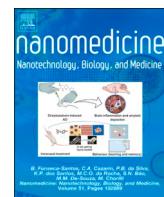




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## Original Article

# Identification of formulation parameters that affect the analgesic efficacy of ProGel-Dex – A thermoresponsive polymeric dexamethasone prodrug for chronic arthritis pain relief



Xin Wei, PhD<sup>a,1</sup>, Gang Zhao, PhD<sup>b,1</sup>, Ningrong Chen, PhD<sup>a</sup>, Xiaoke Xu, BS<sup>a</sup>, Haochen Jiang, BS<sup>a</sup>, Daniel Tran, BS<sup>b</sup>, Evan Glissmeyer, BS<sup>b</sup>, Mary B. Goldring, PhD<sup>c</sup>, Steven R. Goldring, MD<sup>b,c</sup>, Dong Wang, PhD<sup>a,b,d,\*</sup>

<sup>a</sup> Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE, 68198, USA

<sup>b</sup> Ensign Pharmaceutical, Inc., Omaha, NE 68106, USA

<sup>c</sup> Hospital for Special Surgery, New York, NY 10021, USA

<sup>d</sup> Department of Orthopaedic Surgery & Rehabilitation, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA

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## ABSTRACT

The relief of joint pain is one of the main objectives in the clinical management of arthritis. Although significant strides have been made in improving management of rheumatoid and related forms of inflammatory arthritis, there are still major unmet needs for therapies that selectively provide potent, sustained and safe joint pain relief, especially among patients with osteoarthritis (OA), the most common form of arthritis. We have recently developed ProGel-Dex, an *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-based thermoresponsive dexamethasone (Dex) prodrug, which forms a hydrogel upon intra-articular administration and provides sustained improvement in pain-related behavior and inflammation in rodent models of arthritis. The focus of the present study was to investigate the impact of ProGel-Dex formulation parameters on its physicochemical properties and *in vivo* efficacy. The results of this study provide essential knowledge for the future design of ProGel-Dex that can provide more effective, sustained and safe relief of joint pain and inflammation.

## Introduction

Osteoarthritis (OA) is the most common form of arthritis and is the major cause of work disability in the United States.<sup>1–3</sup> The cardinal signs of OA include pain, stiffness, and loss of joint motion that lead to impaired functional activity and reduced quality of life.<sup>4–6</sup> Although the ultimate goal of therapy is to prevent the development of OA joint pathology, no disease modifying agent is available at present.<sup>7–9</sup> Effective control of joint pain represents the primary objective of clinical OA management.<sup>8,10,11</sup> Osteoarthritis Research Society International (OARSI) guidelines recommend intra-articular (IA) injection of glucocorticoids (GCs) for all OA patient subgroups and it has been shown to provide effective short-term pain relief and improved functions.<sup>8</sup> However, the long-term efficacy of IA GCs has been hampered by the short IA half-life and the adverse effects associated with repeated injections. In

2017, the FDA approved triamcinolone acetonide extended-release formulation (Zilretta™) for OA knee pain. While it significantly improved the GC half-life in the joint, post-injection arthralgia and headache were reported in a significant number of subjects.<sup>12–15</sup> Thus, there remains a significant unmet need for more effective and safer IA GC therapies for management of chronic OA pain.

Recently, we discovered the thermoresponsive behavior of a *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-based dexamethasone (Dex) prodrug. When the Dex content was increased to an unusually high level (e.g., 20 wt%), the prodrug aqueous solution showed low viscosity at room temperature, but became a hydrogel at bodily temperature.<sup>16</sup> We have named this Dex prodrug, “ProGel-Dex”. When tested ProGel-Dex in three rodent models of inflammatory arthritis,<sup>16</sup> and most recently in the destabilization of medial meniscus (DMM) model of post-traumatic OA (PTOA),<sup>17</sup> the IA injected ProGel-Dex

\* Corresponding author at: Department of Pharmaceutical Sciences, University of Nebraska Medical Center, 986125 Nebraska Medical Center, PDD 3020, Omaha, NE 68198-6125, USA.

E-mail address: [dwang@unmc.edu](mailto:dwang@unmc.edu) (D. Wang).

<sup>1</sup> These authors contributed equally to this work.

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provided complete and sustained resolution of joint inflammation and pain. In the present study, we investigated the impact of ProGel-Dex formulation parameters on its physicochemical properties and *in vivo* efficacy. The results obtained provide essential guidance for the future clinical translation of ProGel-Dex as an effective and safe therapy for arthritis pain and inflammation.

## Materials and methods

### Materials

*N*-(3-Aminopropyl) methacrylamide (APMA) hydrochloride was purchased from Polysciences (Warrington, PA, USA). *N*-(2-hydroxypropyl)methacrylamide (HPMA), *S,S'*-bis ( $\alpha,\alpha'$ -dimethyl- $\alpha''$ -acetic acid)-trithiocarbonate (CTA, purity >98 %), and Dex-containing monomer (MA-Gly-Gly-NHN=Dex) were prepared as reported previously.<sup>18–21</sup> IRDye 800CW carboxylate was purchased from Li-Cor (Lincoln, NE, USA). All other reagents were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Acros Organics (Morris Plains, NJ, USA) as reagent grade and used without further purification.

### Instrument

<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Varian 500 MHz NMR spectrometer (Palo Alto, CA, USA). The number average molecular weights ( $M_n$ ), weight average molecular weights ( $M_w$ ), and dispersity ( $D$ ) of ProGel-Dex was determined using a gel permeation chromatography (GPC) system from Agilent (Santa Clara, CA, USA) equipped with a PLgel 5  $\mu$ m MIXED-C column, a Wyatt (Santa Barbara, CA, USA) DAWN 8+ multiangle light scattering (MALS) system and an Optilab T-REX refractive index (RI) detector. An Agilent 1260 Infinity II HPLC was used for Dex content analyses. A SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Device, San Jose, CA, USA) was used to determine the IRDye 800 CW content in the prodrug. The rheological properties were assessed using a DHR-2 rheometer from TA Instrument (New Castle, DE, USA). Bone samples were analyzed using a Skyscan 1172 Micro-CT (Bruker, Kontich, Belgium). Near-infrared fluorescence (NIRF)-based optical imaging was accomplished on a Li-Cor Pearl® Impulse Small Animal Imaging System. For pain-related behavior analysis, an incap-incapacitance tester from Columbus Instruments (Columbus, OH, USA) was used.

### Synthesis of ProGel-Dex

ProGel-Dex and IRDye 800CW-labeled ProGel-Dex were synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization and fully characterized, as described previously.<sup>16</sup> Briefly, HPMA and MA-Gly-Gly-NHN=Dex were dissolved in methanol and copolymerized at 55 °C under argon for 48 h with 2,2'-azobisisobutyronitrile (AIBN) as the initiator and *S,S'*-bis ( $\alpha,\alpha'$ -dimethyl- $\alpha''$ -acetic acid)-trithiocarbonate (CTA) as the RAFT agent. The resulting copolymer was purified by LH-20 column, dialyzed, and lyophilized to produce ProGel-Dex. To synthesize IRDye 800CW-labeled ProGel-Dex (ProGel-Dex-IRDye), HPMA, MA-Gly-Gly-NHN=Dex, and *N*-(3-aminopropyl) methacrylamide (APMA) were copolymerized to introduce primary amine to the prodrug. ProGel-Dex-IRDye was then synthesized by reacting the NH<sub>2</sub>-containing prodrug with the IRDye 800CW carboxylate. To quantify Dex content in ProGel-Dex, it was hydrolyzed in 0.2 N HCl (at 2 mg/mL) in 50 % methanol overnight. The resulting solution was neutralized using 0.2 N NaOH in 50 % methanol and analyzed on HPLC.

### In vitro ProGel-Dex activation

ProGel-Dex with different Dex contents were dissolved in saline with different concentrations at 4 °C in 1.5 mL Eppendorf tubes. The polymer

solutions formed hydrogel when placed in a preheated dry block heater at 30 °C.<sup>16</sup> The weights of the hydrogels were measured using a Mettler Toledo analytical balance. The releasing medium (acetated buffer, pH = 5.0) was used to simulate the acidosis associated with inflammation and the acidity within the lysosomes where the polymeric prodrug is processed. The acetated buffer (1 mL, pre-heated to 30 °C) with sodium azide (0.02 w/v%) as preservative was added on top of the hydrogel as the releasing medium. Pluronic F127 (1 w/v %) was added to the releasing buffer to ensure the sink condition. Triplicates were prepared under each concentration for each sample, and agitated in a shaking incubator (60 r/min) at 30 °C. The supernatants (200  $\mu$ L) were withdrawn at pre-designed time points for free Dex extraction with methyl tert-butyl ether (MTBE, 1200  $\mu$ L). MTBE solutions (1000  $\mu$ L) were collected and evaporated using a vacuum evaporator. The residues were reconstituted in H<sub>2</sub>O/MeOH solution (H<sub>2</sub>O/MeOH = 1:1, 100  $\mu$ L) for HPLC analyses under the same conditions used in the Dex content analysis. The MTBE extraction recovery efficiency was assessed with the Dex concentration ranging 1–100  $\mu$ g/mL.

### The rheological analyses of ProGel-Dex

The ProGel-Dex samples were uniformly loaded between the Peltier plate (40 mm parallel plate geometry) of the rheometer and the sample thickness was set at 100  $\mu$ m. As previously described, the linear viscoelastic range (LVR) was pre-determined at 30 °C using the oscillation amplitude function with 1 % strain and the angular frequency of 10 rad/s. The rheological parameters used in all tests were within LVR. The gelation temperature ( $T_{gel}$ ) and syneresis temperature ( $T_{syn}$ ) were determined by measuring the storage modulus ( $G'$ ) and loss modulus ( $G''$ ) of samples in the oscillation temperature ramp (10–45 °C) at a fixed frequency of 10 rad/s and 1 % strain with a heating rate of 0.5 °C/min. The viscosity of the gel phase was assessed at constant temperature by flow shear rate sweep (10, 20, 30 and 40 °C, shear rate from 0.0001 to 1000 s<sup>-1</sup>).

### DMM model establishment

C57BL/6J mice (male, 9-week-old) were purchased from the Jackson Laboratory. After one week of acclimation, the mice were randomized into 18 groups (10 mice/group) as follows: Healthy, Sham, Saline (DMM), ProGel-Dex (DMM, 5 formulations with different Dex contents, each dosed at 1, 5, and 10 mg/kg Dex equivalent). DMM surgery was performed on 16 groups (1 Saline and 15 ProGel-Dex groups) of mice on the right knee joint, as previously reported.<sup>17</sup> Briefly, after anesthesia with 2 % isoflurane, the fur on the right hind limb of mice was removed. A 3 mm longitudinal incision from the distal patella to proximal tibial plateau was made with a #15 blade. After blunt dissection of the fat pad, the intercondylar region was exposed. The medial meniscotibial ligament (MMTL) was subsequently identified and transected. The joint capsule was then closed with an 8–0 absorbable suture. The skin was closed with a 5–0 unabsorbable suture. For the Sham DMM group, a procedure identical to the DMM surgery was performed without transection of MMTL. All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center, performed in accordance with the IACUC-approved protocol.

### Near-infrared optical imaging analysis

To assess the joint retention time of different ProGel-Dex formulations post IA administration, the IRDye-labeled ProGel-Dex administered to mice on the 12th week post DMM surgery via IA injection (IRDye dose =  $4.5 \times 10^{-7}$  mol IRDye/kg). The mice were imaged weekly using a Li-Cor imager under anesthesia to evaluate the distribution and retention of ProGel-Dex. Imaging acquisition conditions were set as dual channel (800 nm and white light) with 85- $\mu$ m resolution. The signal

intensity from the right knee joints was semi-quantitatively analyzed using the resident software. The region of interest (ROI) with identical areas were selected using a drawing tool of the software. After euthanasia on the 15th week post treatment, vital organs and hind limbs were harvested and assessed with the same imager.

#### In vivo pain relief assessment

At week 12 post-DMM surgery, different ProGel-Dex formulations were IA administered to the DMM-operated knee joints with an injection volume of 2–8  $\mu$ L (Dex dose equivalent). From week 8 post-DMM until the experimental end point, incapacitance tests were performed weekly to assess pain-related behavior as reported previously.<sup>16,17</sup> The weightbearing score was expressed as a ratio of the weight placed through the DMM-operated limb *versus* the sum of the weights placed through both the operated and the intact limbs, with a ratio of 50 % representing equal weight distribution across both hind limbs. All major organs, including heart, kidney, liver, spleen, lung, and adrenal gland, were collected and weighed at the experiment end point. The hind limbs were also harvested and processed for micro-CT analyses.

#### Micro-CT evaluation of bone tissue

To assess the potential disease modifying efficacy and toxicity of ProGel-Dex, the DMM-operated hind limbs were isolated and fixed in formalin for micro-CT analysis. The bones were analyzed with the scanning parameters set as 55 kV, 181  $\mu$ A, 8.93  $\mu$ m, 0.5 mm aluminum filter, 0.4 rotation step, 4 frames averaging, and 180° scans. The datasets were reconstructed using NRecon software (Skyscan). All datasets were realigned and 3D-registered before analysis using Dataviewer software (Skyscan). The medial meniscus was digitally isolated from the knee joint using the automatic contouring functions in CTAn software (Skyscan) and the bone volume was quantified. The medial tibial subchondral bone plate was digitally separated from the trabeculae and a region of interest (0.3 mm × 0.7 mm) was chosen to analyze the thickness of the subchondral bone plate using CTAn software. CTvox software (Skyscan) was used to generate the micro-CT images.

#### Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, version 8). Results are expressed as the mean ± standard deviation (SD). The Analysis of Variance (ANOVA) was used to analyze continuous outcomes among three or more groups. Tukey's pairwise post-hoc testing was performed for multiple comparisons. *P*-value <0.05 was considered statistically significant.

## Results

#### The synthesis and characterization of ProGel-Dex

To explore the impact of ProGel-Dex structural and formulation parameters on its physicochemical properties and *in vivo* activity, six ProGel-Dex samples with different Dex contents (17–25 wt%, Table 1) were synthesized. Given the prior finding that the thermoresponsive

**Table 1**  
Characterization of the ProGel-Dex formulations.

Dex monomer feed-in ratio (mol %)	Dex content (wt %)	$M_w$ (kDa)	Dispersity ( $D$ )
9	17.8 ± 0.3	7.1	1.09
10	19.8 ± 0.3	7.1	1.06
11	21.5 ± 0.2	7.1	1.06
12	22.3 ± 0.1	8.9	1.02
13	23.3 ± 0.1	9.3	1.06
14	24.9 ± 0.1	7.0	1.15

phase-transition behavior of ProGel-Dex does not depend on the weight average molecular weight ( $M_w$ ),<sup>16</sup> and our desire to limit its serum half-life when drained from the joints, the  $M_w$  of the ProGel-Dex used in this study was maintained in the range of 7–9 kDa and was not considered as an influential parameter. All ProGel-Dex synthesized had narrow dispersity with  $D$  values mostly less than 1.10.

#### In vitro Dex release from ProGel-Dex

While the hydrazone bond that connects Dex to HPMA copolymer dictates the ProGel-Dex activation, other parameters, including Dex content and ProGel-Dex concentration, may also affect the prodrug activation kinetics. To explore their impact, six ProGel-Dex samples with Dex content ranging from 17.8 to 24.9 wt% were dissolved in water at 15, 20 and 25 w/v% concentrations. The Dex release kinetics from these prodrug hydrogels are shown in Fig. 1. Overall, most of the samples showed slow but linear *in vitro* Dex releasing profiles with approximate daily releasing rates of less than 0.2 %. The increase of the ProGel-Dex concentration further slowed down the release. An increase of the Dex content in the ProGel-Dex formulations also suppressed the Dex release. Among all tested formulations, ProGel-Dex with 24.9 wt% Dex content and 25 w/v% concentration showed the slowest Dex release. Of note, ProGel-Dex with 17.8 wt% Dex content could not form a hydrogel with all concentrations tested. Therefore, it was excluded from further assessment.

#### The impact of Dex content on ProGel-Dex rheological behavior

To understand the impact of Dex content on ProGel-Dex rheological behavior, storage modulus ( $G'$ ) and loss modulus ( $G''$ ) of samples were measured in the oscillation temperature ramp (10–45 °C). All ProGel-Dex samples were prepared with a concentration of 20 w/v%. As shown in Fig. 2, altering the Dex content in ProGel-Dex had a significant impact on the gelation temperature ( $T_{gel}$ ) of ProGel-Dex. For ProGel-Dex with 19.8 and 21.5 wt% of Dex content, their  $T_{gel}$  values were above normal body temperature (37 °C). The  $T_{gel}$  for ProGel-Dex with 22.3 wt% of Dex content was 35.3 °C. Interestingly, with a further increase of Dex content in ProGel-Dex to 23.3 and 24.9 wt%, the  $T_{gel}$  values decreased to ~20 °C. The viscosities of ProGel-Dex with Dex contents ranging from 19.8 to 24.9 wt% all demonstrated shear rate dependency, with drastically reduced viscosity at higher shear rates. The ProGel-Dex viscosities were also temperature dependent. Only the ProGel-Dex with Dex content <22.3 wt% achieved viscosity values  $\leq 10^{-2}$  Pa·s at room temperature.

#### The impact of Dex content and dosing level on the intraarticular retention of ProGel-Dex

The long-term local presence, gradual release and activation post IA injection of ProGel-Dex is essential for maintaining an effective intraarticular Dex concentration, which is required for sustained OA pain relief. To assess the impacts of Dex content and dosing level on local retention of ProGel-Dex, we prepared a series of ProGel-Dex formulations as shown in Table 2. The concentrations of the formulations were all set at 20 w/v%. The ProGel-Dex used in these formulations was covalently labeled with IRDye 800CW and intraarticularly administered to DMM mice. The animals were imaged weekly with the Li-Cor imager. The NIR fluorescent signal intensities in the treated joints were semi-quantitatively analyzed and are shown in Fig. 3. The intraarticular fluorescent signal intensity for all formulations showed a near linear decline over the 3-month study. The results demonstrate that the dosing level of ProGel-Dex has a major impact on its intraarticular presence. The higher the dose, the stronger the residual intraarticular fluorescent signal intensity at the experimental end point. The Dex content also affects the residual ProGel-Dex remaining at the experiment endpoint. Results demonstrate that a higher Dex content resulted in a more

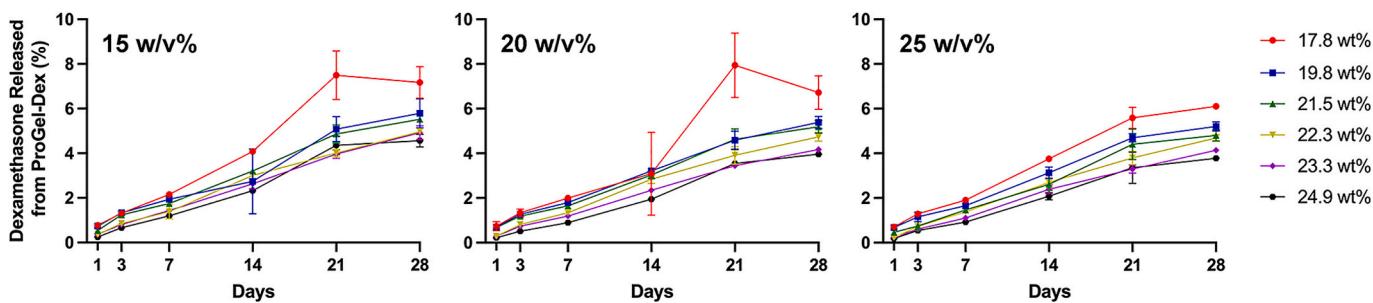


Fig. 1. The *in vitro* Dex releasing kinetics at pH 5.0 from ProGel-Dex formulations with different Dex content and different concentrations.

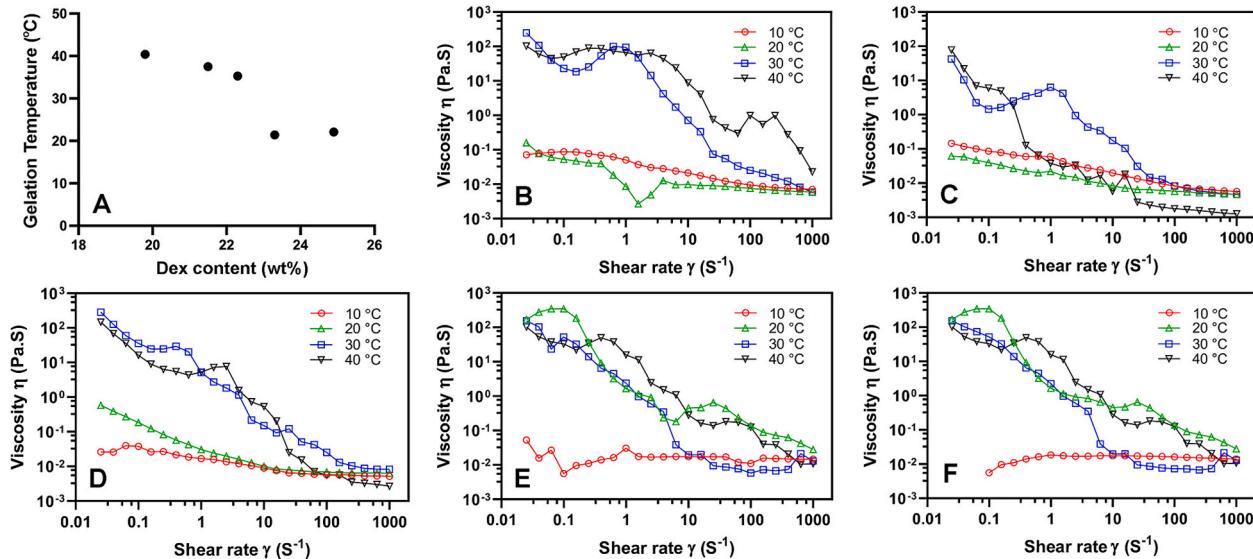


Fig. 2. Characterization of the rheological behavior of the ProGel-Dex formulations. A. The increase of Dex content in ProGel-Dex decreased the gelation temperature ( $T_{gel}$ ). B. ProGel-Dex (Dex content = 19.8 wt%) viscosity vs. shear rate profile at different temperatures. C. ProGel-Dex (Dex content = 21.5 wt%) viscosity vs. shear rate profile at different temperatures. D. ProGel-Dex (Dex content = 22.3 wt%) viscosity vs. shear rate profiles at different temperatures. E. ProGel-Dex (Dex content = 23.3 wt%) viscosity vs. shear rate profiles at different temperatures. F. ProGel-Dex (Dex content = 24.9 wt%) viscosity vs. shear rate profile at different temperatures. All ProGel-Dex formulations were prepared with a concentration of 20 w/v%. All the data presented are of single data points.

**Table 2**  
*In vivo* optical imaging of the IRDye 800CW-labeled ProGel-Dex formulations.

Dex content (wt%)	ProGel-Dex concentration (w/v%)	Dex equivalent dosing level (mg/kg)
A	19.8	20
B	21.5	20
C	22.3	20
D	23.3	20
E	24.9	20
F	19.8	20
G	21.5	20
H	22.3	20
I	23.3	20
J	24.9	20
K	19.8	20
L	21.5	20
M	22.3	20
N	23.3	20
O	24.9	20

durable ProGel-Dex retention in the joint.

In addition to the IA fluorescent signal intensity, we analyzed the end point fluorescent signal intensities of all major organs and tissues to semi-quantitatively assess the biodistribution of ProGel-Dex after IA injection. As shown Fig. 4, all formulations at all dosing levels showed a clear IA joint specificity. The remaining ProGel-Dex levels in the IA

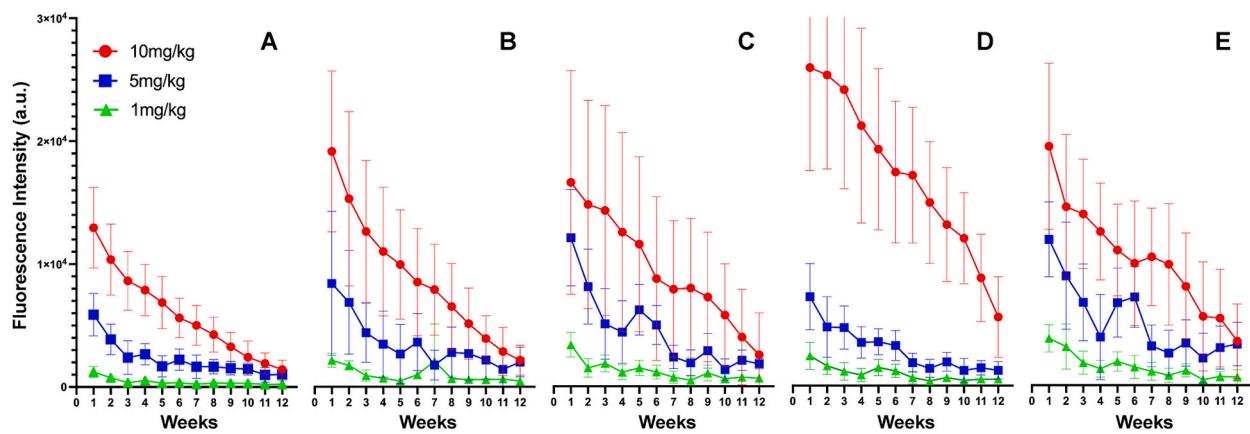
injected joints were at least 10 times higher than the levels found in all the major organs and tissues. However, we noted that when the Dex content in ProGel-Dex was at 19.8 and 21.5 wt%, the joint-specificity of the formulation was not as pronounced as the formulations at a higher Dex content, which correlated with reduced joint/liver and joint/kidney fluorescent signal intensity ratios. These results also validated kidney and liver as the main redistribution sites of ProGel-Dex after IA injection.

#### The impact of Dex content and dosing level on the analgesic effect of ProGel-Dex

Incapacitance testing was used to analyze the impact of Dex content and dosing level on the analgesic effect of ProGel-Dex in the DMM mice. The IA ProGel-Dex treatment started at 12 weeks post-DMM surgery. The percent weightbearing score (%WBS) measured by the incapacitance tester were calculated using the following formula:

$$\%WBS = \left[ \frac{\text{weight on DMM leg}}{\text{weight on DMM leg} + \text{weight on contralateral leg}} \right] \times 100\%$$

As can be seen in Fig. 5, all ProGel-Dex formulations tested at all dosing levels showed immediate improvement of pain-related behavior post IA injection. The DMM-induced weightbearing imbalance was completely mitigated in all tested groups, but the duration varied depending on the Dex content and most significantly on the dosing



**Fig. 3.** Semi-quantitative analysis of near infrared (NIR) fluorescence intensity associated with a single IA of the different ProGel-Dex formulations over 12 weeks. A. ProGel-Dex formulation with Dex content at 19.8 wt%. B. ProGel-Dex formulation with Dex content at 21.5 wt%. C. ProGel-Dex formulation with Dex content at 22.3 wt%. D. ProGel-Dex formulation with Dex content at 23.3 wt%. E. ProGel-Dex formulation with Dex content at 24.9 wt%. All ProGel-Dex formulations tested had a concentration of 20 w/v%.

levels. For ProGel-Dex with Dex content at 19.8 wt%, while the improvement was immediate and sustained, it started to diminish at 7–8 weeks post treatment at all dosing levels. For ProGel-Dex with Dex content at 21.5 wt%, the formulation dosed at 10 mg/kg level provided sustained mitigation of pain-related behavior until the 12-week experimental end point, while the improvement started to decline for the 5 and 1 mg/kg dosing level at around 7–9 weeks post treatment. Similar trends were also observed for the ProGel-Dex formulations with Dex contents at 22.3, 23.3 and 24.9 wt%, where formulations dosed at 10 mg/kg all provided balanced weightbearing for up to 12 weeks. For dosing levels at 5 and 1 mg/kg, the analgesic effects started to fluctuate after 4–6 weeks post IA administration.

#### Micro-CT analysis of the medial meniscus and subchondral bone

Abnormal bone remodeling is an integral feature of OA joint pathology,<sup>22,23</sup> which manifests by the development of a thicker subchondral bone plate and increased bone volume (BV) and calcification of the medial meniscus.<sup>24,25</sup> Representative 3D micro-CT reconstructed images of the knee joint from the different groups are shown in Fig. 6A. As shown in Fig. 6B-F, the medial menisci bone volume from ProGel-Dex groups were all significantly lower than the Saline controls. They were also significantly higher than the Sham and Healthy controls. Interestingly, no significant difference in medial menisci bone volume was observed among all the dosing levels tested.

For subchondral bone analysis, the selected ROIs are illustrated in Fig. 7A. The results of the quantitative micro-CT analysis of subchondral bone plate thickness (*Subcho.BP.Th*) from the different groups are shown in Fig. 7B-F. The *Subcho.BP.Th* values of Sham and Healthy controls are significantly lower than the Saline group. Most of the ProGel-Dex treated mice showed a trend of lower *Subcho.BP.Th* values than the Saline controls, but the differences were not significant. Only ProGel-Dex with Dex content at 22.3 wt%, dosed at 10 mg/kg and ProGel-Dex with Dex content at 23.3 wt%, dosed at 5 mg/kg exhibited significantly lower *Subcho.BP.Th* values compared to the Saline controls.

#### Discussion

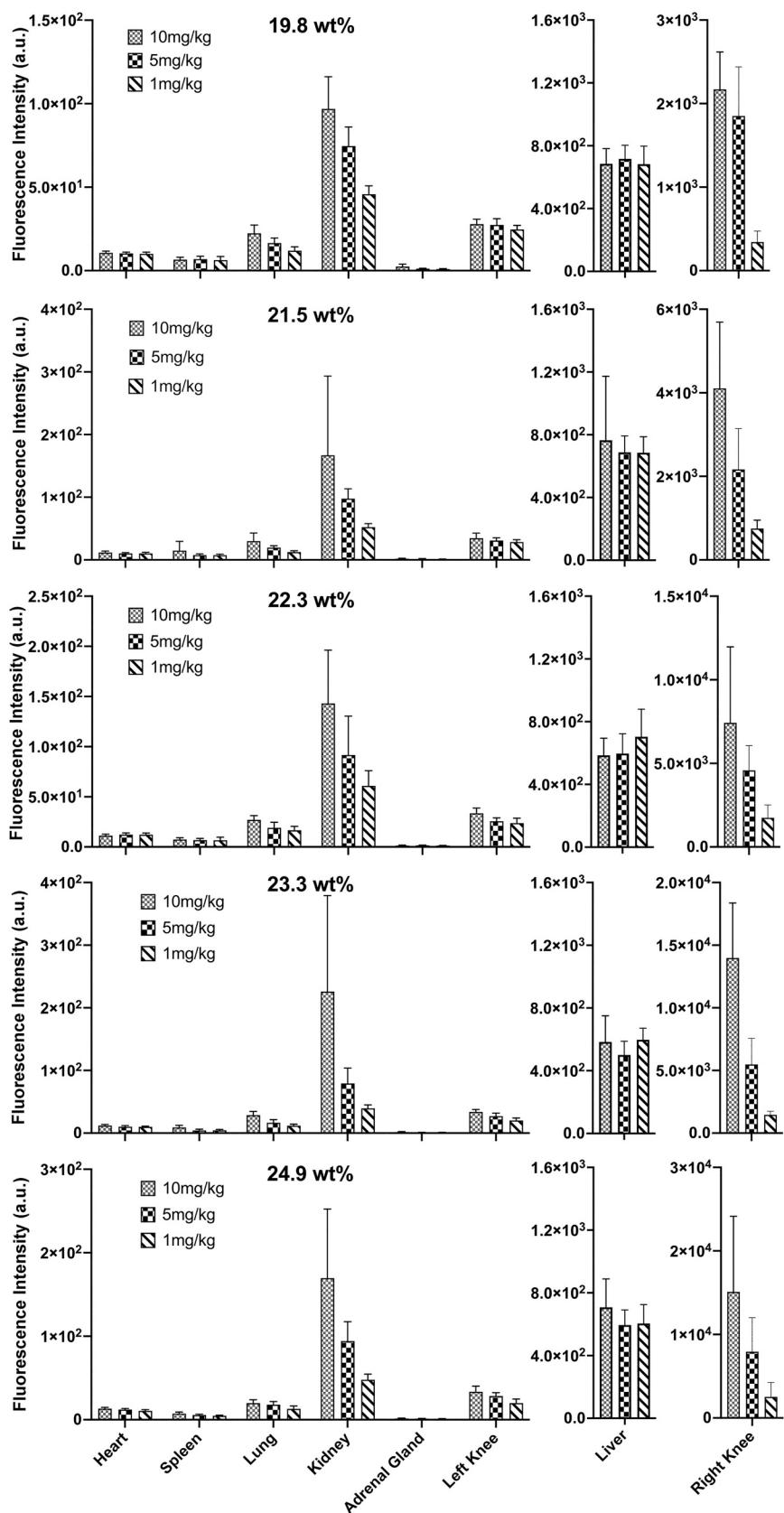
Osteoarthritis (OA) is the most common form of arthritis and it is the major cause of disability in the adult population.<sup>1–3</sup> The main objective of OA clinical management is the alleviation of joint pain. The currently recommended treatments of OA pain include oral nonsteroidal anti-inflammatory drugs (NSAIDs), IA injection of viscosupplements, and although not recommended, the prescription of opioids. IA GCs are

among the most commonly used treatments for OA pain and inflammation and their use is included in all of the guidelines of the major musculoskeletal societies. However, the main limitation of the currently available IA GCs is their relatively short duration of action. Our development of the thermoresponsive HPMA-copolymer-based dexamethasone (ProGel-Dex) was designed to overcome this limitation and mitigate potential GC-associated adverse effects. We recently reported the efficacy of ProGel-Dex in providing sustained mitigation of pain-related behavior in the DMM mouse model of PTOA for 15 weeks.<sup>17</sup> The present study was undertaken to provide a comprehensive analysis of the impact of ProGel-Dex formulation parameters on the physicochemical properties and *in vivo* efficacy of ProGel-Dex.

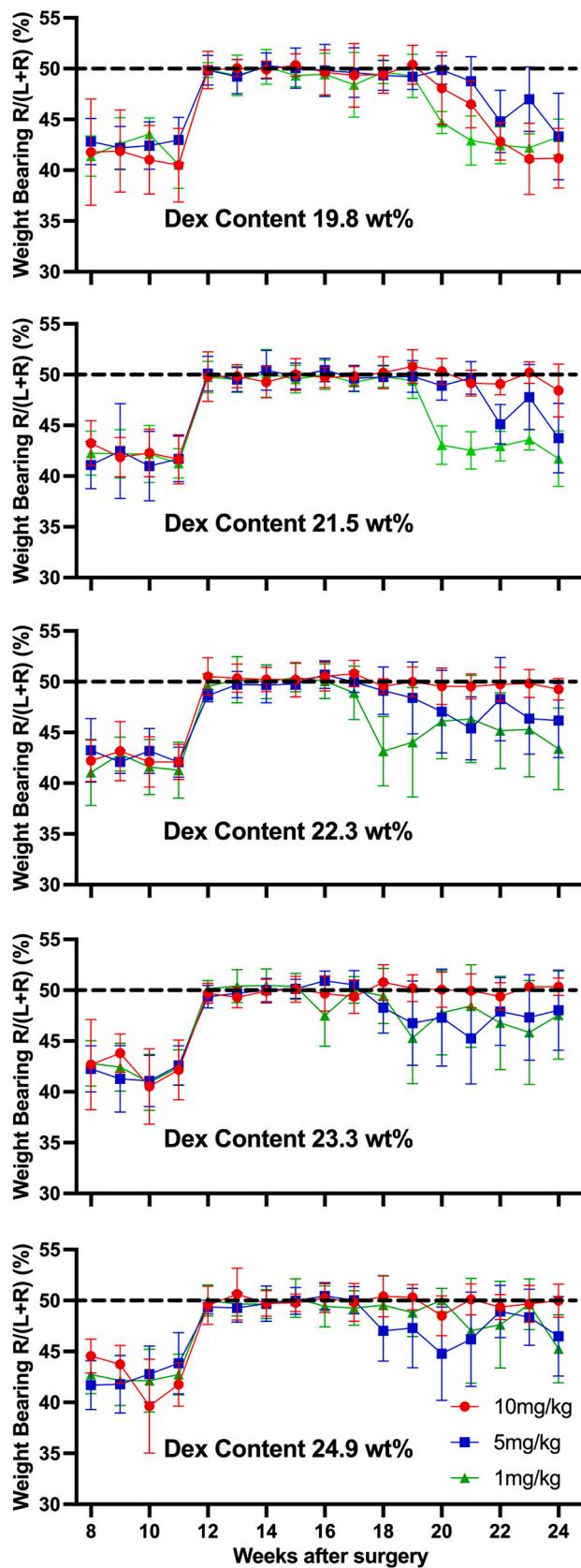
As a water-soluble polymeric prodrug, ProGel-Dex forms hydrogel once administered *in vivo*. This unique property facilitates its physical entrapment at the sites of injection.<sup>16</sup> Upon IA administration, the physically cross-linked ProGel-Dex gradually dissolves from the hydrogel formed with exposure to synovial fluid. It is then activated to release free Dex, either extracellularly *via* local acidosis or intracellularly *via* lysosomal acidity. Given that the gelation temperature of ProGel-Dex is relatively independent of its molecular weight (MW),<sup>16</sup> we purposely used ProGel-Dex with  $M_w$  lower than 10 kDa, resulting in its rapid renal clearance, greatly reduced serum half-life and systemic redistribution when it enters the circulation.

Results from the *in vitro* Dex release study suggest that at pH 5.0, the ProGel-Dex activation is slow. The increase of Dex content in ProGel-Dex was associated with suppression of its activation, but the impact was very limited except at the lowest Dex content. The impact of the ProGel-Dex concentration also had a limited effect. These observations confirmed that the hydrazone bond formed at C3 carbonyl has reasonable stability at pH 5.0 *in vitro* for its intended utility as the activation trigger for a long-lasting Dex prodrug. Once administered *in vivo*, we predict that the ProGel-Dex activation rate may be accelerated upon encountering enzymes that may specifically catalyze the cleavage of the hydrazone bond.<sup>26,27</sup> It is interesting to note that the Dex activation for the non-thermoresponsive HPMA copolymer-based Dex prodrug (P-Dex) was also linear but occurred at a faster rate of ~1 %/day.<sup>28</sup> The much higher Dex content and aggregation-induced physical crosslink in ProGel-Dex may be the main contributor to the delayed activation.

We observed that at 25 w/v% ProGel-Dex samples were relatively viscous. Therefore, it was decided that the ProGel-Dex concentration should be maintained at 20 wt% for all the rheology studies. It has been reported previously that the IA temperatures of OA joints tend to be lower than 35 °C.<sup>29</sup> Of all the ProGel-Dex formulations tested, only the formulation with Dex content at 22.3 wt% exhibited a  $T_{gel}$  around 35 °C. Further increase of Dex content resulted in ProGel-Dex with a  $T_{gel}$



**Fig. 4.** The biodistribution of ProGel-Dex formulations in different organs and tissues at 12 weeks post IA injection. The concentrations of ProGel-Dex in all tested formulations were maintained at 20 w/v%.



**Fig. 5.** Assessment of pain-related behavior improvement after a single IA injection of the ProGel-Dex formulations with dosing levels at 1, 5 and 10 mg/kg, at 12-week post DMM surgery.

around 20 °C. Corresponding to these observations, we also found that for ProGel-Dex formulations with Dex content at 23.3 and 24.9 wt%, their viscosities at 20 °C were high, posing challenges for IA injection.

The *in vivo* optical imaging studies confirmed the joint-specificity of ProGel-Dex after its IA administration, with the liver and kidneys as the main redistribution sites. The signal intensity ratios between IA injected joint and these clearance organs were at least 10-fold higher with most formulations. We found that the higher the Dex content in ProGel-Dex, the longer the ProGel-Dex resident time after IA administration. However, the most significant impact was exerted by the dosing level, with single IA ProGel-Dex dosed at 10 mg/kg providing the most sustained joint retention for up to 3 months. Based on the residue fluorescent signal intensity within the joints at the end point, we predict that the presence of ProGel-Dex formulations dosed at 10 mg/kg may last beyond the tested 3 months duration, which needs to be validated with additional experiments. Reduction in the dosing level to 5 and 1 mg/kg led to faster ProGel-Dex clearance from the joint.

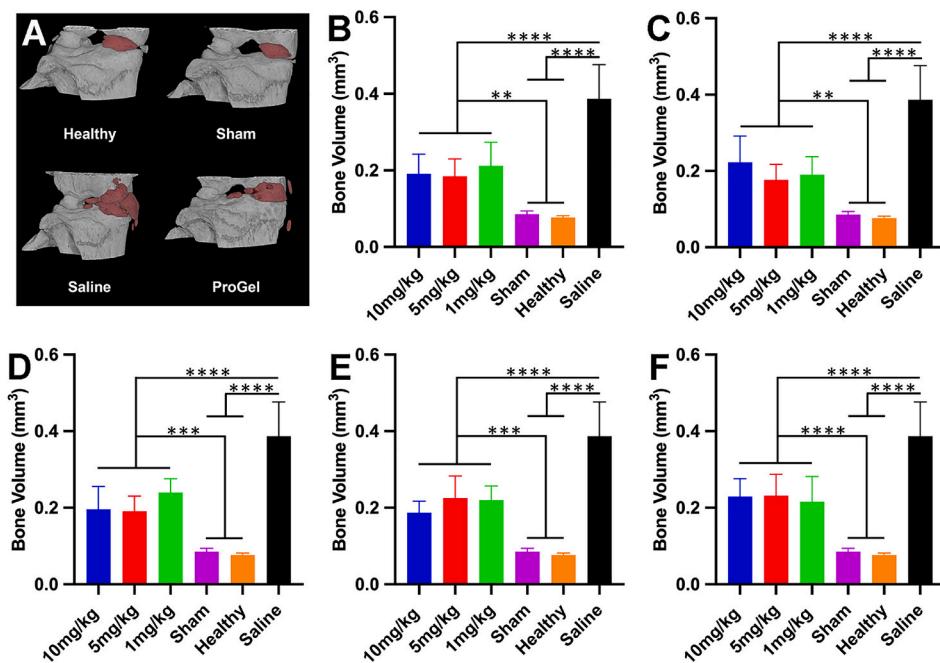
The *in vivo* incapacitance test results were consistent with the imaging findings. The single IA ProGel-Dex formulations dosed at 10 mg/kg all provided sustained mitigation of pain-related behavior for up to 3 months, with the exception of the formulation with 19.8 wt% Dex content. Dose reduction to 5 and 1 mg/kg resulted in fluctuation of joint analgesia prior to the experimental end point. Due to concerns for the potential negative impact of chronic GC exposure to the articular cartilage, IA GC treatments are advised to be given no more than 3–4 times per year, especially prior to TKA and THA.<sup>30</sup> An important recent study in a large cohort of OA patients who had received repeated IA GCs, however, showed no adverse effects of IA GCs on OA cartilage or bone, which refutes the concept that IA GCs adversely affect joint tissues.<sup>31–34</sup> This has led to a reconsideration of the use of frequent IA GC injections in the clinical care of OA patients.

OA joint pathology is often associated with signs of joint inflammation detectable by MRI,<sup>35</sup> and there is evidence that synovitis may contribute to pain and progression of cartilage pathology. It is therefore possible that the suppression of synovial inflammation by IA ProGel-Dex may in part be related to the anti-inflammatory effects of Dex released from the prodrug. As previously reported,<sup>16,17</sup> no apparent injection site reaction, such as inflammation was observed in the present study with all the formulations tested. Further histological analyses of the synovial tissues are necessary to investigate the potential impact of ProGel-Dex on synovitis and to validate the absence of a microscopic injection site reaction.

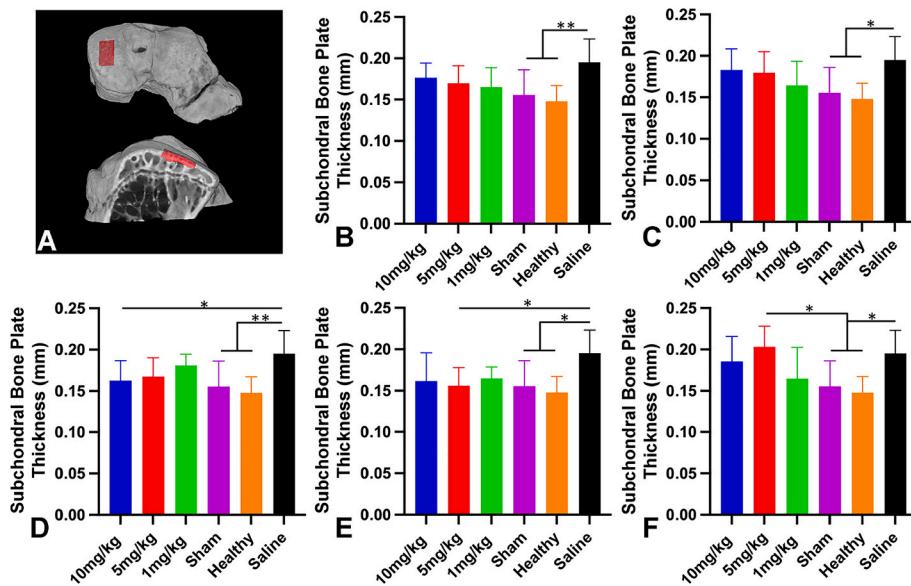
The single IA injection of ProGel-Dex showed some effect on subchondral bone and menisci based on the results of the micro-CT analysis. All ProGel-Dex treated DMM mice, regardless of dosing levels and Dex content, showed significant improvement over Saline controls in reducing the size of the calcified medial menisci. However, all treatment groups showed significantly higher values than the Sham and the Healthy controls. Importantly, the size of the calcified medial menisci among the different dosing levels showed similar values, suggesting that while the medial menisci calcification can be affected by IA GC, it may be an early event and not affected by the IA GC levels nor the long-term presence of GC in the joints. Unlike the calcified medial menisci, differences in Dex content and dosing levels of ProGel-Dex showed a very limited impact on the medial tibial subchondral bone plate thickness. Further histological analysis is necessary to assess the potential impact of ProGel-Dex on articular cartilage.

## Conclusion

In this study, we comprehensively analyzed the impact of different ProGel-Dex formulation parameters on its physicochemical properties, and *in vivo* biological effects. We found that the Dex content, ProGel-Dex concentration and the dosing levels are important parameters to be considered in the design of the formulation. Based on these experimental findings, we have identified ProGel-Dex with Dex content at around



**Fig. 6.** Micro-CT analysis of medial meniscal bone volume. A. Representative 3D micro-CT reconstructed OA knee joint images from the different treatment groups. Calcified medial menisci are digitally highlighted in burgundy. B. Comparison of the medial menisci bone volume of DMM mice treated with different doses of ProGel-Dex (Dex content = 19.8 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. C. Comparison of the medial meniscal bone volume of DMM mice treated with different doses of ProGel-Dex (Dex content = 21.5 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. D. Comparison of the medial meniscal bone volume of DMM mice treated with different doses of ProGel-Dex (Dex content = 22.3 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. E. Comparison of the medial meniscal bone volume of DMM mice treated with different doses of ProGel-Dex (Dex content = 23.3 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. F. Comparison of the medial meniscal bone volume of DMM mice treated with different doses of ProGel-Dex (Dex content = 24.9 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. \*\*, P < 0.01, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.



**Fig. 7.** Micro-CT analysis of subchondral bone plate thickness. A. Representative 3D micro-CT reconstructed images of the knee joint showing the selected subchondral bone plate region of interest (ROI, highlighted in burgundy). B. Comparison of the subchondral bone plate thickness in DMM mice treated with different doses of ProGel-Dex (Dex content = 19.8 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. C. Comparison of subchondral bone plate thickness in DMM mice treated with different doses of ProGel-Dex (Dex content = 21.5 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. D. Comparison of the subchondral bone plate thickness in DMM mice treated with different doses of ProGel-Dex (Dex content = 22.3 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. E. Comparison of the subchondral bone plate thickness in DMM mice treated with different doses of ProGel-Dex (Dex content = 23.3 wt %, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. F. Comparison of the subchondral bone plate thickness in DMM mice treated with different doses of ProGel-Dex (Dex content = 24.9 wt%, concentration = 20 w/v%) vs. Saline, Sham and Healthy controls. \*, P < 0.05, \*\*, P < 0.01.

22.3 wt% and a concentration of 20 w/v% as the formulation candidate for future *in vivo* large animal assessment. This is an important step forward to facilitate ProGel-Dex' clinical translation as a highly effective and safe intervention for OA joint pain and inflammation.

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## CRediT authorship contribution statement

**Xin Wei:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gang Zhao:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ningrong Chen:** Investigation, Data curation. **Xiaoke Xu:** Investigation, Data curation. **Haochen Jiang:** Investigation, Data curation. **Daniel Tran:** Validation, Formal analysis, Data curation. **Evan Glissmeyer:** Validation, Formal analysis, Data curation. **Mary B. Goldring:** Writing – review & editing, Validation, Formal analysis. **Steven R. Goldring:** Writing – review & editing, Validation, Formal analysis, Conceptualization. **Dong Wang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

D. Wang, S. R. Goldring, G. Zhao, and X. Wei are co-inventors of a PCT patent application covering ProGel technology. D. Wang and S. R. Goldring hold equity positions in Ensign Pharmaceutical, Inc., a start-up company which has licensed ProGel technology for further preclinical and translational development. The other coauthors declare no competing interest.

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