change of AA rats' spleen weight after the treatment with different HPMA copolymer conjugates. The average spleen weight of healthy male Lewis rats of matching age is ~ 0.5 g.

sone 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 Supporting Information). 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 $AUC_{\text{kidney}}/AUC_{\text{blood}}$ 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's accumulation in kidney. This is clearly demonstrated in the case of P–L- M_w , which has the high Dex content, the 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 that for 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 postadministration 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-Asp₈) of 24,000 g/mol showed a higher kidney distribution than P–D-Asp₈ of 46,000 g/mol but lower distribution than P–D-Asp₈ 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

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Table 2. The Pharmacokinetic (PK) Parameters of Different HPMA Copolymer Conjugates after Their Systemic Administration to AA Rats^a

	P-L-M _w	P-M-M _w	P-H-M _w /Dex	P-M-Dex	P-L-Dex
β^b	0.053 ± 0.007	0.038 ± 0.003	0.022 ± 0.002	0.022 ± 0.002	0.040 ± 0.004
α^b	2.17 ± 0.18	1.46 ± 0.24	1.24 ± 0.34	0.85 ± 0.16	0.88 ± 0.13
$t_{1/2(\beta)}^b$	13.0 ± 1.7	18.2 ± 1.4	31.6 ± 3.2	31.0 ± 2.4	17.2 ± 1.6
$t_{1/2(\alpha)}^b$	0.32 ± 0.03	0.48 ± 0.08	0.56 ± 0.15	0.82 ± 0.15	0.78 ± 0.12
CL ^b	18.8 ± 1.6	3.6 ± 0.2	1.1 ± 0.1	1.4 ± 0.1	4.6 ± 0.3
V _c ^b	47.4 ± 2.9	37.0 ± 2.2	24.8 ± 1.9	27.7 ± 1.3	35.8 ± 1.7
V _{ss} ^b	297.6 ± 27.1	91.0 ± 4.9	49.4 ± 3.1	61.8 ± 3.0	102.5 ± 6.4
AUC _{blood} ^b	5.3 ± 0.5	27.6 ± 1.6	90.6 ± 6.6	69.9 ± 3.8	21.8 ± 1.4
AUC _{leftankle} /AUC _{blood}	3.3	2.1	1.6	1.3	1.9
AUC _{rightankle} /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

^a A two-compartmental model with 1/y weighting was used in the calculation. ^b 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.

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 different 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 postinjection 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's potential therapeutic benefit in reducing the size

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of enlarged spleen in RA patients with splenomegaly or Felty's syndrome, cautions must be taken to avoid oversuppression 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 monitored 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. The increase of both the MW and Dex content will facilitate P–Dex's 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.

Abbreviations Used

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 antirheumatic drug; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; HPMA, *N*-(2-hydroxypropyl)-methacrylamide; ID/g, injected dose per gram of tissue; iv, intravenous; LA, *N,N*-dioctadecyl-*N',N'*-bis(2-hydroxyethyl)-1,3-propanediamine; LPS, lipopolysaccharide; MA-Dex, *N*-methacryloylglycylglycylhydrazinyl dexamethasone; MA-FITC, *N*-methacryloylaminopropyl fluorescein thiourea; MA-Tyr-NH₂, *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-NH₂, 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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Supporting Information Available: A representative SPECT/ μ -CT movie depicting the biodistribution of ¹²⁵I-labeled P–H- M_w /Dex in an AA rat at 8 days post-treatment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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