Treatment of experimental arthritis with stealth-type polymeric nanoparticles encapsulating betamethasone phosphate

Tsutomu Ishihara, Tetsushi Kubota, Tesu Choi, Megumu Higaki

Institute of Drug Delivery System, Research Center for Medical Sciences, The Jikei University School of Medicine, 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan (T.I., T.K., T.C., M.H.)
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Treatment of arthritis with steroid loaded nanoparticles

b) **Address correspondence to:**

Prof. Megumu Higaki M.D., Ph.D.

Institute of Drug Delivery System, Research Center for Medical Sciences, The Jikei University School of Medicine 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan

Tel: +81-6-6466-1111 (ext.2430) Fax: +81-3-3438-2557

E-mail: mh0402@jikei.ac.jp

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d) **Abbreviations**

BP, betamethasone disodium 21-phosphate; RA, rheumatoid arthritis; MPS, mononuclear phagocyte system; PLGA, poly (D, L-lactic/glycolic acid); PLA, poly(D, L-lactic acid); PEG, poly(ethylene glycol); AA rat, adjuvant arthritis rat; AbIA mice, mice with anti-type II collagen antibody-induced arthritis; Pluronic F68, polyoxyethylene-polyoxypropylene block copolymer; DEA, diethanolamine; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio] -1-propanesufonate; Cy, cyanidine; NHS, N-hydroxy succinimide; Twenn 80, polyethylene glycol sorbitan monooleate; DMSO, dimethyl sulfoxide; TR-FIA, time resolved fluoroimmunoassay; NS, non stealth;

e) **Section;** Inflammation, Immunopharmacology, and Asthma
Abstract

We examined the therapeutic activity of betamethasone phosphate (BP) encapsulated in biocompatible and biodegradable blended nanoparticles of PLGA/PLA homopolymers and PEG-block-PLGA/PLA copolymers (stealth nanosteroid) in experimental arthritis models. Various stealth nanosteroids with a size of 45 - 115 nm were prepared, and then intravenously administered to rats with adjuvant arthritis (AA rats) and mice with anti-type II collagen antibody-induced arthritis (AbIA mice). The accumulation of stealth nanoparticles with Cy7 in inflamed joints was determined using an in vivo imaging system. The Type A stealth nanosteroid composed of PLA (2.6 kD) and PEG (5kd)-PLA(3kd), with a PEG content of 10%, a diameter of 115 nm, exhibited the highest anti-inflammatory activity. In AA rats, a 35% decrease in paw inflammation was obtained in 1 day and maintained for 9 days with a single injection of the Type A stealth nanosteroid (40 μg of BP), while the same does of non-stealth nanosteroid and 3 times higher free BP showed a significantly weaker response. In AbIA mice, a single injection of the Type A stealth nanosteroid (3 μg of BP) resulted in complete remission of the inflammatory response after 1 week. Furthermore, in AbAI mice, the accumulation of Type A stealth nanoparticles in inflamed joints was shown to parallel the severity of inflammation. The observed strong therapeutic benefit obtained with the Type A stealth nanosteroid in experimental arthritis may have been due to prolonged blood circulation and targeting to the inflamed joint in addition to its sustained release in situ.
Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint synovial inflammation and progressive cartilage and bone destruction; however, its pathogenesis is not yet clearly understood (Harris, 1990).

Glucocorticoids can be highly effective in treating joint inflammation, but their systemic application is limited because of a high incidence of serious adverse effects, particularly in long-term treatment (Saag, 2002; Rhen and Cidlowski, 2005). As intravenously administered glucocorticoids are distributed throughout the whole body and rapidly cleared, high and frequent dosing is necessary to achieve an effective concentration at inflamed target sites. Moreover, the profound physiological activity of glucocorticoids in many different tissues increases the risk of adverse effects in patients. Thus we need to develop a drug delivery system in particular nanocarriers, with enhanced localization to the target site and sustained drug release (Torchilin, 2005; Yih and Al-Fandi, 2006; Peer et al., 2007).

We previously reported PLGA/PLA nanoparticles encapsulating hydrophilic betamethasone disodium 21-phosphate (BP) at a high efficiency prepared in the presence of zinc by an oil-in-water solvent diffusion method (Ishihara et al., 2005). Although these nanoparticles encapsulating BP had strong anti-inflammatory activity (Higaki et al., 2005; Sakai et al., 2006), they were rapidly removed from circulation by the mononuclear phagocyte system (MPS), resulting in accumulation in the liver and spleen. Alternatively, poly (ethylene glycol) (PEG) is an attractive material for surface modification of nanocarriers to reduce opsonization and to prevent interactions with the MPS (Bazile et al., 1995). Although PEG-liposomes containing prednisolone phosphate for the treatment of arthritis have been reported (Metselaar et al., 2003; Metselaar et al., 2004; Teshima et al., 2006), allergic reactions to liposome preparations, even in the PEGylated form (Chanan-Khan et al., 2003), in addition to unstable incorporation, are
problematic. In addition, polymeric micelles formed from self-assembled PEG-block copolymers have also been reported (Lavasanifar et al., 2002; Park and Yoo, 2006; Torchilin, 2007), but they have become an orphaned technology due to poor loading. Here, we have prepared polymeric nanoparticles formed from a blend of PLGA/PLA hydrophobic homopolymers and PEG-PLGA/PLA block copolymers with BP, since PLGA/PLA and PEG-PLGA/PLA are potential materials for drugs carriers due to their biodegradability, biocompatibility and low toxicity (Okada, 1997). Various Types of nanoparticles with different properties, such as drug encapsulation efficiency, diameter and PEG length/density on the surfaces, can be easily prepared by controlling the blend ratio and compositions/molecular weights of the polymers (Mosquiera et al., 2001; Avgoustakis, 2004; Beletsi et al., 2005; Sasatsu et al., 2006; Ishihara et al, 2008).

In the present study, we examined the anti-inflammatory activity of stealth nanosteroids in experimental arthritis models, namely rats with adjuvant arthritis (AA rats) and mice with anti-Type II collagen antibody-induced arthritis (AbIA mice), and then determined the distribution of stealth nanoparticles in the inflamed joints.
METHODS

Materials
Poly (D, L-lactic acid) (PLA) and poly (D, L-lactic/glycolic acid) (PLGA) with a lactic/glycolic acid ratio of 50/50 were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Poly (ethylene glycol)-block-poly (D, L-lactide) (PEG-PLA) and poly (ethylene glycol)-block-poly(L-lactide-co-glycolide) (PEG-PLGA) were synthesized by a ring-opening polymerization of D, L-lactide and glycolide (Purac America, IL, USA), which had been purified by recrystallization in ethyl acetate, in the presence of monomethoxy-PEG (5580 kDa, Mn5390; NOF Co., Tokyo, Japan). BP, polyoxyethylene-polyoxypropylene block copolymer (Pluronic F68), diethanolamine (DEA), acetonitrile, and 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma Chemical Co., St. Louis, MO, USA); 4-(dimethylamino)-pyridine (MERCK, Hohenbrunn, Germany); and cyanidine (Cy7) mono-N-hydroxy succinimide (NHS) ester (GE Healthcare, Buckinghamshire, UK) were used. Zinc chloride, polyethylene glycol sorbitan monooleate (Tween 80), acetic acid, dimethyl sulfoxide (DMSO), and n-dodecylamine hydrochloride were purchased from Wako Pure Chemicals. All reagents were dissolved in distilled water, unless otherwise noted.

Preparation of nanoparticles
Nanoparticles were prepared by the oil-in-water solvent diffusion method as reported previously (Ishihara et al., 2008). In brief, a mixture (50 mg) of PLGA/PLA homopolymers and PEG-PLGA/PLA block copolymers was dissolved in 1 ml of acetone. The blending ratio of homopolymers and copolymers is shown as the PEG content in the mixture. A 500-μl aliquot of 15 mg/ml DEA in acetone, 68 μl of 1 M zinc chloride (pH 1.9), and 28 μl of 350 mg/ml BP were added to the mixture in order and
allowed to stand for 30 min at room temperature. A 26-G needle was used to add the mixture dropwise to 25 ml of distilled water stirred at 1,000 rpm at a rate of 48 ml/h. During diffusion of the organic solvent into water, nanoparticles formed rapidly along with the solidification of polymers. A 1-ml aliquot of 0.5 M citrate (pH 7.0) and 125 μl of 200 mg/ml Tween 80 were immediately added to chelate BP-zinc complexes and enhance the stable dispersion of the nanoparticles, respectively. Finally, the nanoparticles were purified and concentrated by ultrafiltration (Centriprep YM-50, Amicon, Millipore, Bedford, MA, USA). Vehicle-only nanoparticles without BP were also prepared as controls. Conventional PLA nanoparticles formed from homopolymers alone were also prepared by the addition of 4.5 ml of acetone solution with 50 mg PLA (4.9), 7.5 mg DEA, 68 μl of 1 M zinc chloride and 28 μl of 350 mg/ml BP to 0.5% Pluronic F68 (Ishihara et al., 2005).

Cy7-dodecylamine conjugate was synthesized by mixing Cy7-NHS ester, n-dodecylamine hydrochloride, and 4-dimethylamino-pyridine in DMSO, and was then purified by HPLC (Agilent 1100 series, Agilent, Waldbornn, Germany) with a ZOEBAX Eclipse XDB-C18 column (2.1 × 150 mm, Agilent). Nanoparticles encapsulating BP with Cy7-dodecylamine conjugate were similarly prepared by the addition of dyes (100 μl of 2.5 mM Cy7-dodecylamine conjugate). Furthermore, 50 mM CHAPS was added to prevent the precipitation of dyes, and the nanoparticles were stratified on 800 mg/ml sucrose solution before gel filtration (Sephadex G-100, GE Healthcare) and ultrafiltration (YM-50).

**Animals**

Lewis rats (7-week-old males weighing 200 - 250 g) and BALB/c mice (6-week-old females weighing 20 - 25 g) obtained from SLC (Shizuoka, Japan) were used in this study. The animals were housed in a specific pathogen-free environment and allowed
free access to food and water. The experimental protocols were approved by the Jikei University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

**Induction of adjuvant arthritis**

Arthritis was induced in Lewis rats by injecting 50 μl of incomplete Freund's adjuvant (DIFCO, Detroit, MI, USA) containing 6 mg/ml of *Mycobacterium butyricum*, under ether anesthesia, into the subplantar region of the left hind paw as reported (Higaki et al., 2005). Fourteen days after administration of the adjuvant, when the joint inflammation in all rats reached a maximum in the experiment, the animals were divided into groups (n = 7 in each group) so that there were no significant differences between the groups with respect to the volume of the left hind leg of the animals. In addition, a single dose (40 μg as BP/500 μl saline) of the stealth nanosteroids (Type A, B, C, or D) or conventional PLA nanosteroi (Type NS) was administered intravenously to each group. As controls, a single dose of Type A vehicle-only nanoparticles or BP (40 μg or 120 μg) was administered to the respective groups of rats (n = 7 in each group). The development of arthritis in the left hind paw was monitored plethysmographically by recording changes in paw volume by water displacement using a model TK-101 unit (Muromachi Co Ltd, Tokyo, Japan). The inflammation rate was calculated using the following equation:

\[
\text{Inflammation rate (\%) = (measured leg volume - leg volume without adjuvant)/(leg volume on Day 0 - leg volume without adjuvant) \times 100.}
\]

The average ± S.D. leg volume without adjuvant was 1.5 ± 0.2 ml. Body weights were also monitored daily during the experiment.

**Biodistribution of nanoparticles in AA rats**
Following intravenous administration of nanoparticles encapsulating BP, the tissue distribution of the nanoparticles was determined. Rats (n = 4 in each group) were injected in the tail vein with Type A or NS nanoparticles (40 μg as BP/500 μl saline). After 24 h, rats were anesthetized and blood was taken from the retroorbital plexus. Then they were sacrificed by cervical dislocation and the major organs including the liver and spleen in addition to the inflamed paw were excised and washed quickly with cold water to remove surface blood. The concentration of BP was quantified using a time-resolved fluoroimmunoassay (TR-FIA) kit for betamethasone supplied by Shionogi & Co., Ltd. (Osaka, Japan) as described previously (Ishihara et al., 2005). The detection limit of BP for this assay was 0.01 μg/ml.

**Induction of antibody induced arthritis (AbIA) mice**

Arthritis was induced using the reported methods (Terato et al., 1995; Higaki et al., 2005). BALB/c mice were injected intravenously with 2 mg/kg of an arthritogenic monoclonal antibody cocktail (Chondrex, LLC, Seattle, WA) on Day -5, and lipopolysaccharide (LPS; 2.5 mg/kg) was intraperitoneally injected on Day -2. This protocol induces severe arthritis within 48 h after the LPS injection and the arthritis persists for more than 1 week. Intravenous treatment with Type A or Type B stealth nanosteroid (3 μg as BP/100 μl saline), BP (9 μg in 100 μl saline), or saline (n = 7 in each group) was started 2 days after LPS administration (on Day 0). Mice were weighed daily and examined for visual signs of inflammation. The clinical severity was graded as follows: 0, normal; 1, erythema; 2, slight swelling; and 3, severe swelling or deformity. Each limb was graded, allowing a maximum clinical score of 12 for each animal.

**Biodistribution of nanoparticles in AbIA mice**
Mice (n = 5 in each group) were injected in the tail vein with Type A or NS nanoparticles (3 μg as BP/100 μl saline) on Day 0. After 24 h, mice were anesthetized and blood was taken from the retroorbital plexus. Then they were sacrificed by cervical dislocation and the liver and spleen in addition to the inflamed paw were excised and washed quickly with cold water to remove surface blood. The concentration of BP was quantified using a TR-FIA kit as described above. The detection limit was 0.01 μg/ml. The accumulation of Type A nanoparticles encapsulating Cy7 in the inflamed joints was also evaluated by fluorescence imaging. A 500 μl aliquot of Type A or Type NS nanoparticles encapsulating Cy7-dodecylamine conjugate (Cy7-dodecylamine, 2.6 μg/ml) or free Cy7-dodecylamine was injected into the tail vein of AbIA mice (n=3 in each group) on day 0. After 24 h, the mice were anaesthetized with sodium pentobarbital (45 mg/kg, intraperitoneally), placed into a whole-body animal in vivo imaging system (Optix, GE Healthcare), equipped with band-pass excitation at 750 nm and long-pass emission filters at 770 nm, in order to obtain near-infrared fluorescence images of the paws.

Statistics

The Mann-Whitney U-test was used for statistical analysis. Values of p < 0.05 were considered to indicate statistical significance.
RESULTS

Preparation of Nanoparticles

Several Types of nanoparticles encapsulating BP were prepared from the mixtures of PLGA/PLA homopolymers and PEG-PLGA/PLA block copolymers by the solvent diffusion method. The composition was shown to be PLA(Mw kD) and PEG (Mw kD)-PLA/PLGA (Mw kD). The basic physicochemical characteristics of the nanoparticles with approximately the same diameter (Types A, C, and D), the smaller nanoparticles with higher PEG content (Type B), and the conventional PLA nanoparticles (Type NS; not stealth) are shown in Table 1.

AA rats

Three days after administration of the adjuvant, the first signs of joint inflammation became apparent (paw volume; 3.0 ± 0.2 ml), in conjunction with a loss of body weight due to the onset of adjuvant-induced arthritis. Fourteen days later, the disease reached its maximum severity (paw volume 4.8 ± 0.3 ml), after which the inflammation gradually resolved. Although Type A vehicle-nanoparticles without BP did not show any anti-inflammatory effect, Type A stealth nanosteroid exhibited, as early as 1 day after administration, a high anti-inflammatory effect (62 ± 3%) and the effect continued over the succeeding 9 days. Meanwhile, the anti-inflammatory effect of free BP, even at a dose 3 times higher, was lower (73 ± 2%) than that of Type A stealth nanosteroid (p < 0.05) (Fig. 1A).

As shown in Figure 1B, the anti-inflammatory effects of all stealth nanosteroids were better than that of conventional nanosteroid (p < 0.01, D1). Type A stealth nanosteroid exhibited the strongest and longest anti-inflammatory effect (D2-D9, P < 0.01, vs. Type NS nanosteroid), possibly because it accumulates in the inflammatory region and slowly releases BP. Although the smallest nanoparticle, Type B stealth nanosteroid, showed a
longer blood half-life in normal mice (Type B, 24.2 h vs. Type A, 7.5 h), its in vitro release was relatively fast (Type B, 10 d vs. Type A, 36 d). The sustained release of Type A stealth nanosteroid at the inflammatory lesion is important for a strong and sustained anti-inflammatory effect. Furthermore, the BP encapsulation efficiency of Type A nanoparticles (7.5%) was higher than that of Type B (2.5%). Type C stealth nanosteroid exhibited only a weak anti-inflammatory effect, because it did not accumulate in the inflammatory region due to fast PEG release from nanoparticles, as suggested by its early blood clearance (t1/2; 2.3 h). Type D exhibited only a transitory anti-inflammatory effect, because it quickly released BP at the inflammatory region, as suggested by its having the fastest in vitro drug release (half time; 3 d).

There were no differences in body weight change among the groups.

Conventional Type NS nanoparticles accumulated in the liver 24 h after intravenous administration (4.49 ± 0.43% injection dose (ID)/g tissue). However, the accumulation of Type A stealth nanosteroid in the liver was significantly lower (0.53 ± 0.01% ID/g) (p < 0.01, vs. Type NS) (Fig. 2A). The extended residence in the blood of Type A nanosteroid (Type A, 9.83 ± 0.43% ID/g vs. Type NS, 0.12 ± 0.02% ID/g) might reflect lower liver uptake.

Interestingly, Type A nanosteroid had a tendency to accumulate in the spleen (20.21 ± 3.22%ID/g) as has been previously observed (Ishihara et al., submitted).

Type A nanosteroid also accumulated in the inflamed joint significantly more than conventional Type NS nanosteroid (2.60 ± 0.65% ID/g vs. 0.30 ± 0.12% ID/g) (p < 0.01) (Fig. 2B). No nanoparticles, either steroid-containing or vehicle only, accumulated in the muscle, kidneys, bones, or lungs (data not shown).

**AbIA mice**

AbIA developed rapidly in mice, and clinical signs (periarticular erythema and
oedema) of the disease first appeared in the hind paws 2 days after the LPS challenge (Day 0) with a 100% incidence. Erythema and swelling in the hind and front paws increased in frequency and severity in a time-dependent manner, reaching maximum arthritis indices of 5.5 (100%) on Day 2 in the saline-treated mice. The clinical score of the Type A vehicle nanoparticle-treated mice was as severe as that of the saline-treated mice (data not shown). In contrast, Type A and Type B stealth nanosteroid-treated mice on Day 2 had macroscopic evidence of disease with a reduced score of 25% for Type A and 43% for Type B on Day 2 (p < 0.01 vs. BP 3× dose). In addition, stealth nanosteroid- treated mice demonstrated a significant reduction in joint inflammation over the succeeding 6 days (p < 0.01 vs. BP 3× dose) (Fig. 3). The rate of incidence and the absolute increase in body weight were comparable in normal mice and AbIA mice during the experiment.

Markedly increased blood circulation time and reduced liver uptake of the stealth nanosteroids compared with Type NS conventional nanosteroid was also observed in the AbIA mice. Type NS conventional nanosteroid accumulated in the liver 24 h after intravenous administration (56.7 ± 15.2% ID/g) at a higher rate than Type A stealth nanosteroid (28.3 ± 4.8% ID/g) (p < 0.01). Type A nanosteroid also had a tendency to accumulate in the spleen in AbAI mice (52.3 ± 26.9% ID/g vs. Type NS, 33.1 ± 27.0% ID/g) (not significant). The average BP concentration in inflamed paw (inflammation score, 1.5) was 3.92% ID/g for Type A stealth nanosteroid compared with 0.13% ID/g for Type NS conventional nanosteroid (p<0.01).

In addition, in vivo imaging analysis showed the accumulation of Cy7-labeled Type A stealth nanoparticles in the inflamed joint from 3 h up to 96 h after administration. The accumulation of Cy7-labeled Type A stealth nanoparticles, but neither Cy7-labeled Type NS conventional nanoparticles nor free Cy7, in the inflamed paws 24h after administration was shown (Figure 4A).
stealth nanoparticles in the inflamed paw paralleled the inflammation score. A representative data showed strong accumulation in the right hind paw (inflammation score, 1.5) and moderate accumulation in the left hind paw (inflammation score, 0.8), but no accumulation in the normal paws (Figure 4B).
DISCUSSION

We prepared nanoparticles with varying properties (particle diameter, drug encapsulation efficiency and release rate, and PEG density and release) by blending PLGA/PLA homopolymers and PEG-PLGA/PLA block copolymers at various blend ratios and compositions/molecular weights of polymers. We then examined the basic characteristics of several blended nanoparticles encapsulating BP to determine the therapeutic potential of these stealth nanosteroids in arthritic animal models.

In AA rats, about 30 - 40% remission of joint inflammation was accomplished within 1 day of treatment with stealth nanosteroids, and the therapeutic benefit of the injection lasted for up to 9 days with Type A stealth nanosteroid, whereas the equivalent dose of conventional Type NS nanosteroid and free BP gave 15% remission at the same dose.

In AbIA mice, extended sustained anti-inflammatory effect was obtained within 2 days and lasted for 8 days with the stealth nanosteroids. A significant therapeutic difference between Type A and Type B nanosteroids was not observed in AbIA mice.

The inhibitory effect on the production of inflammatory cytokines and serum amyloid A in sera of AbIA mice in the course of the treatment was not clear because of low sensitivity in a preliminary experiment, although the increase of IL-6 in the inflamed joints of AbIA mice (on Day 8) was significantly suppressed with the treatment of Type A stealth nanosteroid (data not shown).

Further experiments for time-dependent BP distribution are necessary to clarify the effect of particle size, although the AbIA mice model is less severe than the AA rat model. Nonetheless, a single intravenous injection of Type A stealth nanosteroid was able to induce a strong, rapid, and long-lasting therapeutic benefit in both arthritic models.

Inflammation-dependent accumulation of Type A nanosteroid in AbIA mice, was also demonstrated using in vivo imaging, as we knew that stealth nanoparticles escape from
hepatic uptake and have a prolonged blood half-life (Ishihara et al., submitted).

Following introduction into the systemic circulation, non-stealth conventional nanoparticles are rapidly and predominantly intercepted by the hepatic Kupffer cells and to a lesser extent by the splenic marginal zone and red pulp macrophages. The inability of the liver Kupffer cells to remove Type A stealth nanosteroid allow nanoparticleless circulate for extended periods of time but might be eventually cleared by the splenic red pulp macrophages due to filtration at the interendothelial cell slits at venous walls. Moreover, Type A stealth nanoparticles have been shown to accumulate more in the spleen of AbIA mice than in the spleen of normal mice in a preliminary study, the inflammatory process might also enhance the splenic accumulation of Type A stealth nanoparticles.

In a preliminary experiments, the animals tolerated the repeated injection (3 μg as BP for mice and 40 μg as BP for rats, twice a month) of Type A stealth nanosteroid with no change in body weight or general condition for 6 months. And the serum Glu/Gla-osteocalcin levels did not alter in treated-rats, although the titers of corticosterone remained lower than normal levels as with animals treated with free BP. In addition, no histopathological change in liver and spleen was observed.

The data indicate that in order to obtain superior anti-inflammatory activity in AA rats, nanoparticles should exhibit not only prolonged blood circulation, but also sustained drug release at the lesion. These properties of nanoparticles appear to be influenced by surface PEG content, the size of the nanoparticles and the rate of PEG loss from the nanoparticles, as suggested (Mosquiera et al., 2001; Beletsi et al., 2005). Polymeric nanoparticles are attractive vehicles for vascular drug delivery and our preparation of blended nanoparticles could enhance the loading efficiency. Taken together with the biodegradable, biocompatible and low toxic properties of the nanoparticles, these results suggest a strong potential for use of these stealth blended
nanoparticles as novel pharmacological agents for anti-inflammatory therapy in clinical practice. Stealth nanosteroids may not only enhance the concentration of the drug at the target site but may also lower the drug concentration at non-target tissues. The possibility of reduced toxicity may further improve the therapeutic index of glucocorticoids upon stealth nanosteroids. This new approach may offer important advantages over the existing treatments for arthritis, such as pulse therapy, intra-articular injection, and liposome preparations of steroids. And these drug carriers could be further targeted by conjugating them with specific ligands. Additionally, these results might apply to other inflammatory or immune diseases for which a steroid is effective.
ACKNOWLEDGEMENTS

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References


Legends for Figures

Figure 1 Paw inflammation rate of AA rats.

Arthritis was induced in Lewis rats as described in the “Materials and Methods” section.

Inflammation rate (%) = (measured leg volume - leg volume without adjuvant)/ (leg volume on Day 0 - leg volume without adjuvant) × 100.

The mean (point) ± S.D. (line) of 7 rats from Days 0 - 9 in each group is shown.

(A) ○; Type A stealth nanosteroid (40 μg),●; Type A vehicle, Δ; BP (40 μg), ▽;BP (120 μg), **p < 0.01 (40 μg Type A vs. 120 μg BP)

(B) ○ ; Type A stealth nanosteroid
Δ; Type B stealth nanosteroid
▽; Type C stealth nanosteroid,
□; Type D stealth nanosteroid
■; Type NS nanosteroid
**p < 0.01 (Type A vs. Type NS)
††p < 0.01 (Type B, C, D vs. Type NS)

Figure 2 Biodistribution of BP 24 h after i.v. administration to AA rats.

Arthritis was induced in Lewis rats and BP concentration was measured as described in the “Materials and Methods” section. Columns and bars represent the mean ± S.D. (n = 4).

(A) liver, spleen, blood and (B) inflamed foot

Black, Type NS nanosteroid; White, Type A stealth nanosteroid

**p < 0.01 (Type A vs. Type NS)
Figure 3 Paw inflammation score of AbIA mice.
Arthritis was induced in BALB/c mice as described in the “Materials and Methods” section. The average clinical score is shown. S.D. is within 10%.
○; Type A stealth nanosteroid (3 μg),
△; Type B stealth nanosteroid (3 μg)
▼; BP (9 μg), ■; saline
**p < 0.01 (3 μg Type A or B nanosteroid vs. 9 μg BP)

Figure 4 Accumulation of nanoparticles containing Cy7-dodecylamine conjugate in the inflamed joint.
Cy7-dodecylamine conjugate-encapsulated nanoparticles were intravenously administrated in AbIA mice and observed with an explore Optix in vivo fluorescence imaging system as described in the “Materials and Methods” section. Representative data are shown.

(A) A; Control Cy7, B; Type NS conventional nanoparticles, C; Type A stealth nanosteroid

(B) Signal intensity and inflammation score of each paw with Type A stealth nanosteroid
The bar indicates the photon count.
Table 1 Nanoparticle characteristics

<table>
<thead>
<tr>
<th>Type</th>
<th>PLGA/PLA homopolymers (Mw)</th>
<th>PEG-PLGA/PLA block copolymers (Mw)</th>
<th>PEG content (wt. %)</th>
<th>Diameter (nm)</th>
<th>BP encapsulation efficiency (wt. %)</th>
<th>In vitro (d)</th>
<th>In vivo (h)</th>
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<tr>
<td>A</td>
<td>PLA(6)</td>
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<td>10</td>
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<td>PEG(6)-PLA(9)</td>
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<td>PEG(6)-PLA(9)</td>
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<td>7.5</td>
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<tr>
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<td>PLA(16)</td>
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<td>0</td>
<td>178</td>
<td>4.8</td>
<td>&gt; 60</td>
<td>1.1</td>
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Figure 3

Inflammation rate (%) vs. Time (days)

- Treatment

0 2 4 6 8

**
Figure 4

A

B

C

![Images showing fluorescence with signal intensity graph](image)

<table>
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<tr>
<th></th>
<th>Signal intensity (Counts)</th>
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<tr>
<td>[ ]</td>
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<tr>
<td>[ ]</td>
<td>Right front paw</td>
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<tr>
<td>[ ]</td>
<td>Left hind paw</td>
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<td>Right hind paw</td>
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Inflammation score:

- (0.0)
- (0.0)
- (0.8)
- (1.5)