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Degradation, Intra-Articular Biocompatibility, Drug Release, and Bioactivity of Tacrolimus-Loaded Poly(D-L-lactide-PEG)-*b*-poly(L-lactide) Multiblock Copolymer-Based Monospheres

Maria J. Sandker,[*,†,#] Luisa F. Duque,[‡] Everaldo M. Redout,[§] Evelien C. Klijnstra,[‡] Rob Steendam,[‡] Nicole Kops,[†] Jan H. Waarsing,[†] Rene van Weeren,[§] Wim E. Hennink,[∥] and Harrie Weinans[⊥,□]

[†]Department of Orthopaedics, Erasmus Medical Centre, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands

[‡]InnoCore Pharmaceuticals, L.J. Zielstraweg 1, 9713 GX Groningen, The Netherlands

[§]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

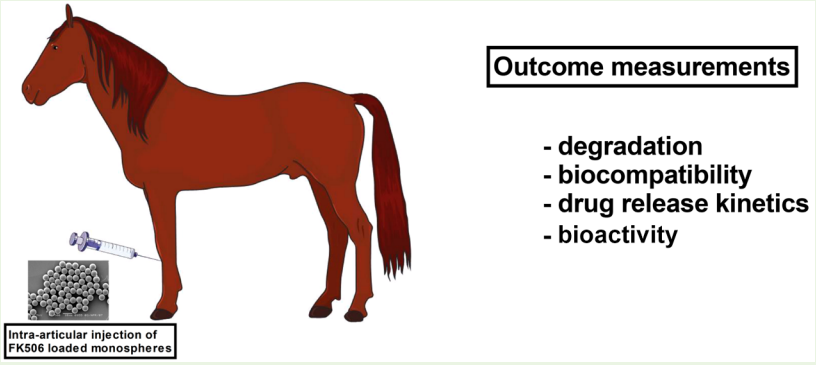
[∥]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3512 JE Utrecht, The Netherlands

[⊥]Department of Orthopaedics and Department of Rheumatology, UMC Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

[□]Department of Biomechanical Engineering, TUDelft, Mekelweg 2, 2628 CD Delft, The Netherlands

[#]Department of Orthopaedics, UMC Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Supporting Information



ABSTRACT: The aim of this study was to develop a formulation with a sustained intra-articular release of the anti-inflammatory drug tacrolimus. Drug release kinetics from the prepared tacrolimus loaded monodisperse biodegradable microspheres based on poly(D-L-lactide-PEG)-*b*-poly(L-lactide) multiblock copolymers were tunable by changing polymer composition, particularly hydrophobic−hydrophilic block ratio. The monospheres were 30 μm and released the drug, depending on the formulation, in 7 to >42 days. The formulation exhibiting sustained release for 1 month was selected for further in vivo evaluation. Rat knees were injected with three different doses of tacrolimus (10 wt %) loaded monospheres (2.5, 5.0, and 10 mg), contralateral control knees with saline. Micro-CT and histology showed no negative changes on cartilage, indicating good biocompatibility. Minor osteophyte formation was seen in a dose dependent fashion, suggesting local drug release and therapeutic action thereof. To investigate in vivo drug release, tacrolimus monospheres were injected into horse joints, after which multiple blood and synovial fluid samples were taken. Sustained intra-articular release was seen during the entire four-week follow-up, with negligible systemic drug concentrations (<1 ng/mL), confirming the feasibility of local intra-articular drug delivery without provoking systemic effects. Intra-articular injection of unloaded monospheres led to a transient inflammatory reaction, measured by total synovial leucocyte count (72 h). This reaction was significantly lower in joints injected with tacrolimus loaded monospheres, showing not only the successful local tacrolimus delivery but also local anti-inflammatory action. This local anti-inflammatory potential without systemic side-effects can be beneficial in the treatment of inflammatory joint diseases, among which is osteoarthritis.

KEYWORDS: *monodisperse microspheres, intra-articular, in vivo release, tacrolimus, biodegradable polymers*

INTRODUCTION

Osteoarthritis is a common joint disease with a complex, multifactorial pathogenesis that causes pain and loss of joint function in patients. The disequilibrium between anabolic and

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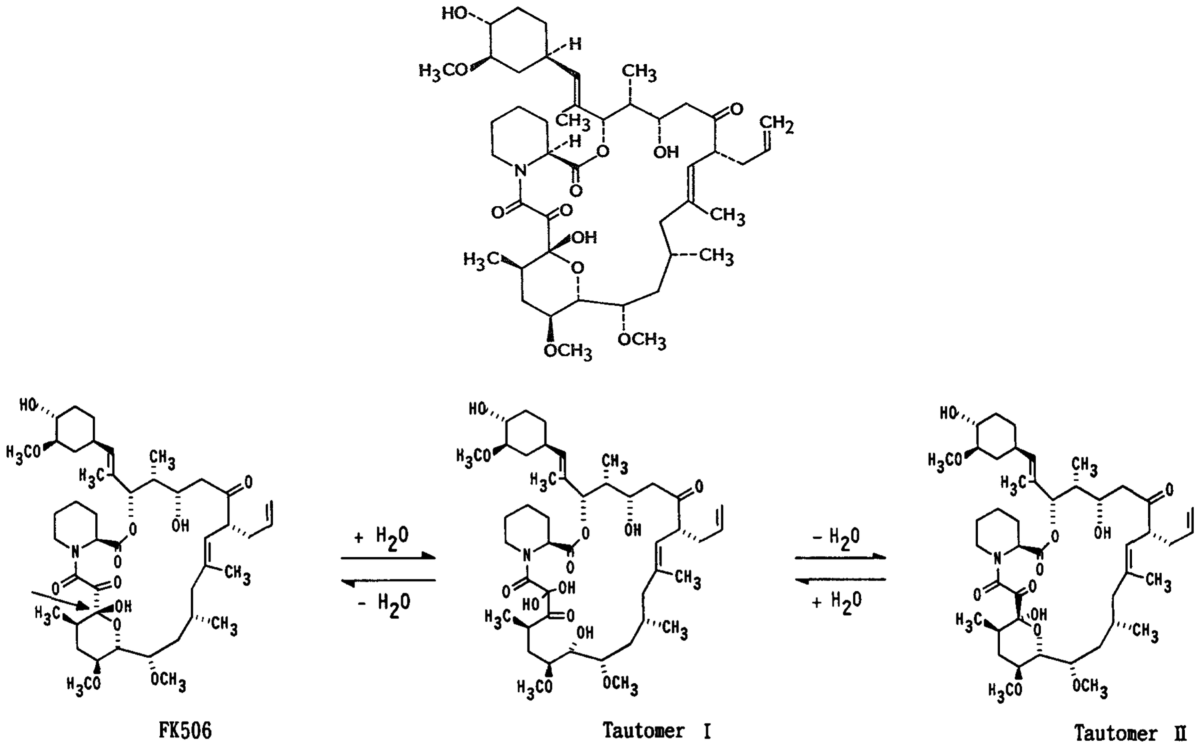

Figure 1. Chemical structure of tacrolimus[38] and its tautomers as reported by Akashi et al.[39] Reproduced with permission from ref 39. Copyright Elsevier.

catabolic activities leads to up-regulation of catabolic enzymes and pro-inflammatory cytokines, eventually resulting in cartilage breakdown and joint destruction.[1,2] No disease modifying osteoarthritic drugs are yet available, and the current standard of care consists largely of pain management using oral medication.[3] Consequently, there is a constant search for new treatment options. A potential drug for osteoarthritis is tacrolimus (FK506, Figure 1), a calcineurin inhibitor that has been used as an immunosuppressant in transplantation surgery to prevent graft rejection.[4,5] Tacrolimus has also shown to be a treatment option for patients with active rheumatoid arthritis, a chronic systemic inflammatory disease leading to joint destruction.[6] In patients with rheumatoid arthritis, synovial fluid concentrations of catabolic (inflammatory) cytokines such as TNF (tumor necrosis factor), IL-1b (interleukin 1), and IL-6 (interleukin 6), and several matrix metalloproteinases (MMPs) are elevated.[7] Although the pathogenesis of osteoarthritis has not been fully unraveled and the stimuli leading to the initial onset are different from rheumatoid arthritis, the process of cartilage degeneration involves the same loop of cytokines and MMPs as seen in rheumatoid arthritis, and synovial inflammation is also common in osteoarthritis.[8−11] The suppressive effect on catabolic cytokines of tacrolimus as well as of another (less potent) calcineurin inhibitor such as e.g. cyclosporin-A has been demonstrated both in vitro[12,13] and in vivo.[14−16] It has also been shown that calcineurin inhibition leads to less cartilage destruction in rheumatoid arthritis of in vivo animal models.[13,17,18] It is likely that this encouraging effect is (partially) achieved through the suppression of the catabolic cytokines and that a similar positive effect can be accomplished in osteoarthritis. Furthermore, calcineurin inhibition induced chondrogenic differentiation of clonal mouse embryonic carcinoma cells[19] and improved regeneration of cartilage defects,[13] which is likely related to its stimulation of endogenous TGFb.[15] These features potentially pose great opportunities for cartilage regeneration and the treatment of osteoarthritis. However, oral administration of tacrolimus in doses high enough to reach therapeutic levels in the knee joint is problematic due to the presence of severe toxic side effects, especially renal toxicity, which makes monitoring of the concentration of tacrolimus in blood important.[20] Further, the mean oral bioavailability is ∼25% and also is highly variable (5−93%),[21] which is a serious drawback for the clinical application of this drug. Tacrolimus is hence a very attractive drug for local intra-articular sustained delivery, as it is a potent immunosuppressor with low aqueous solubility and poor and strongly variable oral bioavailability.[22−24] To circumvent the problems encountered with oral administration, the best option would be to administer tacrolimus directly to the joint via intra-articular injections. This route of drug administration has, however, major disadvantages because the intra-articular drug turnover is rapid,[25−27] necessitating repeated intra-articular injections which carry the risk of cartilage damage and/or infections and are not patient friendly.[25,26,28,29] The osteo-arthritis community is therefore urgently seeking for appropriate drug delivery systems (DDSs) for local, controlled intra-articular delivery of therapeutic drugs with sustained drug retention times.[30−34] We previously showed the suitability and potential capacity of monospheres (microspheres with a narrow particle size distribution) based on poly(D-L-lactide-PEG)-*b*-poly(L-lactide) multiblock copolymers due to their excellent intra-articular biocompatibility and their retention in the knee joint for several weeks.[35] These polymers are composed of a semicrystalline (poly(L-lactide)) and an amorphous (poly(D-L-lactide-PEG)) block, allowing a more controlled polymer swelling and sustained drug release kinetics than the commonly used PLGA, where dugs are released in a bi- or triphasic manner due to its degradation properties.[36,37]

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In this study, we investigated the potential of poly(D-L-lactide-PEG)-*b*-poly(L-lactide)-based monospheres for intra-articular drug delivery of tacrolimus as potential formulation for clinical treatment of osteoarthritis. We therefore developed and characterized a suitable tacrolimus-loaded monosphere formulation and investigated its intra-articular biocompatibility and bioactivity and in vivo release kinetics in both rats and horses.

MATERIALS AND METHODS

Materials. Tacrolimus (FK506) was purchased from LC Laboratories. [PDLA−PEG_{1000}]−*b*-[PLLA] multiblock copolymers were synthesized by InnoCore Pharmaceuticals as described previously.[35] In short, low molecular weight poly(L-lactide) [PLLA] (M_w 4000 g/mol) and poly(D-L-lactide)-polyethylene $glycol_{1000}$-poly(D-L-lactide) [PDLA−PEG_{1000}] (M_w 2000 g/mol) prepolymers were synthesized by standard stannous octoate catalyzed ring-opening polymerization. PLLA and PDLA−PEG_{1000} prepolymers were then chain-extended in dioxane using 1,4-butanediisocyanate to yield *xx*[PDLA−PEG_{1000}]−*yy*[PLLA] multiblock copolymers with [PDLA−PEG_{1000}]/[PLLA] block ratios (*xx*/*yy*) of 10/90, 16/84, and 20/80 (%w/w). The characteristics of the polymers used in this study are shown in Table 1. The number-average molecular weight (M_n) and the weight-average molecular weight (M_w) of synthesized polymers were determined as described previously.[35]

Table 1. Properties of *xx*[PDLA−PEG_{1000}]−*yy*[PLLA] Multiblock Copolymers Used in the Present Study

#	polymer composition	PEG content (wt %)	M_w (Da)	M_n (Da)	T_g (°C)	T_m (°C)	ΔH_m (J/g)
1	10[PDLA−PEG_{1000}]−90[PLLA]	5	49 600	28 800	55	136	67.5
2	16[PDLA−PEG_{1000}]−84[PLLA]	8	36 100	17 700	42	130	41.9
3	20[PDLA−PEG_{1000}]−80[PLLA]	10	33 600	19 200	39	130	43.9

Poly(vinyl alcohol) (PVA, molecular weight 13−23 kDa), sodium dodecyl sulfate (SDS), acetone, sodium chloride (NaCl), and phosphoric acid were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Carboxymethyl cellulose (CMC, type 7HF-PH) was purchased from Aqualon (Barendrecht, The Netherlands).

Dichloromethane (DCM, p.a. stabilized with EtOH), dimethyl sulfoxide (DMSO), sodium azide (NaN_3), Brij-35, and Tween-20 were purchased from Across (Geel, Belgium). Ultrapure water was purchased from B. Braun Medical B.V. (Oss, The Netherlands). Mannitol was purchased from Fagron (Barbsbütel, Germany). Hyaluronidase and ascomycin were both purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Hexabrix 320, a clinical iodine-based contrast agent, was obtained from Guerget, The Netherlands. All reagents were used as received. Saline was purchased from Gibco (Bleiswijk, The Netherlands).

Preparation of Tacrolimus Loaded Microspheres. Microspheres with a target diameter of 30 μm and with a narrow size distribution (referred to as "monospheres") were prepared under best clean conditions by a membrane emulsification-based solvent extraction/evaporation process using microfabricated microsieve membranes with uniformly sized pores of 20 μm (Iris-20) (Nanomi BV, The Netherlands) as described in detail elsewhere.[30] Selection was based on findings of our previous study[35] in which this size showed to be most suitable for intra-articular delivery due to its retention for several weeks and only limited phagocytosis.[35] In short, 50 mg of tacrolimus and 450 mg of the respective polymer were codissolved in 1.5 mL of dichloromethane (DCM) to obtain a 20% w/w polymer solution followed by filtration through a 0.2 μm PTFE filter. Using 35 mbar air pressure, the filtered polymer solution was processed through the microsieve membrane into a sterile aqueous solution containing 4% w/v PVA and 5% NaCl. The formed emulsion was stirred for 3 h at room temperature to extract and evaporate DCM. Hardened monospheres were collected by centrifugation, washed twice with demiwater and twice with 0.05% w/v aqueous Tween 20 solution, and finally lyophilized. Prior to intra-articular injection in rats, monospheres were reconstituted in injection medium (0.4 wt % CMC, 0.1 wt % Tween 20, and 5.0 wt % mannitol sterile solution). For injection in horses, the microspheres were reconstituted in saline.

Microsphere Characterization. Monospheres were visually examined by optical microscopy. Particle size was determined by dispersing 5−10 mg of monospheres in 50−100 mL of electrolyte composition solution (Beckman Coulter, Woerden, The Netherlands) and measuring the particle size distribution (PSD) with a Multisizer 3 Coulter Counter (Beckman Coulter, Woerden, The Netherlands) equipped with a 200 μm measuring cell. The volume average particle size (D50) and coefficient of variance (CV%) were determined from the PSD. Surface morphology of the monospheres was assessed by scanning electron microscopy (JCM-5000 Neoscope, Jeol, Germany). Samples were sputtered with a thin layer of gold using the JFC Neocoater (Jeol, Germany).

To determine the drug content of the obtained microspheres, 10 mg monospheres was incubated in 1.0 mL of an acetone−ethanol mixture (2:1) for 1 h at room temperature to extract Tacrolimus. Previously conducted experiments in our lab confirmed that 1 h was sufficient to fully extract the loaded drug (extraction and HPLC methods for tacrolimus quantification after extraction are shown in Supporting Information). A 0.9 mL aliquot was withdrawn and replaced by 0.9 mL of a 5 w/v% Brij-35 aqueous solution to preserve the stability of the dissolved drug and avoid its tautomerization. The 0.9 mL withdrawn samples showed no recovered drug. Avoiding tautomerization[39] was important to avoid peak shifting and erroneous integration during analysis. The tautomers have a different retention time and less stability, which could lead to less recovery of the drug. These samples containing Brij solution were then centrifuged at 15 000 rpm for 5 min, and the tacrolimus-containing supernatant was subsequently filtrated through a 0.2 μm filter to prevent blocking of the column. The drug concentrations determined by HPLC are described below.

The drug loading and encapsulation efficiency (EE) was calculated as follows:

(1) Drug loading (%) = (drug mass in the monospheres/mass of monospheres) × 100%.

(2) Encapsulation efficiency (EE) (%) = (drug $loading_{experimental}$/drug $loading_{theoretical}$) × 100%.

The concentration of tacrolimus in the different samples was determined by RP-HPLC using an Alliance 2695 HPLC system (Waters Chromatography BV, The Netherlands) equipped with a UV/vis detector PDA 2998 (Waters Chromatography BV, The Netherlands). A C18 Waters Xterra LC/MS reversed phase column (3.5 μm 150 × 4.6 mm column) at a temperature of 50 °C was used in isocratic mode using 70/29/1 v/v/v acetonitrile/water/phosphoric acid using a flow rate of 1.7 mL/min. Detection of tacrolimus was done spectrophotometrically at a wavelength of 210 nm. A calibration curve of tacrolimus in the range of 0.1−120 μg/mL was prepared using the acetone/ethanol (2:1) mixture system as described before. The calibration standards and samples were stabilized in a 5 w/v% Brij-35 solution to avoid tacrolimus tautomerization prior analysis.[39]

Tacrolimus monospheres (5−10 mg) were suspended in 2 mL of 100 mM PBS + 0.1 wt/v % SDS (added to allow resuspension of monospheres, avoid caking, and solubilize the released drug, pH 7.4) in glass tubes that were placed in a shaking water bath thermostated at 37 °C. At predetermined time intervals 1.8mL of release buffer was drawn and replaced by 1.8mL fresh buffer. From the drawn samples, tacrolimus concentration was determined by HPLC as described before. Sink conditions were maintained by refreshing 90% of the release buffer after each sampling point.

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Residual DCM content of lyophilized microspheres was determined by GC-FID using a TraceGC gas chromatograph (Thermo Finnigan, Rodano, Milan, Italy) equipped with a CombiPal headspace module (CTC Analytics AG, Zwingen, Switzerland) and an Agilent Column, DB-624/30 m/0.53 mm and using dichloromethane standards in dimethyl sulfoxide in the range of 0−2000 ppm. Endotoxin concentrations in the different microsphere batches was performed by Limulus amoebocyte lysate assay according to the chromogenic end point standard procedure with a lower limit of detection of 0.05 EU/mL.[40]

In Vivo Biocompatibility Study in Rats. The Animal Ethics Committee of the Erasmus Medical Center, Rotterdam, The Netherlands, approved all procedures (agreement number EMC2255-(116-11-02). A total of 20 16-week-old (400−450 g) male Wistar rats (Charles River Nederland BV, Maastricht, The Netherlands) were housed in the animal facility of the Erasmus Medical Center with a 12-h light-dark regimen at 21 °C. Animals were fed standard food pellets and water ad libitum. Experiments started after an acclimatization period of 2 weeks. For the first experiment, in which we assessed in vivo biocompatibility of tacrolimus loaded monospheres, all rats received an IA injection of 50 μL tacrolimus microsphere dispersion in saline (n = 5 low dose, n = 5 medium dose, n = 5 high dose) in the left knee; contralateral 50 μL of saline was injected and served as a control. The different groups represent the following injected dosages. Low dose: 2.5 mg monospheres (8.8% loading) containing a total of 220 μg tacrolimus; medium dose: 5 mg monospheres (8.8% loading) containing a total of 440 μg tacrolimus; and high dose: 10 mg monospheres (8.8% loading) containing a total of 880 μg tacrolimus. Micro-CT scans of all knees were acquired at 3 different time points (t = 0, t = 6, and t = 12 weeks) as specified below. Following the last scan, knees were harvested, and EPIC μCT-scanning was performed at 18 μm resolution as a quantitative measure of sulfated glycosaminoglycan content in the cartilage[41−44] followed by histology. The tissue surrounding the injected rat knees, including synovial tissue, was harvested after the last μCT scan and used to evaluate possible remains of monospheres and tacrolimus locally. For this, the samples were vacuum-dried at 30 °C overnight. Presence of monospheres was evaluated by scanning electron microscopy, and remaining tacrolimus was extracted and detected through the use of HPLC as described in the Supporting Information (Figure S.1 and S.2). At 0, 6, and 12 weeks, the weight of the rats was measured as a clinical outcome parameter for systemic toxicity of the drug. Only for the weight experiment, as a negative control, a group of n = 10 rats (equal in strain, gender, and age) was used. These rats were treated with a daily oral dosage of 3.2 mg tacrolimus/kg bodyweight (suspended in NaCl 0.9%/ethanol), a proven tolerated dose.[45] As a positive control for all experiments, a group from a previously conducted study, consisting of rats of the same strain, age, and gender but with osteoarthritic knee joints[46] was used.

μCT scans of both knees from all groups were performed to measure bone changes at the three time points (t = 0, 1, and 6 weeks). Scans were made using an in vivo scanner (Skyscan model 1176, Skyscan, Kontich, Belgium). Rats were placed in a custom-made scanner bed under isoflurane anesthesia, fixing the hind limb in extended position. Scanner settings were the following: isotropic voxel size of 18 μm, 67 kV, 380 mA, 35 mm field of view, 1.0 mm Al filter, 0.5 rotation step over 198°, frame averaging of 2.

Following the last in vivo μCT scan, EPIC-CT scanning (equilibrium partitioning of an ionic contrast agent) of the joints was performed.[43,42] Rats were killed, and the knee joints were dissected into femur, tibia, and patella with removal of the surrounding soft tissue. These samples were incubated for 24 h in 40% Hexabrix solution (Hexabrix 320, Mallinckrodt, Hazelwood, MO, United States) at room temperature to achieve equilibrium between the contrast agent and the sGAG (sulfated glycosaminoglycans) content of the cartilage,[47] which is a direct measurement for cartilage quality. The influx of Hexabrix into cartilage correlates well with the inverse of sGAG content; therefore, cartilage degeneration is measurable using μCT (expressed as attenuation).[43,44] Samples were scanned using the following settings: isotropic voxelsize of 18 μm, 60 kV, 170 mA, 885 ms exposure time, 35 mm field of view, 0.5 mm Al filter, 0.8 rotation step over 198°, frame averaging of 3.

The obtained images were converted into 3D reconstructions using nRecon version 1.5 (Skyscan). All in vivo bone scan data sets were segmented with a local threshold algorithm; the tibial epiphysis was selected, and cortical and trabecular bone were automatically separated, after which the subchondral plate thickness of the medial and lateral compartment of the tibial plateau were measured as described previously.[46] In all EPIC-μCT data sets, X-ray attenuation (arbitrary gray values inversely related to sGAG content) and thickness (μm) were calculated separately for cartilage of the medial and lateral tibial plateau.[46] Using Skyscan analysis software (CT-An), all data sets were segmented using a fixed attenuation threshold between air (attenuation 30) and subchondral bone (attenuation 120). In the segmented μCT data sets, regions of interest (150 slices) were drawn around the cartilage of the medial and lateral plateau of the tibia separately, and for these regions, cartilage attenuation and thickness (μm) were calculated. As stated before, osteoarthritic knee joints of rats of the same strain, age, and gender from a previous study were used in which cartilage damage was generated using a strenuous running protocol combined with three unilateral i.a. papain injections as a positive control.[46]

Following EPIC scans, the knee parts were fixed with formalin, decalcified, and embedded in paraffin. Six μm sections for histology were cut sagitally at 300 μm intervals and stained with Safranin-O and Hematoxylin-Eosin (HE) to assess cartilage quality and synovium tissue.

Equine in Vivo Study. The study design was approved by the Ethics Committee on the Care and Use of Experimental Animals of Utrecht University in compliance with Dutch legislation on animal experimentation. Three milliliters of saline was used to disperse 200 mg of unloaded monospheres and was administered into the right middle carpal joint of three healthy horses with normal carpal and talocrural joints, as determined by radiographic and clinical evaluation. Three milliliters of saline (control) was injected into the contralateral middle carpal joint. Additionally, 3 mL of saline was used to disperse 200 mg tacrolimus (8.8% loading; 17.6 mg tacrolimus) loaded monospheres and was injected into the right talocrural joint. Three milliliters of saline was injected into the left talocrural joint to serve as a control. Lameness examinations (scored on a standardized 0 to 5 scale[48]) were conducted at 0, 8, 24, 72 h and 1, 2, 3, 4 weeks postinjection. Horses were monitored throughout the study for signs of discomfort.

From the horses, synovial fluid samples as well as blood samples were taken at the same predetermined time points when the lameness examinations were performed (0, 8, 24, 72 h and 1, 2, 3, 4 weeks postinjection), and tacrolimus concentrations were measured to establish the local and systemic drug concentrations that were achieved. To determine the tacrolimus concentrations in whole blood samples, tacrolimus was extracted as described earlier.[39] Synovial fluid samples were pretreated with hyaluronidase before performing tacrolimus extraction using the same method. In short, the precipitation reagent methanol/1.125 M $ZnSO_4$ in water (66/34, v/v) containing 20 ng/mL ascomycin as internal standard was prepared immediately before sample preparation. Blood samples (100 μL) were transferred into a 1.5 mL test tube, and 200 μL precipitation reagent was added. Samples were subsequently vortexed for 30 s and left 5 min at room temperature. After being vortexed for an additional 5 s, the tubes were centrifuged for 10 min at 15 000g at 4 °C. The supernatant was transferred into an autosampler vial, and a 5 μL sample was injected onto a HyPURITY C_{18} (50 × 2.1 mm, particle size of 3 μm) analytical column (Thermo Fisher Scientific, Utrecht, NL). Separation was performed at a flow rate of 500 μL/min with a total run time of 3 min. The mobile phases consisted of 10 mM ammonium acetate pH 3.5 in water (A) and 10 mM ammonium acetate pH 3.5 in methanol (B). Samples were separated using the following gradient A/B vol/vol: 0−0.8 min, 65/35; 0.8−0.9 min, 21/79; 0.9−2.0 min, 21/79 to 13/87; 2.0−2.1 min, 13/87 to 0/100; 2.1−2.6 min, 0/100; 2.6−2.7 min, 0/100 to 65/35; 2.7−3.2 min, 65/35 at a column temperature of 40 °C. The first 0.8 min of the column effluent was discarded to prevent

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Table 2. Polymer Composition and Characteristics of Tacrolimus-Loaded Monospheres (Data Acquired by Coulter Counter)

	polymer ratio (%)			tacrolimus monosphere characteristics		
formulation #	20[PDLA–PEG_{1000}]–80[PLLA]	10[PDLA–PEG_{1000}]–90[PLLA]	PEG content (wt %)	average particle size (μm)	particle size CV (%)	loading (EE %)
F1	100	0	10	30.1	14.2	79.3
F2	80 (18)	20	9	28.9	10.4	70.6
F3	70 (17)	30	8.5	29.2	10.9	72.0
F4	60 (16)	40	8	33.4	11.5	72.7
F5	50 (15)	50	7.5	32.0	12.1	70.0
F6	0	100	5	33.2	16.4	80.1

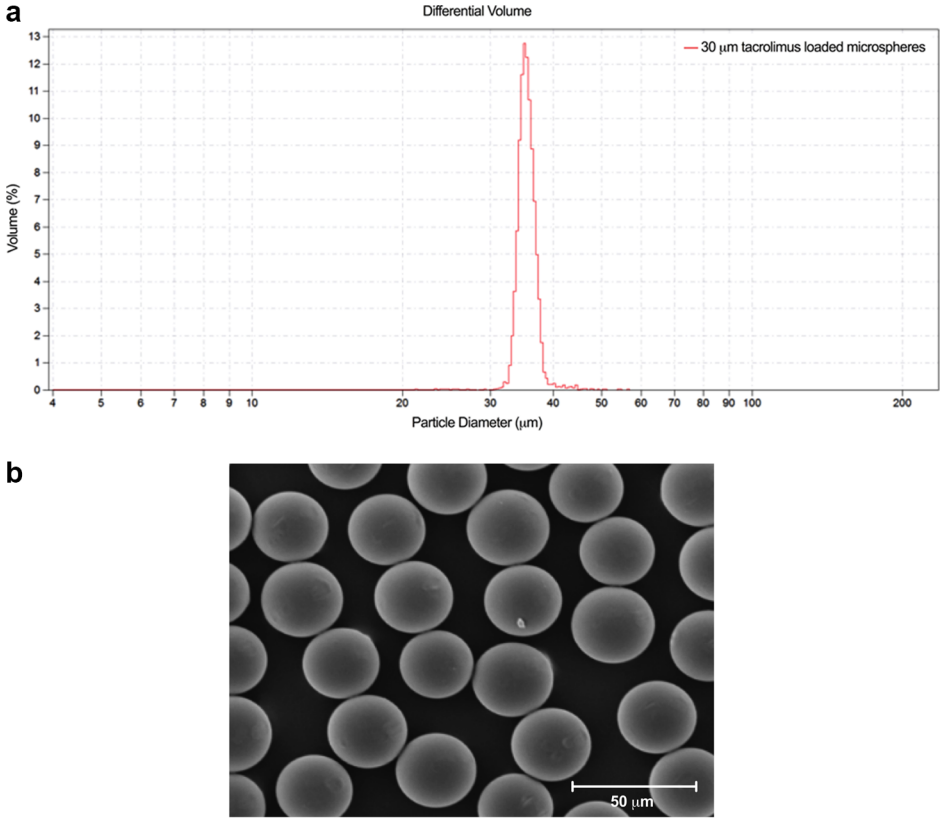

Figure 2. (a) Particle size distribution of tacrolimus loaded monospheres. (b) SEM image of tacrolimus loaded monospheres.

nonvolatile components to enter the ionization interface, where after the effluent was introduced by an electospray ionization (EPI) interface (Sciex, Toronto, ON) into a 4000 Q TRAP mass spectrometer. For maximal sensitivity and for linearity of the response, the mass spectrometer was operated in multiple-reaction monitoring (MRM) mode at unit mass resolution. Peaks were identified by comparison of retention time and mass spectra of standards. For each component, one ion transition was monitored, ascomycin: 809.5 → 756.5 (collision energy: 21 V), and FK-506:821.5 → 768.4 (collision energy: 26 V). The following MS parameters were used: curtain gas: 10 psi; ion spay voltage: 5500 V; source temperature: 360 °C; gas flow 1:50 psi; gas flow 2:40 psi; decluster potential: 80 V and entrance potential: 10 V. Data were analyzed with Analyst software version 1.6.2 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). FK-506 peak areas were corrected for the ascomycin recovery, and concentrations were calculated using a FK-506 reference line ranging from 0.5 ng to 1000 ng/mL which was linear in this range (r = 0.9997).

A second portion of synovial fluid was processed for white blood cell (WBC) count and total protein measurement, which are known to be elevated during inflammatory responses.[49] The remaining volume was centrifuged, and the supernatant was stored at −80 °C until further analysis. Analyses consisted of measuring the glycosaminoglycan (GAG) concentrations using a modified 1,9-dimethylmethylene blue dye-binding assay used previously in our laboratory[50] and of the C2C epitope concentration, which are markers for proteoglycan and collagen damage, respectively.[51] All values were compared to saline injected joints as well as to the response after intra-articular injections of unloaded monospheres to check for possible anti-inflammatory action of the locally released tacrolimus.

Statistical Analysis. Differences in μCT data between the tacrolimus loaded microsphere-injected knees and the contralateral knees were assessed using type-1, two-tailed, paired t tests. For the measured weight gain, differences between t = 0 and 6 weeks were analyzed for each independent group using a paired t test. For the difference in weight gain (6 w − t = 0) between the different groups, a one-way ANOVA with a posthoc Bonferroni correction was used. For all tests, p values <0.05 were considered statistically significant. All data are presented as mean ± SD.

RESULTS AND DISCUSSION

Preparation and in Vitro Characterization of Tacrolimus Monospheres. The different tacrolimus loaded microsphere formulations were obtained in a high yield (>75%) with average particle size in a narrow range from 28−33 μm (see

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Table 2 and Figure 2a) and smooth and nonporous surface (Figure 2b). An encapsulation efficacy of ≥70% tacrolimus was obtained for the different formulations prepared.

Analysis of the in vitro release of these different formulations showed that variations in polymer composition allowed tuning the release kinetics of tacrolimus over a period of 4−6 weeks (Figure 3). Small variations in the content of the relatively

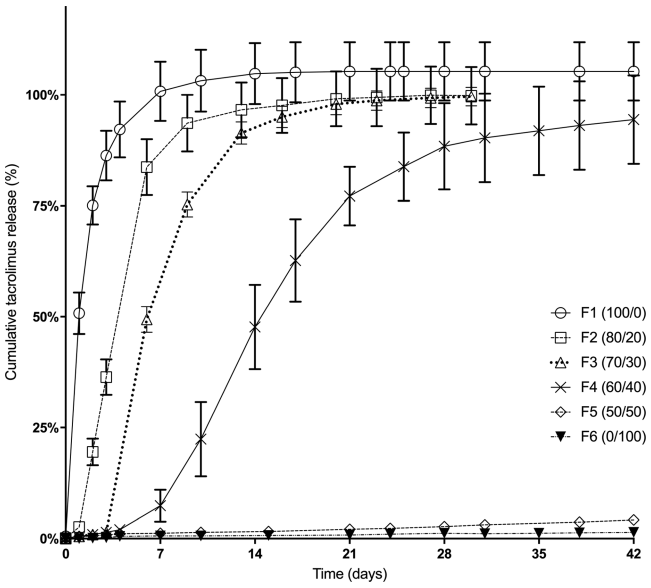

Figure 3. In vitro release of different tacrolimus loaded *xx*[PDLA−PEG_{1000}]−*yy*[PLLA] based monospheres (n = 3 per time point). Composition of each monosphere formulation is shown in Table 2.

hydrophilic amorphous block, (PDLA−PEG_{1000}) of the multiblock copolymer had an impact on the release kinetics of the tacrolimus loaded monospheres. Tacrolimus was released faster when there was an increase in this hydrophilic block, most likely due to greater swelling of the matrix caused by an overall increase of PEG content within the polymer matrix, which facilitates diffusion of tacrolimus out of the polymer.[35] Tacrolimus is most probably released by a combination of diffusional and polymer degradation mechanisms as well as limited solubility of tacrolimus. Formulation F1 with 10 PEG % (see Table 2) showed a fast and complete release of tacrolimus within 7 days. In contrast, formulation F6 0/100 (having the lowest PEG content) was extremely stable and showed minor release (≤2.0%) during 42 days. Interestingly, blends of 20[PDLA−PEG_{1000}]−80[PLLA] and 10[PDLA−PEG_{1000}]−90-[PLLA] in different ratios (Table 2) allowed control of the lag phase time and release rate, as shown in Figure 3. Monospheres based on the more hydrophilic 20[PDLA−PEG_{1000}]−80-[PLLA] polymer with a higher PEG-content showed a faster release, probably because these particles swell more in buffer, facilitating the diffusion of tacrolimus to the release medium and the influx of water to the matrix that promotes its degradation and pore formation when PEG leaves the matrix.[52−54] Pore formation likely occurred in all formulations; however, we do not have data about the pore size of the monospheres during drug release and cannot exclude the fact that the size of the pores and the rate of their formation would differ between formulations based on the polymer composition. Therefore, differences in tacrolimus release rate between formulations could also be influenced by pore formation.

There is no literature about the in vivo use of tacrolimus for the treatment of osteoarthritis; therefore, the most ideal release pattern is not known. Most articles focusing on the treatment of osteoarthritis via intra-articular drug delivery systems aim at a sustained release of several weeks[33] because shorter release periods would necessitate multiple injections, which increases the risk of infection. Based on this information, formulation F4 showed the most suitable profile because it presented a continuous in vitro release of tacrolimus for over 5 weeks without showing a burst release. Formulations F1, F2, and F3 showed drug releases that were much faster than our aimed release period, with >75% being released within the first 10 days. Whereas formulations F5 and F6 were releasing the drug at a very slow rate, with only <5% of the drug being released after 42 days; this would lead to minimal local drug concentrations, which would very likely not be in the therapeutic range. Therefore, we chose to work with formulation F4 (blend of ratio of 60:40 of 20[PDLA−PEG_{1000}]−80[PLLA] over 10[PDLA−PEG_{1000}]−90[PLLA]). Sixty percent of a polymer containing 20% [PDLA−PEG_{1000}] and 40% of a polymer containing 10% [PDLA−PEG_{1000}] will give 16% of this multiblock in the final blend. Hence, a polymer presenting this ratio (16[PDLA−PEG_{1000}]−84[PLLA]) was synthesized and used to produce microspheres suitable for in vivo evaluation. The properties of this polymer are summarized in Table 1. The decision to synthesize a new polymer instead of using a blend of the two was made to facilitate easier manufacturing and up-scaling for clinical translation.

Three batches with the same composition were prepared under best clean conditions for in vivo evaluation. They had a residual dichloromethane content of <600 ppm and very low endotoxins content <0.01 EU/mg, complying with the acceptance limits for parenteral administration.[55] Monospheres with smooth and nonporous surfaces as observed by SEM (Figure 4) and with an average diameter between 31.8 and 33.8

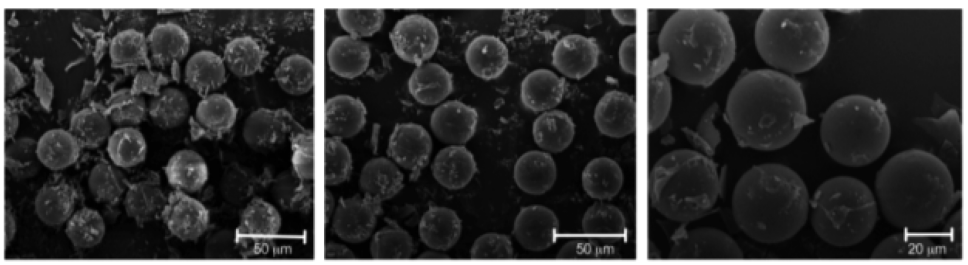

Figure 4. SEM picture of 30 μm 16[PDLA−PEG_{1000}]−84[PLLA] monospheres. This picture shows the size uniformity of the batches. Mannitol was used during the washing and before freezing of the monospheres, explaining the irregularities seen in the picture. The surface of the monospheres appears smooth and without pores.

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ACS Biomater. Sci. Eng. 2018, 4, 2390−2403

μm (CV 13−21%) were obtained. The size uniformity and absence of very small and very big microspheres represent additional advantages because possible particle-induced immunoactivation is avoided.[56−58] Moreover, more reproducible and predictable in vivo pharmacokinetics are obtained,[56,59] and injection is less painful due to the possibility to use smaller needles. The drug encapsulation efficiency was >85%, and the release kinetics was comparable to the release kinetics observed for monospheres of the 60:40 blend of 20[PDLA−PEG_{1000}]−80[PLLA] and 10[PDLA−PEG_{1000}]−90[PLLA] (Figure 5),

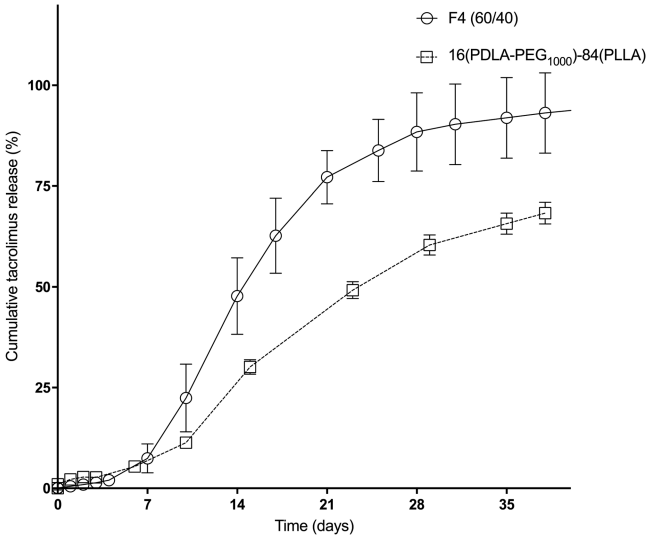

Figure 5. In vitro release (IVR) kinetics of 7.4−7.9 wt % tacrolimus loaded monopsheres composed of 16[PDLA−PEG_{1000}]−84[PLLA] used for in vivo studies and the 60:40 20[PDLA−PEG_{1000}]−80[PLLA]:10[PDLA−PEG_{1000}]−90[PLLA].

showing a lag phase of 1 week followed by a sustained release for over a period of 5 weeks. This lag phase could have an impact on the efficacy of tacrolimus in the joint. Nonetheless, it is known that differences between in vitro and in vivo release kinetics exist for polymer-based formulations due to the in vivo environment.[60] As a consequence of the presence of enzymes as well as cellular responses in the tissues, changes of polymer degradation and overall drug release (e.g., absence of lag phase) could be expected.[58,61] We recently showed in an article regarding PCLA−PEG-PCLA that after in vivo injection, the polymer molecular weight decreased, and composition determined by ^{1}H NMR significantly changed over time during in vivo degradation. This indicates chain scission at the PEG−PCLA bonds and dissolution of the PEG-rich chains due to enzymatic degradation.[62] Because of expected differences between in vitro and in vivo release, an in vivo evaluation is required to assess the efficacy of the sustained release formulation. Consequently, further evaluation in two animal models was undertaken to evaluate the in vivo performance of the developed monospheres.

In Vivo Tolerance Study in Rats. Neither local toxic responses (e.g., changed locomotion, joint redness/swelling) nor systemic effects occurred during the 12-week observation period after intra-articular injection.

Oral treatment with tacrolimus (Figure 6: oral tacrolimus (OA)) led to a stagnation of growth (nonsignificant difference between weight at t_0 and after 6 weeks), whereas the intra-articular administration of tacrolimus loaded monospheres

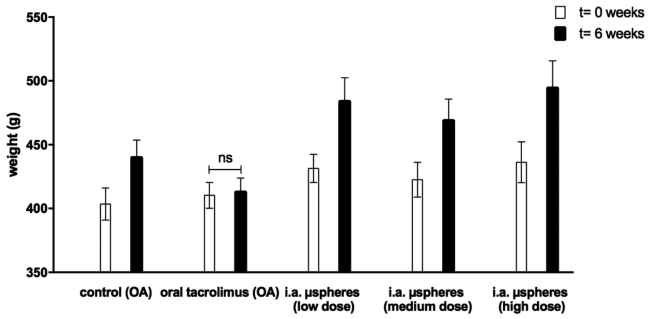

Figure 6. Weight gain of rats injected with intra-articular monospheres containing a low/medium or high dose of tacrolimus. As a control, untreated rats with induced OA and animals treated with daily oral tacrolimus administration (also induced OA) are shown. As can be seen, the oral treatment with tacrolimus led to a stagnation of growth (nonsignificant difference between weight at t_0 and after 6 weeks), whereas the intra-articular administration of tacrolimus loaded monospheres (by which in comparison to the oral administration much higher intra-articular concentrations could be achieved) did not hamper growth of the animals.

(Figure 6: i.a. μ-spheres) did not hamper growth of the animals, indicating the absence of systemic toxic effects when administering the drug locally. A nonsignificant (only significant for highest loading) difference between growth of both OA induced groups (control (OA) and oral tacrolimus (OA)) versus the healthy animals injected with intra-articular tacrolimus-loaded monospheres was seen as well, which is likely due to the retarded growth of animals suffering from pain caused by OA.

Based on several studies in which oral bioavailability of tacrolimus was measured in rats,[63−65] the oral dosage of 3.2 mg/kg/day would lead to a maximal systemic plasma concentration of 50 ng/mL, which in a steady state situation could maximally lead to 50 ng/mL intra-articularly. Those studies all showed that after oral administration, the plasma peak concentration occurs within the first 2 h after administration, followed by a quick decrease to below detection level (thus <0.3−1.0 ng/mL) within the first 24 h. The total tacrolimus loads of the intra-articularly administered microspheres were respectively 220 μg (low), 440 μg (medium), and 880 μg (high). These loadings should, after local release, likely lead to very high intra-articular concentrations compared to what would be feasible with oral administration with no systemic side effects (as shown in this study). However, as sequential synovial fluid sampling is impossible in rats without greatly influencing the pharmacokinetics of the drug delivery system, we were not able to generate data regarding this topic. Therefore, we did these measurements in horses, as described in In Vivo Tacrolimus Release Kinetics.

Intra-Articular Retention of Monospheres and Tacrolimus in Rats. In a previous study, it has been shown that unloaded monospheres retain within the knee joint for at least three months.[35] Here, we examined the period that drug loaded monospheres retained in the joint and whether sustained intra-articular presence of the drug could be demonstrated. In samples of the surrounding tissue, including the synovium harvested at the 12-week end-point, SEM imaging revealed the presence of monospheres (see Figure 7a). Also, tacrolimus could be detected in four out of six samples by UV-HPLC, as shown by the overlapping chromatogram (Figure 7b). The sustained release of at least 12 weeks is longer than what would

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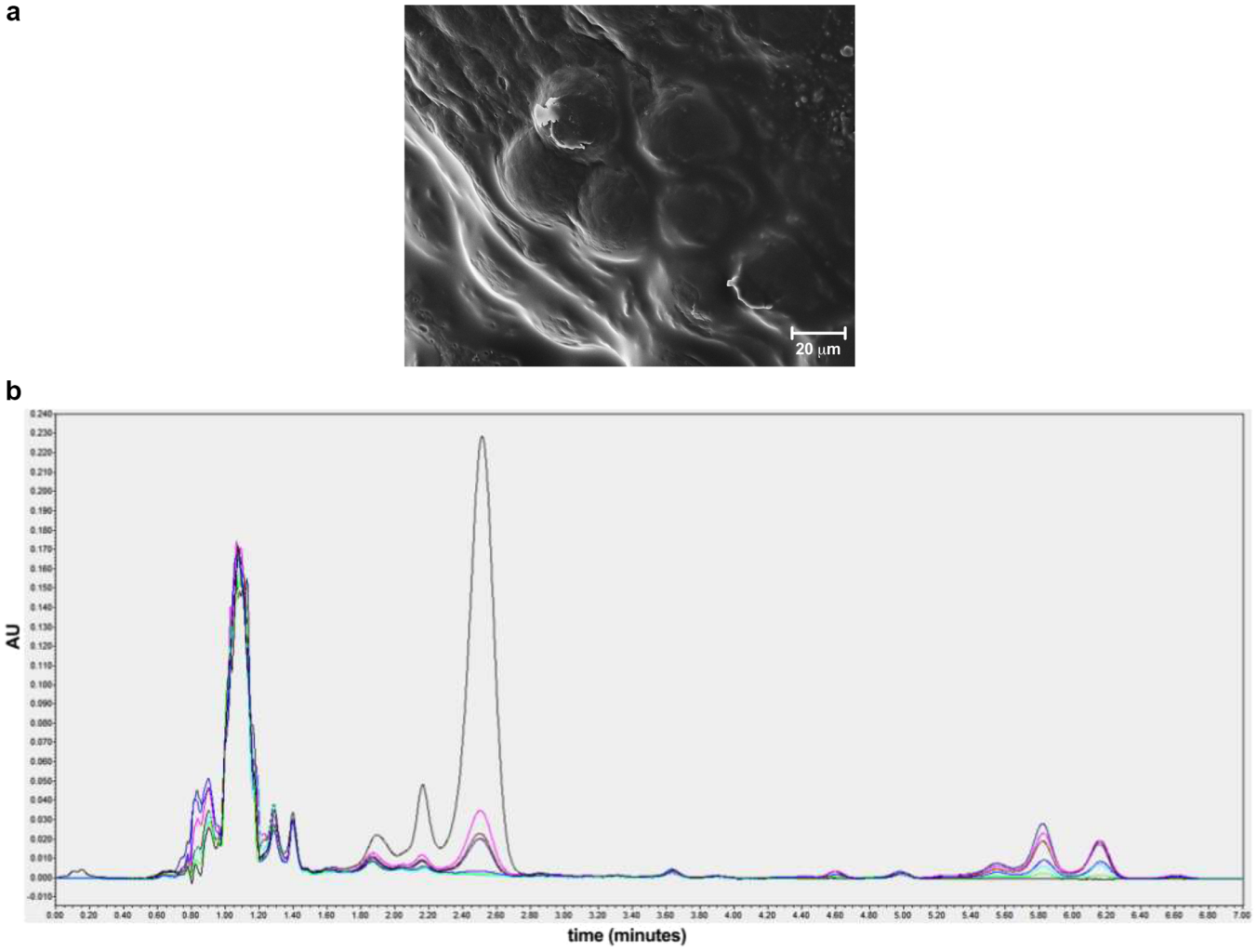

Figure 7. (a) SEM image of tissue surrounding the knee joint of a rat injected with tacrolimus loaded monospheres 12 weeks after injection. Monospheres are still present in the joint and are partly taken up by the surrounding tissue. (b) Overlap chromatograms of tacrolimus in retrieved tissue 12 weeks postinjection. The black line (highest curve) represents a sample of pure FK-506; all other lines are samples derived from the FK506 injected rat joints. Four out of the six harvested knees showed presence of tacrolimus; in the joints of two rats (green and blue line), no tacrolimus was detected.

be expected from the in vitro release data (Figure 5). However, it is a well-known phenomenon for polymer-based sustained release formulations that in vivo and in vitro releases may differ significantly due to differences in the environmental conditions.[60] Anyhow, a longer release is a favorable feature for a local drug delivery system. The inability to detect tacrolimus in two of the six samples (1 low dosage, 1 medium dosage) may be due to either full release or harvesting an insufficient amount of tissue or tissue from a wrong area because only a part of the tissue surrounding the joint was used for this analysis.

Intra-Articular Biocompatibility in Rats. *Subchondral Plate Thickness.* When longitudinally comparing healthy control knees (saline injected) to tacrolimus loaded monospheres injected knees, normal gain in subchondral plate thickness of both the lateral and medial compartment was seen for all groups, which is related to normal growth of the rat (Figure 8). In contrast, we also correlated them to values found for osteoarthritic knee joints of rats (of the same strain, sex, and age) from a previous study.[46] In these rats, the subchondral bone plate thickness of the medial tibia compartment in OA knees was slightly reduced after 6 and 12 weeks of follow up, while for the lateral compartment, the thickness clearly increased over time compared to healthy control knees (saline injected).

Osteophytes are bony changes associated with osteoarthritis,[66,67] and therefore μCT scans were also inspected for these entities. A dose-dependent effect was seen, with no osteophyte formation for the lowest dose of tacrolimus loaded monospheres and some osteophyte formation in all knees injected with the highest dosage (Figure 10, see μCT in high dose row). All osteophytes were very small and not visible during histological evaluation of the joints.

Correlation of Clinical Outcome with Bone Changes. While there was no significant difference between healthy (saline injected) and tacrolimus loaded microsphere injected knees, there seemed to be a dose-dependent trend toward less increase of subchondral plate thickness for the tacrolimus loaded microsphere injected knees. This is in agreement with an earlier study in which we found that orally administered tacrolimus in an OA model led to a reduction of sclerotic bone formation, e.g. less thickening of the subchondral plate, when compared to untreated OA controls.[68] Tacrolimus is known to act directly on osteoclast and osteoblast activity, which can lead to bone changes. Indeed, tacrolimus was shown to be able to

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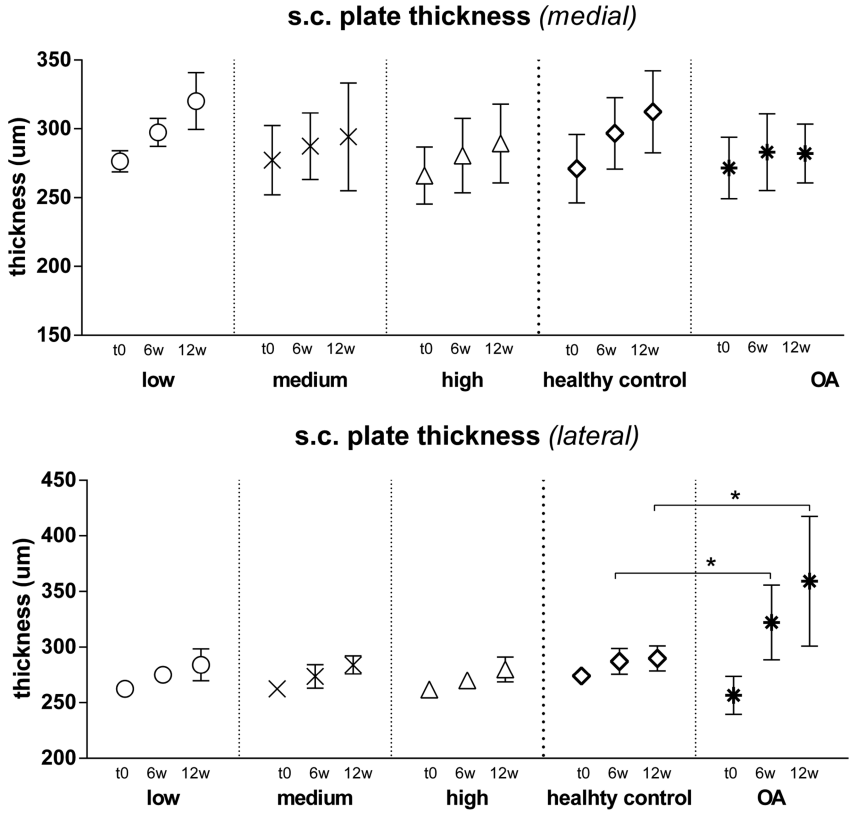

Figure 8. Subchondral plate thickness of knee joints injected with saline (healthy control) or low/medium or high dose of tacrolimus loaded monospheres. Longitudinal in vivo scanning was performed at t_0 (before injection), 6 weeks, and 12 weeks after injection. As a reference, values of OA knees are also shown. Generalized estimating equations were used to calculate differences between groups and over time; data is shown as mean ± SD, $p < 0.05$ (*) is considered significant.

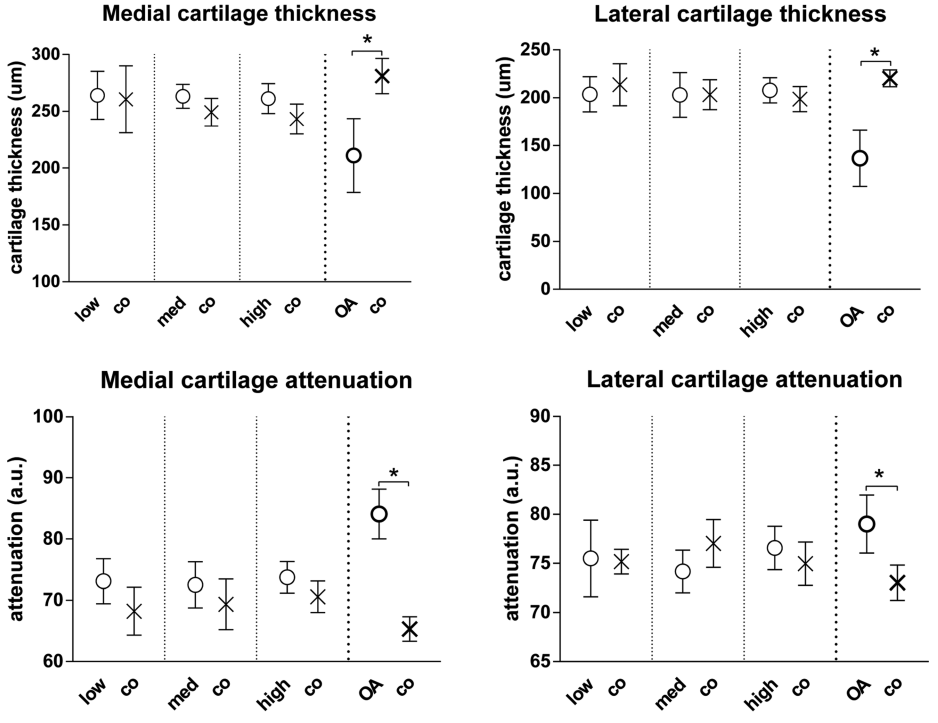

Figure 9. EPIC-μCT scanning to determine cartilage thickness (μm) and attenuation (arbitrary units) of knee joints injected with a low, medium, or high dose of tacrolimus loaded monospheres 12 weeks after injection vs contralateral control knees (co). As a reference, values of OA knees are also shown. Data are shown as mean ± SD; $p < 0.05$ is considered significant. *: significant differences were found for all measurements of the OA knees when compared to the contralateral control knees and when compared to all doses of tacrolimus loaded monospheres.

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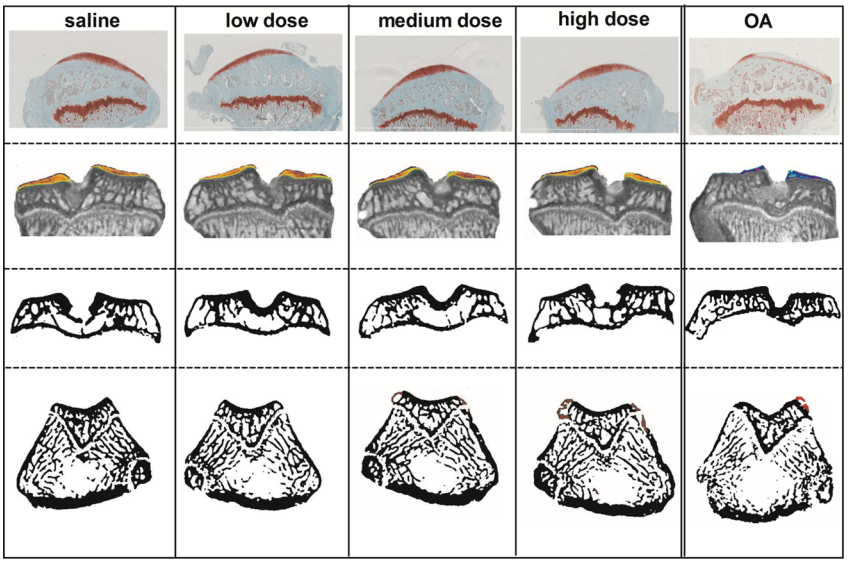

Figure 10. Histology and CT images of saline injected knees compared with low, medium, and high doses of intra-articular injected tacrolimus loaded microspheres. Histologically and based on EPIC scanning, no differences are seen in cartilage thickness and quality. Also, for subchondral bone thickness, no differences are seen between saline injected or tacrolimus microsphere injected knees. On the right, as a comparison, images of an osteoarthritic joint are shown. Both cartilage quality and quantity are affected. Also, a difference (be it subtle) can be seen in terms of subchondral bone thickness of the lateral compartment. The lowest row of this figure shows the osteophyte formation, which is highlighted in red. No osteophyte formation is seen for the knees injected with saline and the low dose, whereas small osteophytes can be seen for the medium and high dose of tacrolimus microspheres and the osteoarthritic knee.

induce loss of bone density through antianabolic effects on osteoblastic cells[69] as well as by inhibiting osteoblast differentiation.[70,71] In contrast, there are studies suggesting the opposite, where tacrolimus treatment increased bone formation in patients with rheumatoid arthritis.[72] Further investigation of this topic is needed to clarify the direct actions of tacrolimus on bone, but with the doses used in our current study, these actions are not relevant and were of no concern.

The formation of osteophytes is definitely not a desired effect and suggests that the medium and higher dosages of tacrolimus are probably not the best to be used in the treatment of osteoarthritis. It does, however, in combination with the fact that the osteophyte formation happened in a dose-dependent fashion, show that the monospheres are indeed a good drug delivery system and that the intra-articular administration led to local dosages (high enough to facilitate an effect) with low systemic concentrations.

Using EPIC-μCT (Figure 9), we evaluated cartilage proteoglycan content and its quality. Knee joints injected with a low, medium, or high dose of tacrolimus loaded monospheres showed no differences with respect to either attenuation (cartilage quality) or thickness (cartilage quantity) when compared to the contralateral control knee. All values were within the range of what was found for healthy cartilage in a previous study using the same technique.[73] When comparing our results to the knees in which osteoarthritis was induced in this same previously conducted study,[73] we see that significantly ($p < 0.01$) worse attenuation and cartilage thickness were found.

The histological images correlated nicely to the EPIC-μCT images (see Figure 10, rows 1 and 2). No differences in Saf-O staining were found between tacrolimus loaded monospheres injected knees (low, medium and high dosage) and controls. Morphologically, the cartilage appeared healthy in all groups. These results, together with the earlier shown harmlessness of the intra-articular injection of unloaded monospheres,[35] pave the way to the possible exploration of the effectiveness of the intra-articular delivery of tacrolimus loaded monospheres for the treatment of osteoarthritis. However, the caveat with respect to osteophyte formation (see earlier) suggests the use of even lower dosages of tacrolimus in future experiments.

In Vivo Tolerance in Horses. There were no signs of lameness in either the saline injected or the tacrolimus loaded monospheres injected joints at any of the time points, indicating that the presence of these monospheres did not have any negative clinical effect on locomotion. In vivo tolerance in horses can hence be classified as excellent.

In Vivo Tacrolimus Release Kinetics. From the local synovial fluid and systemic plasma analyses in the three horses, we demonstrate that tacrolimus was indeed being released after the tacrolimus loaded monospheres were injected intra-articularly (Figure 11). Horse 2 was probably not injected properly into the joint because tacrolimus was not detected from 167 h postinjection on. In the other two horses, a sustained local release (in the synovial fluid) was seen during the entire follow-up period of 4 weeks (672 h). The three horses showed measurable systemic concentrations for the first 24 h postinjection that were substantially lower compared to the intra-articular concentrations at those time points, indicating that most of the tacrolimus was released locally. From 72 h after injection until the end of the experiment, systemic concentrations were below detection limits (<1.5 ng/mL). Clinically, this was indirectly confirmed by the absence of any systemic side-effect. In vivo, we did not observe the lag phase, which was observed during the in vitro release (Figure 5). As stated before, differences between in vitro and in vivo release kinetics are a well-known phenomenon. Contributing environmental factors are for instance the different composition of synovial fluid compared to PBS, which could influence drug solubility, the presence of proteins/enzymes that may change polymer degradation properties, and volume available to dissolve tacrolimus (sink conditions).[60,61] To minimize the

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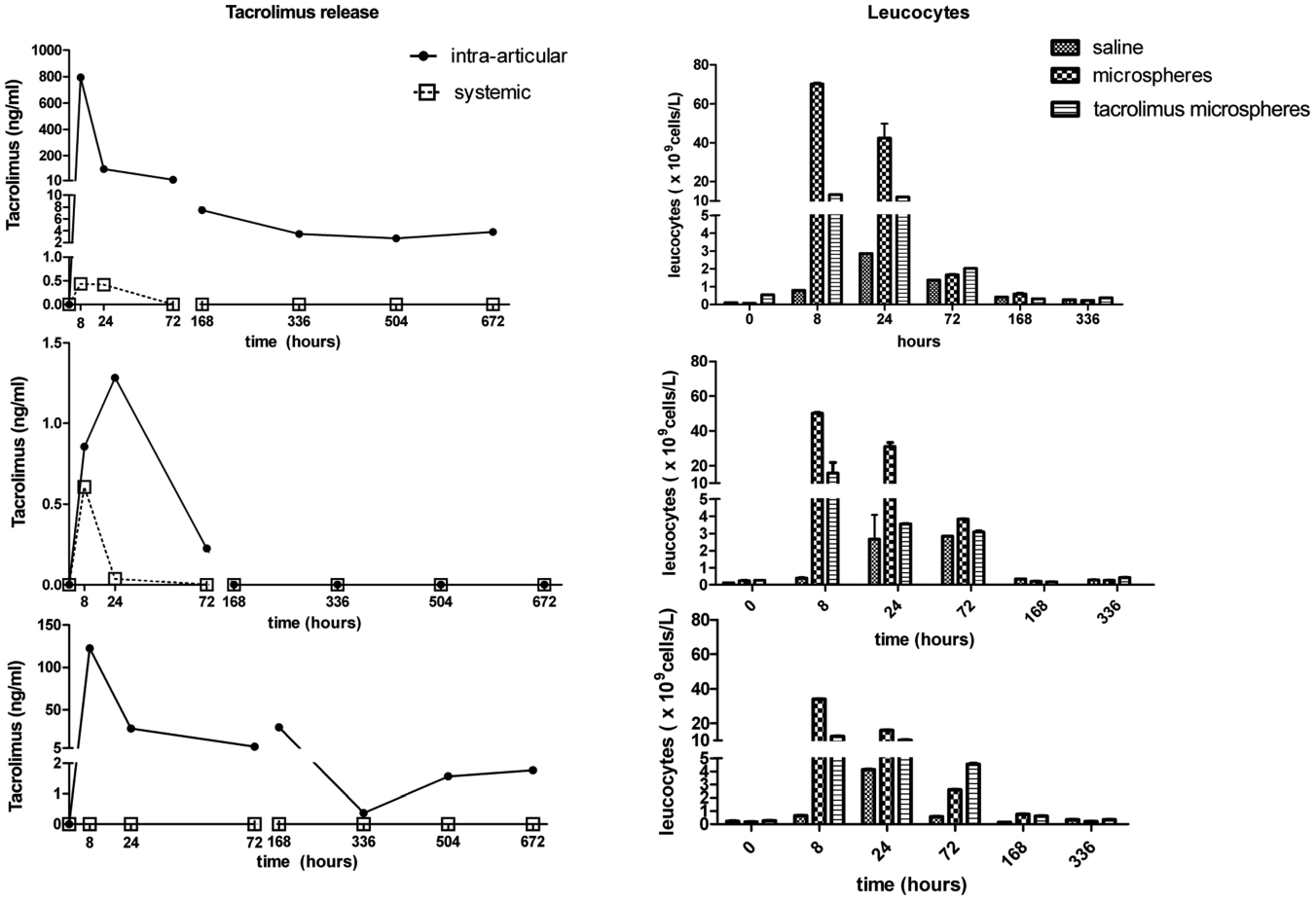

Figure 11. Tacrolimus concentrations in synovial fluid (intra-articular) and plasma (systemic) after intra-articular administration of tacrolimus loaded monospheres over time per individual horse (left). Right: for the same horse, the amount of intra-articular leucocytes, a measure of inflammatory reaction, for joints injected with saline (negative control), unloaded monospheres (positive control), and tacrolimus loaded monospheres.

effect of tacrolimus solubility on the release kinetics, in vitro release experiments were performed under sink conditions. In vivo, however, solubility might play a role in drug release due to the limited fluid volume and less predictable replenishment conditions (synovial fluid turnover). Synovial fluid turnover for healthy joints takes around 1 h.[74] There is no literature yet about the solubility of tacrolimus in synovial fluid. Clearance of other substances from the synovial fluid have been tested and vary from 2 h for proteins to 5 h for NSAIDS.[33] Based on this, we hypothesize that synovial fluid turn-over would not be a key factor in the in vitro/in vivo differences in release pattern because clearance of intra-articular drugs appears to happen in a matter of hours, whereas our in vitro conditions were refreshed no more than once every 24 h. Restricted amount of synovial fluid within the joint, however (~50 μL for rat knees, 2−5 mL for talocrural horse joints), could have a significant influence on drug solubility. Although the effects of synovial fluid volume and turnover are intertwined, and rapid turnover could diminish the effect of restricted volume, the amount of synovial fluid could have played an important role in the differences between in vitro and in vivo tacrolimus release.

In Vivo Bioactivity of Released Tacrolimus. After injection of the unloaded monospheres in the horse joints, a transient inflammatory reaction occurred (increased WBC and total protein in the synovial fluid), which lasted for 72 h, as reported in our previous study.[35] This transient inflammatory reaction was also observed after the intra-articular injection of the same amount of tacrolimus loaded monospheres when compared to joints injected with saline alone. Macrophages within the synovial lining are activated by the presence of particles as well as by the (minimal) trauma of injecting the joint. These macrophages, whose primary role is to maintain the joint's homeostasis, attract inflammatory cells (among which are WBC).[75] A short period of leukocytosis (elevated leucocytes) is part of a healthy immune response. However, a chronic inflammatory state would eventually lead to a disruption of the joint's homeostasis and might cause cartilage and bone damage, leading to osteoarthritis.[76]

In a previous study, leucocyte count in synovial fluid of horses was measured after injection of different actives, including Hyanote.[77] Injection of Hyonate led to increased leucocyte counts (>10^{10}/L) at 8 and 24 h postinjection, which returned to normal at 72 h postinjection. Because Hyonate is clinically approved for intra-articular use in both humans and horses, we conclude that mild inflammatory reaction seen in our current study is not a harmful one. Moreover, the inflammatory reactions of all joints injected with tacrolimus loaded monospheres were much less than for the joints injected with unloaded monospheres, indicating that the induced transient inflammatory reaction was less prominent (the same trend was seen for total protein count, see Supporting Information, Figure S.3). At t = 8 h, for all horses the leucocyte

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count of the tacrolimus loaded microspheres injected joints was less than 50% of the leucocyte count for joints injected with unloaded microspheres. This reflects intra-articular release and a local anti-inflammatory action of tacrolimus.[38] In all horses, tacrolimus release lasted for at least the entire duration of the transient inflammatory reaction (72 h) and showed the potential of this medication to act on intra-articular inflammation, a hallmark of many joint diseases. The prolonged release as seen in two of the three horses shows the potential of the platform-active combination to influence any long-term inflammatory status of a joint, as for instance seen in OA.

Intra-Articular Biocompatibility in Horses. Chronic joint inflammation seems to play a major role in the structural changes of synovium, bone, and cartilage seen in osteoarthritis, mainly through enzymes, growth factors, and cytokines produced by activated macrophages.[11,78] It is therefore important to determine whether the mild transient inflammatory reaction in the first two weeks caused by the injected monospheres had any negative effect on the joints. Recently, we showed that the administration of unloaded microspheres in the horse joint did not have negative effects on the cartilage.[35] The intra-articular doses of tacrolimus would also not be likely to have a negative effect on the cartilage because it was shown previously in our lab that tacrolimus, with doses up to 1000 ng/mL, did not affect cell proliferation and viability in vitro in human articular chondrocytes.[15] Moreover, a dose of 500 ng/mL was tested more extensively and shown to have anticatabolic and even anabolic effects on chondrocytes.[16]

Additionally, we analyzed GAG release and collagenase-cleavage fragments of type II collagen (C2C assay) in the synovial fluid samples of the horses, which can be considered indicative for cartilage breakdown. Neither of these two markers was elevated, and the GAG marker showed even a trend toward less GAG release in the tacrolimus-treated joints compared to the unloaded microsphere treated joints (see Supporting Information, Figure S.3). This correlates well with the positive effect of the tacrolimus loaded monospheres on the inflammatory reaction postinjection, and it could be hypothesized that the decrease of GAG release might be linked to this phenomenon.

It can be concluded that no negative changes in the cartilage occurred due to the injection of tacrolimus loaded monospheres, which is in line with the biocompatibility results in the rats.

CONCLUSIONS

Poly(D-L-lactide-PEG)-*b*-poly(L-lactide) multiblock copolymers provide a suitable platform for the development of injectable tacrolimus-loaded monospheres for intra-articular controlled drug delivery in the treatment of joint diseases such as osteoarthritis. Tacrolimus-loaded monospheres were shown to be safe for intra-articular use while being retained in the synovial fluid and synovial lining of the rat knee and in the equine carpal joint for at least four weeks. Most importantly, local sustained drug release was achieved by the monospheres because the drug was measurable in high concentrations in the joint without measurable systemic concentrations or toxic side effects. Effects of local tacrolimus delivery on peri-articular bony osteophytes were seen in a dose-dependent manner, indicating that indeed local release can be tuned and thereby achieving the desired local concentrations of an incorporated drug based on the therapeutic window and specific needs in different diseases. The locally released tacrolimus partially counteracted intra-articular inflammation, an action that is potentially beneficial in the treatment of arthritides. Not only have we shown tacrolimus to be a very interesting drug for further investigation in the field of osteoarthritis, but we also have shown monospheres to be excellent drug carriers for intra-articular delivery of potentially interesting drugs in general.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.8b00116.

Figure S.1: Calibration curve of tacrolimus quantification (HPLC); Figure S.2: Results of testing different extraction solvents for tacrolimus; Figure S.3: Synovial fluid measurements in horses after i.a. injection with tacrolimus (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel.: 31 10 7043463; Fax: 31 10 7044690; E-mail: m.sandker@erasmusmc.nl.

ORCID

Maria J. Sandker: 0000-0001-7697-6438
Wim E. Hennink: 0000-0002-5750-714X
Harrie Weinans: 0000-0002-2275-6170

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Pelletier, J. P.; Martel-Pelletier, J.; Abramson, S. B. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum.* **2001**, *44* (6), 1237−1247.

(2) Goldring, M. B.; Berenbaum, F. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. *Clin. Orthop. Relat. Res.* **2004**, *427*, S37−S46.

(3) McAlindon, T. E.; Bannuru, R. R.; Sullivan, M. C.; Arden, N. K.; Berenbaum, F.; Bierma-Zeinstra, S. M.; Hawker, G. A.; Henrotin, Y.; Hunter, D. J.; Kawaguchi, H.; Kwoh, K.; Lohmander, S.; Rannou, F.; Roos, E. M.; Underwood, M. OARSI guidelines for the non-surgical management of knee osteoarthritis. *Osteoarthritis Cartilage* **2014**, *22* (3), 363−388.

(4) Hooks, M. A. Tacrolimus, a new immunosuppressant–a review of the literature. *Ann. Pharmacother.* **1994**, *28* (4), 501−511.

(5) Kelly, P. A.; Burckart, G. J.; Venkataramanan, R. Tacrolimus: a new immunosuppressive agent. *Am. J. Health Syst. Pharm.* **1995**, *52* (14), 1521−1535.

(6) Lee, Y. H.; Woo, J. H.; Choi, S. J.; Ji, J. D.; Bae, S. C.; Song, G. G. Tacrolimus for the treatment of active rheumatoid arthritis: a systematic review and meta-analysis of randomized controlled trials. *Scand. J. Rheumatol.* **2010**, *39* (4), 271−278.

(7) van Leeuwen, M. A.; Westra, J.; Limburg, P. C.; van Riel, P. L.; van Rijswijk, M. H. Interleukin-6 in relation to other proinflammatory cytokines, chemotactic activity and neutrophil activation in rheumatoid synovial fluid. *Ann. Rheum. Dis.* **1995**, *54* (1), 33−8.

DOI: 10.1021/acsbiomaterials.8b00116
ACS Biomater. Sci. Eng. 2018, 4, 2390−2403

(8) Fernandes, J. C.; Martel-Pelletier, J.; Pelletier, J. P. The role of cytokines in osteoarthritis pathophysiology. *Biorheology* **2002**, *39* (1−2), 237−246.
(9) Goldring, M. B.; Otero, M. Inflammation in osteoarthritis. *Curr. Opin. Rheumatol.* **2011**, *23* (5), 471−478.
(10) Sellam, J.; Berenbaum, F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat. Rev. Rheumatol.* **2010**, *6* (11), 625−35.
(11) Lopes, E. B. P.; Filiberti, A.; Husain, S. A.; Humphrey, M. B. Immune Contributions to Osteoarthritis. *Curr. Osteoporos Rep* **2017**, *15* (6), 593−600.
(12) Sakuma, S.; Kato, Y.; Nishigaki, F.; Sasakawa, T.; Magari, K.; Miyata, S.; Ohkubo, Y.; Goto, T. FK506 potently inhibits T cell activation induced TNF-alpha and IL-1beta production in vitro by human peripheral blood mononuclear cells. *Br. J. Pharmacol.* **2000**, *130* (7), 1655−1663.
(13) Yoo, S. A.; Park, B. H.; Yoon, H. J.; Lee, J. Y.; Song, J. H.; Kim, H. A.; Cho, C. S.; Kim, W. U. Calcineurin modulates the catabolic and anabolic activity of chondrocytes and participates in the progression of experimental osteoarthritis. *Arthritis Rheum.* **2007**, *56* (7), 2299−2311.
(14) Fuseler, J. W.; Hearth-Holmes, M.; Grisham, M. B.; Kang, D.; Laroux, F. S.; Wolf, R. E. FK506 attenuates developing and established joint inflammation and suppresses interleukin 6 and nitric oxide expression in bacterial cell wall induced polyarthritis. *J. Rheumatol* **2000**, *27* (1), 190−199.
(15) van der Windt, A. E.; Jahr, H.; Farrell, E.; Verhaar, J. A.; Weinans, H.; van Osch, G. J. Calcineurin inhibitors promote chondrogenic marker expression of dedifferentiated human adult chondrocytes via stimulation of endogenous TGFbeta1 production. *Tissue Eng., Part A* **2010**, *16* (1), 1−10.
(16) van der Windt, A. E.; Haak, E.; Kops, N.; Verhaar, J. A.; Weinans, H.; Jahr, H. Inhibiting calcineurin activity under physiologic tonicity elevates anabolic but suppresses catabolic chondrocyte markers. *Arthritis Rheum.* **2012**, *64* (6), 1929−1939.
(17) Magari, K.; Nishigaki, F.; Sasakawa, T.; Ogawa, T.; Miyata, S.; Ohkubo, Y.; Mutoh, S.; Goto, T. Anti-arthritic properties of FK506 on collagen-induced arthritis in rats. *Inflammation Res.* **2003**, *52* (12), 524−529.
(18) Sasakawa, T.; Sasakawa, Y.; Ohkubo, Y.; Mutoh, S. FK506 ameliorates spontaneous locomotor activity in collagen-induced arthritis: implication of distinct effect from suppression of inflammation. *Int. Immunopharmacol.* **2005**, *5* (3), 503−510.
(19) Nishigaki, F.; Sakuma, S.; Ogawa, T.; Miyata, S.; Ohkubo, T.; Goto, T. FK506 induces chondrogenic differentiation of clonal mouse embryonic carcinoma cells, ATDC5. *Eur. J. Pharmacol.* **2002**, *437* (3), 123−128.
(20) Staatz, C. E.; Tett, S. E. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. *Clin. Pharmacokinet.* **2004**, *43* (10), 623−53.
(21) MacFarlane, G. D.; Shaw, L. M.; Venkataramanan, R.; Mullins, R.; Scheller, D. G.; Ersfeld, D. L. Analysis of whole blood tacrolimus concentrations in liver transplant patients exhibiting impaired liver function. *Ther. Drug Monit.* **1999**, *21* (6), 585−592.
(22) Kaplan, B.; Lown, K.; Craig, R.; Abecassis, M.; Kaufman, D.; Leventhal, J.; Stuart, F.; Meier-Kriesche, H. U.; Fryer, J. Low bioavailability of cyclosporine microemulsion and tacrolimus in a small bowel transplant recipient: possible relationship to intestinal P-glycoprotein activity. *Transplantation* **1999**, *67* (2), 333−335.
(23) Yokogawa, K.; Takahashi, M.; Tamai, I.; Konishi, H.; Nomura, M.; Moritani, S.; Miyamoto, K.; Tsuji, A. P-glycoprotein-dependent disposition kinetics of tacrolimus: studies in mdr1a knockout mice. *Pharm. Res.* **1999**, *16* (8), 1213−8.
(24) Saitoh, H.; Saikachi, Y.; Kobayashi, M.; Yamaguchi, M.; Oda, M.; Yuhki, Y.; Achiwa, K.; Tadano, K.; Takahashi, Y.; Aungst, B. J. Limited interaction between tacrolimus and P-glycoprotein in the rat small intestine. *Eur. J. Pharm. Sci.* **2006**, *28* (1−2), 34−42.
(25) Bellamy, N.; Campbell, J.; Robinson, V.; Gee, T.; Bourne, R.; Wells, G. Intraarticular corticosteroid for treatment of osteoarthritis of the knee. *Cochrane Db Syst. Rev.* **2005**, No. 2, 1.
(26) Hunter, D. J. In the clinic. Osteoarthritis. *Ann. Int. Med.* **2007**, *147* (3), 1−16.
(27) Bahadir, C.; Onal, B.; Dayan, V. Y.; Gurer, N. Comparison of therapeutic effects of sodium hyaluronate and corticosteroid injections on trapeziometacarpal joint osteoarthritis. *Clin. Rheumatol.* **2009**, *28* (5), 529−533.
(28) Charalambous, C. P.; Tryfonidis, M.; Sadiq, S.; Hirst, P.; Paul, A. Septic arthritis following intra-articular steroid injection of the knee - a survey of current practice regarding antiseptic technique used during intra-articular steroid injection of the knee. *Clin. Rheumatol.* **2003**, *22* (6), 386−390.
(29) Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines. *Arthritis Rheum.* 2000, *43* (9), 1905−1915.
(30) Gerwin, N.; Hops, C.; Lucke, A. Intraarticular drug delivery in osteoarthritis. *Adv. Drug Delivery Rev.* **2006**, *58* (2), 226−242.
(31) Edwards, S. H. R. Intra-articular drug delivery: The challenge to extend drug residence time within the joint. *Vet. J.* **2011**, *190* (1), 15−21.
(32) Larsen, C.; Ostergaard, J.; Larsen, S. W.; Jensen, H.; Jacobsen, S.; Lindegaard, C.; Andersen, P. H. Intra-articular depot formulation principles: role in the management of postoperative pain and arthritic disorders. *J. Pharm. Sci.* **2008**, *97* (11), 4622−4654.
(33) Kang, M. L.; Im, G. I. Drug delivery systems for intra-articular treatment of osteoarthritis. *Expert Opin. Drug Delivery* **2014**, *11* (2), 269−282.
(34) Bajpayee, A. G.; Grodzinsky, A. J. Cartilage-targeting drug delivery: can electrostatic interactions help? *Nat. Rev. Rheumatol.* **2017**, *13* (3), 183−193.
(35) Sandker, M. J.; Duque, L. F.; Redout, E. M.; Chan, A.; Que, I.; Lowik, C. W.; Klijnstra, E. C.; Kops, N.; Steendam, R.; van Weeren, R.; Hennink, W. E.; Weinans, H. Degradation, intra-articular retention and biocompatibility of monospheres composed of [PDLLA-PEG-PDLLA]-b-PLLA multi-block copolymers. *Acta Biomater.* **2017**, *48*, 401−414.
(36) Wischke, C.; Schwendeman, S. P. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. *Int. J. Pharm.* **2008**, *364* (2), 298−327.
(37) Siepmann, J.; Streubel, A.; Peppas, N. A. Understanding and predicting drug delivery from hydrophilic matrix tablets using the ″sequential layer″ model. *Pharm. Res.* **2002**, *19* (3), 306−314.
(38) Kino, T.; Hatanaka, H.; Hashimoto, M.; Nishiyama, M.; Goto, T.; Okuhara, M.; Kohsaka, M.; Aoki, H.; Imanaka, H. FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. *J. Antibiot.* **1987**, *40* (9), 1249−1255.
(39) Akashi, T.; Nefuji, T.; Yoshida, M.; Hosoda, J. Quantitative determination of tautomeric FK506 by reversed-phase liquid chromatography. *J. Pharm. Biomed. Anal.* **1996**, *14* (3), 339−346.
(40) Liu, Y.; Schwendeman, S. P. Mapping microclimate pH distribution inside protein-encapsulated PLGA microspheres using confocal laser scanning microscopy. *Mol. Pharmaceutics* **2012**, *9* (5), 1342−1350.
(41) Siebelt, M.; Waarsing, J. H.; Kops, N.; Piscaer, T. M.; Verhaar, J. A.; Oei, E. H.; Weinans, H. Quantifying osteoarthritic cartilage changes accurately using in vivo microCT arthrography in three etiologically distinct rat models. *J. Orthop. Res.* **2011**, *29* (11), 1788−1794.
(42) Piscaer, T. M.; Waarsing, J. H.; Kops, N.; Pavljasevic, P.; Verhaar, J. A.; van Osch, G. J.; Weinans, H. In vivo imaging of cartilage degeneration using microCT-arthrography. *Osteoarthritis Cartilage* **2008**, *16* (9), 1011−1017.
(43) Palmer, A. W.; Guldberg, R. E.; Levenston, M. E. Analysis of cartilage matrix fixed charge density and three-dimensional morphology via contrast-enhanced microcomputed tomography. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (51), 19255−19260.
(44) van Tiel, J.; Siebelt, M.; Reijman, M.; Bos, P. K.; Waarsing, J. H.; Zuurmond, A. M.; Nasserinejad, K.; van Osch, G. J.; Verhaar, J. A.;

DOI: 10.1021/acsbiomaterials.8b00116
ACS Biomater. Sci. Eng. 2018, 4, 2390−2403

Krestin, G. P.; Weinans, H.; Oei, E. H. Quantitative in vivo CT arthrography of the human osteoarthritic knee to estimate cartilage sulphated glycosaminoglycan content: correlation with ex-vivo reference standards. *Osteoarthritis Cartilage* **2016**, *24* (6), 1012−1020.
(45) Magari, K.; Nishigaki, F.; Sasakawa, T.; Ogawa, T.; Miyata, S.; Ohkubo, Y.; Mutoh, S.; Goto, T. Anti-arthritic properties of FK506 on collagen-induced arthritis in rats. *Inflammation Res.* **2003**, *52* (12), 524−529.
(46) Siebelt, M.; Groen, H. C.; Koelewijn, S. J.; de Blois, E.; Sandker, M.; Waarsing, J. H.; Muller, C.; van Osch, G. J.; de Jong, M.; Weinans, H. Increased physical activity severely induces osteoarthritic changes in knee joints with papain induced sulphate-glycosaminoglycan depleted cartilage. *Arthritis Res. Ther* **2014**, *16* (1), R32.
(47) Silvast, T. S.; Jurvelin, J. S.; Lammi, M. J.; Toyras, J. pQCT study on diffusion and equilibrium distribution of iodinated anionic contrast agent in human articular cartilage–associations to matrix composition and integrity. *Osteoarthritis Cartilage* **2009**, *17* (1), 26−32.
(48) Ross, M. W. The lameness score: quantification of lameness severity. In *Diagnosis and Management of Lameness in the Horse*, SJ, D., Ed. W.B. Saunders: Philadelphia, 2003; pp 66−67.
(49) Bertone, A. L.; Palmer, J. L.; Jones, J. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Vet Surg* **2001**, *30* (6), 528−538.
(50) de Grauw, J. C.; van de Lest, C. H. A.; Brama, P. A. J.; Rambags, B. P. B.; van Weeren, P. R. In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine Vet J.* **2009**, *41* (7), 693−699.
(51) de Grauw, J. C.; van de Lest, C. H.; van Weeren, P. R. Inflammatory mediators and cartilage biomarkers in synovial fluid after a single inflammatory insult: a longitudinal experimental study. *Arthritis Res. Ther* **2009**, *11* (2), R35.
(52) Petit, A.; Muller, B.; Meijboom, R.; Bruin, P.; van de Manakker, F.; Versluijs-Helder, M.; de Leede, L. G.; Doornbos, A.; Landin, M.; Hennink, W. E.; Vermonden, T. Effect of polymer composition on rheological and degradation properties of temperature-responsive gelling systems composed of acyl-capped PCLA-PEG-PCLA. *Biomacromolecules* **2013**, *14* (9), 3172−3182.
(53) Stankovic, M.; Tomar, J.; Hiemstra, C.; Steendam, R.; Frijlink, H. W.; Hinrichs, W. L. Tailored protein release from biodegradable poly(epsilon-caprolactone-PEG)-b-poly(epsilon-caprolactone) multi-block-copolymer implants. *Eur. J. Pharm. Biopharm.* **2014**, *87* (2), 329−337.
(54) Moshtagh, P. R.; Rauker, J.; Sandker, M. J.; Zuiddam, M. R.; Dirne, F. W.; Klijnstra, E.; Duque, L.; Steendam, R.; Weinans, H.; Zadpoor, A. A. Nanomechanical properties of multi-block copolymer microspheres for drug delivery applications. *J. Mech Behav Biomed Mater.* **2014**, *34*, 313−319.
(55) Kumar, R.; Palmieri, M. J., Jr. Points to consider when establishing drug product specifications for parenteral microspheres. *AAPS J.* **2010**, *12* (1), 27−32.
(56) Siepmann, J.; Faisant, N.; Akiki, J.; Richard, J.; Benoit, J. P. Effect of the size of biodegradable microparticles on drug release: experiment and theory. *J. Controlled Release* **2004**, *96* (1), 123−134.
(57) Tran, V. T.; Benoit, J. P.; Venier-Julienne, M. C. Why and how to prepare biodegradable, monodispersed, polymeric microparticles in the field of pharmacy? *Int. J. Pharm.* **2011**, *407* (1−2), 1−11.
(58) Mao, S.; Guo, C.; Shi, Y.; Li, L. C. Recent advances in polymeric microspheres for parenteral drug delivery–part 1. *Expert Opin. Drug Delivery* **2012**, *9* (9), 1161−1176.
(59) De La Vega, J. C.; Elischer, P.; Schneider, T.; Hafeli, U. O. Uniform polymer microspheres: monodispersity criteria, methods of formation and applications. *Nanomedicine (London, U. K.)* **2013**, *8* (2), 265−285.
(60) Martinez, M.; Rathbone, M.; Burgess, D.; Huynh, M. In vitro and in vivo considerations associated with parenteral sustained release products: a review based upon information presented and points expressed at the 2007 Controlled Release Society Annual Meeting. *J. Controlled Release* **2008**, *129* (2), 79−87.
(61) Zolnik, B. S.; Burgess, D. J. Evaluation of in vivo-in vitro release of dexamethasone from PLGA microspheres. *J. Controlled Release* **2008**, *127* (2), 137−145.
(62) Sandker, M. J.; Petit, A.; Redout, E. M.; Siebelt, M.; Muller, B.; Bruin, P.; Meyboom, R.; Vermonden, T.; Hennink, W. E.; Weinans, H. In situ forming acyl-capped PCLA-PEG-PCLA triblock copolymer based hydrogels. *Biomaterials* **2013**, *34* (32), 8002−8011.
(63) von Suesskind-Schwendi, M.; Gruber, M.; Touraud, D.; Kunz, W.; Schmid, C.; Hirt, S. W.; Lehle, K. Pharmacokinetics of a self-microemulsifying drug delivery system of tacrolimus. *Biomed. Pharmacother.* **2013**, *67* (6), 469−473.
(64) Nassar, T.; Rom, A.; Nyska, A.; Benita, S. Novel double coated nanocapsules for intestinal delivery and enhanced oral bioavailability of tacrolimus, a P-gp substrate drug. *J. Controlled Release* **2009**, *133* (1), 77−84.
(65) Wang, Y. J.; Sun, J.; Zhang, T. H.; Liu, H. Z.; He, F. C.; He, Z. G. Enhanced oral bioavailability of tacrolimus in rats by self-microemulsifying drug delivery systems. *Drug Dev. Ind. Pharm.* **2011**, *37* (10), 1225−1230.
(66) Grynpas, M. D.; Alpert, B.; Katz, I.; Lieberman, I.; Pritzker, K. P. Subchondral bone in osteoarthritis. *Calcif. Tissue Int.* **1991**, *49* (1), 20−26.
(67) Barr, A. J.; Campbell, T. M.; Hopkinson, D.; Kingsbury, S. R.; Bowes, M. A.; Conaghan, P. G. A systematic review of the relationship between subchondral bone features, pain and structural pathology in peripheral joint osteoarthritis. *Arthritis Res. Ther* **2015**, *17*, 228.
(68) Siebelt, M.; van der Windt, A. E.; Groen, H. C.; Sandker, M.; Waarsing, J. H.; Muller, C.; de Jong, M.; Jahr, H.; Weinans, H. FK506 protects against articular cartilage collagenous extra-cellular matrix degradation. *Osteoarthritis Cartilage* **2014**, *22* (4), 591−600.
(69) McCauley, L. K.; Rosol, T. J.; Capen, C. C. Effects of cyclosporin A on rat osteoblasts (ROS 17/2.8 cells) in vitro. *Calcif. Tissue Int.* **1992**, *51* (4), 291−297.
(70) Sun, L.; Blair, H. C.; Peng, Y.; Zaidi, N.; Adebanjo, O. A.; Wu, X. B.; Wu, X. Y.; Iqbal, J.; Epstein, S.; Abe, E.; Moonga, B. S.; Zaidi, M. Calcineurin regulates bone formation by the osteoblast. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102* (47), 17130−5.
(71) Winslow, M. M.; Pan, M.; Starbuck, M.; Gallo, E. M.; Deng, L.; Karsenty, G.; Crabtree, G. R. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev. Cell* **2006**, *10* (6), 771−782.
(72) Kang, K. Y.; Ju, J. H.; Song, Y. W.; Yoo, D. H.; Kim, H. Y.; Park, S. H. Tacrolimus treatment increases bone formation in patients with rheumatoid arthritis. *Rheumatol. Int.* **2013**, *33* (8), 2159−2163.
(73) Siebelt, M.; Groen, H. C.; Koelewijn, S. J.; de Blois, E.; Sandker, M.; Waarsing, J. H.; Muller, C.; van Osch, G. J.; de Jong, M.; Weinans, H. Increased physical activity severely induces osteoarthritic changes in knee joints with papain induced sulfate-glycosaminoglycan depleted cartilage. *Arthritis Res. Ther* **2014**, *16* (1), R32.
(74) Goldring, M. B.; Goldring, S. R. *Biology of the Normal Joint, in Kelley and Firestein's Textbook of Rheumatology* **2013**, 1.
(75) Kurowska-Stolarska, M.; Alivernini, S. Synovial tissue macrophages: friend or foe? *RMD Open* **2017**, *3* (2), e000527.
(76) Houard, X.; Goldring, M. B.; Berenbaum, F. Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. *Curr. Rheumatol. Rep.* **2013**, *15* (11), 375.
(77) Petit, A.; Redout, E. M.; van de Lest, C. H.; de Grauw, J. C.; Muller, B.; Meyboom, R.; van Midwoud, P.; Vermonden, T.; Hennink, W. E.; Rene van Weeren, P. Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses. *Biomaterials* **2015**, *53*, 426−436.
(78) Bondeson, J.; Wainwright, S. D.; Lauder, S.; Amos, N.; Hughes, C. E. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res. Ther* **2006**, *8* (6), R187.

DOI: 10.1021/acsbiomaterials.8b00116
ACS Biomater. Sci. Eng. 2018, 4, 2390−2403