Intra-articular Treatment of Osteoarthritis with Diclofenac-Conjugated Polymer Reduces Inflammation and Pain

Adrian Sulistio,†,§,∥ Friederike M. Mansfeld,†,§,∥ Felisa Reyes-Ortega,†,§ Asha M. D’Souza,§,⊥ Sarah M. Y. Ng,§,⊥ Stephen Birkett,§,⊥ Anton Blencowe,‡ Greg G. Qiao,‖ Christopher B. Little,‖ Cindy C. Shu,‖ Alison M. Bendele,⭐ David Valade,§,⊥ Andrew C. Donohue,§,⊥ John F. Quinn,*†,∥ Michael R. Whittaker,† Thomas P. Davis,†,**,∥ and Russell J. Tait*§,⊥

†ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Royal Parade, Parkville, Victoria 3052, Australia
‡Children’s Cancer Institute, Australian Centre for NanoMedicine and ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, The University of New South Wales, Sydney, New South Wales 2052, Australia
§PolyActiva Pty Ltd., Level 9, 31 Queen Street, Melbourne, Victoria 3000, Australia
⊥Monash Institute of Pharmaceutical Sciences, Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, Royal Parade, Parkville, Victoria 3052, Australia
∥School of Pharmacy and Medical Sciences, University of South Australia, Mawson Lake, South Australia 5095, Australia
☆Department of Chemical and Biomolecular Engineering, University of Melbourne, Parkville, Victoria 3010, Australia
††Raymond Purves Bone and Joint Research Laboratories, Kolling Institute, University of Sydney at Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia
‡‡Bolder BioPATH Inc.,5541 Central Avenue, Suite 160, Boulder, Colorado 80301, United States
▲Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, Queensland, 4072, Australia

ABSTRACT: The most common treatment for osteoarthritis is daily oral administration of a nonsteroidal anti-inflammatory drug such as diclofenac. This daily dosage regime is often associated with severe side effects. In this study, we explored the potential of utilizing a high molecular weight cross-linked polyurethane polymer covalently linked to diclofenac (C-DCF-PU) for intra-articular administration. We aim to exploit the advantages of local drug delivery by developing an implant with improved efficacy and reduced side effects. The polymer was synthesized from a diclofenac-functionalized monomer unit in a simple one-pot reaction, followed by cross-linking. In vitro drug release studies showed zero-order drug release for 4 days, followed by a gradual decline in drug release rate until diclofenac was depleted after 15 days. The cross-linked polymer was triturated to yield an injectable microgel formulation for administration. Whole animal fluorescence imaging of the rhodamine-labeled C-DCF-RH-PU showed good retention of the polymer in the knee joints of healthy rats, with approximately 30% of the injected dose still present 2 weeks post intra-articular administration. In a reactivation arthritis animal model, the C-DCF-RH-PU formulation reduced pain and significantly reduced inflammation after a short lag phase, showing that this drug delivery system warrants further development for long-term treatment of osteoarthritis with the benefit of reduced side effects.

KEYWORDS: osteoarthritis, intra-articular injection, NSAID, polyurethane, controlled drug release, local drug delivery

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by gradual loss of articular cartilage and bone, causing inflammation, pain, loss of mobility, and chronic disability.1,2 OA can affect any joint of the body, but hip and knee joints are most commonly affected. In the absence of curative options, current treatments are limited to symptomatic relief, and the most common approach is daily oral administration of nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac (DCF).3 This, however, is often accompanied by
severe side effects, mainly gastrointestinal, vascular, and coronary complications. As the disease is localized to the joint, interest in intraarticular (i.a.) intervention has been increasing. Local administration of NSAIDs can minimize the amount of drug delivered while maximizing the concentration of drug at the site of action, leading to a significant reduction in systemic exposure and related severe side effects. However, diclofenac, like other small molecules and proteins, is cleared rapidly from the synovial cavity with a half-life of around 5 h, after i.a. injection. Frequent i.a. injections are not desirable due to the risk of infection and discomfort they can cause the patient.

To this end, a variety of delivery vehicles that aim to prolong the residence time of NSAIDs in the joint have been developed and tested, including liposomes, hydrogels, and polymeric microspheres, all of which achieved drug loading by physical encapsulation. This drug loading method can be associated with an initial burst release of drug, and often relatively short treatment windows, as the drug depletes from its carrier after only a few days. Thus, an i.a. drug delivery system with a covalently linked NSAID that increases drug residence time and provides sustained drug release would be of great therapeutic benefit in OA treatment.

We therefore developed a polymeric drug delivery vehicle that can be retained in the knee joint and provide sustained release of NSAID (e.g., diclofenac) after i.a. injection for at least 2 weeks. We recently developed DCF-conjugated polyurethane-based polymers that could give sustained \textit{in vitro} drug release from several weeks to 150 days or more, depending on the amount and MW of polyethylene glycol (PEG) incorporated. However, these polymers have low molecular weight and broad MW distribution (\(M_w = 5.05 - 13.5\) kDa, \(D = 1.35 - 3.56\), which might lead to rapid clearance from the synovial cavity. Hence, to achieve our goals, these polymers were modified through cross-linking using orthogonal chemistry to increase molecular weight and thereby their retention time in the synovial cavity to enable sufficient residence time for drug release to be efficacious. To study the retention of the polymers in the knee joint after i.a. injection, a small amount of rhodamine was covalently incorporated in the polymer backbone to aid the visualization of the polymers with a small animal imaging device once they had been injected into rat knees. Although \textit{in vitro} characterization showed that the cross-linking process reduced the drug release time frame, we were able to formulate the cross-linked polymer for i.a. administration and showed that it was effective in treating pain and inflammation in \textit{in vivo} studies in a rat arthritis inflammation model induced with streptococcus cell wall (SCW) peptidoglycan polysaccharide (PGPS). 

## Experimental Details

### Materials

Diclofenac was purchased from Beta Pharm Company Ltd. (batch number, 201006280; assay, 99.12%; Shanghai, China). Ethyl L-lysine diisocyanate (ELDI) was kindly donated by Commonwealth Science and Industrial Research Organisation (CSIRO). 4-Hydroxybenzenoic acid (≥99%), cis-1,3-O-benzylidene glycerol (97%) poly(ethylene glycol) (PEG, \(M_w = 400\) and 3000 Da), dibutylin dilurate (DBTDL) (95%), N,N,N′,N′-tetramethyl-O-(1H-benzoctiol-1-yl)uronium hexafluorophosphate (HBUT) (≥98%), sodium azide (≥99%), 5-hexynoic acid (97%), rhodamine B, ammonium acetate (≥98%), N,N′-diclohexylglycylol with sodium chloride (≥98%), and triethylene glycol (≥99%), pyridine (≥99%), lithium bromide (≥99%), hydrochloric acid (37%), and acetic acid (glacial, ≥99.8%) were obtained from Sigma-Aldrich and used as received. Sodium bicarbonate (NaHCO\(_3\)) and sodium sulfate (NaSO\(_4\)) were obtained from Ajax Finechem and used as received. Tetrahydrofuran (≥99.9%, LiChrosolv), methanol (≥99.8%, LiChrosolv), dichloromethane (99.8%, Chem Supply), diethyl ether (99.5%, Chem Supply), toluene (EMPURE) and petroleum benzine 60–80 (EMPURE), and deuterated chloroform (chloroform-d) (99.8%, Cambridge Isotope Lab Inc.) were used as received. PGPS 100P fraction (Lot #32291) was purchased from Lee Laboratories, and triaminocine (Kenalog-10, Lot #4D80330) was obtained from Bristol-Myers Squibb. 1,3-Dihydroxypropan-2-yl (2-(2-(2,6-dichlorophenyl)amino) phenyl)acetate benzoate (DCF-PHB-MG, Scheme S1, Supporting Information) was synthesized according to a previously published procedure.

### Synthesis of Rhodamine-MG (Rhodamine-1,3-dihydroxy glycerol ester) (Scheme S2, Supporting Information).

(i) Triethylamine (0.406 g, 4.00 mmol), HBTU (0.569 g, 1.50 mmol), and cis-1,3-O-benzylidene glycerol (0.364 g, 2.02 mmol) were added to a stirred suspension of rhodamine-B (0.479 g, 1.00 mmol) in anhydrous dichloromethane (10 mL). The reaction was stirred under \(N_2\) at room temperature with exclusion of light for 3 days. The mixture was then washed with saturated NaHCO\(_3\) (3 × 100 mL), followed by dilute HCl (pH ≈ 3, 3 × 100 mL) and water (3 × 100 mL). The organic layer was dried over Na2SO4 and filtered through a short plug of silica. The solvents were removed in \textit{vacuo}, and the crude product was purified by multiple rounds of column chromatography on silica gel using 2% methanol in dichloromethane to give the intermediate benzylidene protected rhodamine-MG as a purple solid (0.436 g). (ii) To remove the benzylidene protecting group, the intermediate (0.436 g, 0.720 mmol) was stirred in 80% acetic acid at 45 °C for 20 h and dried in \textit{vacuo} to give a purple paste. This crude product was purified via column chromatography using 2% methanol in dichloromethane to give the desired product as a dark purple solid (0.134 g). See Supporting Information for characterization.

### Synthesis of Alkyne-MG (1,3-Dihydroxypropan-2-yl 5-hexynoate) (Scheme S3, Supporting Information).

A solution of DCC (2 eq., 2.29 g, 11.1 mmol) in 10 mL of anhydrous THF was added dropwise over 30 min into a mixture of cis-1,3-O-benzylidene glycerol (1 eq., 1.00 g, 5.55 mmol), hexynoic acid (2 eq., 1.24 g, 11.1 mmol), and DMAP (0.2 eq., 0.136 g, 1.11 mmol) in 20 mL of anhydrous THF at 0 °C. The reaction was stirred for 24 h at room temperature. The solid reaction byproduct DCU was removed by filtration through a thin bed of Celite, and solvent was removed in \textit{vacuo}. The crude material was purified via column chromatography on silica gel (1:1 ethyl acetate/hexane, followed by 1:1 ethyl acetate/hexane as eluents) to yield the intermediate benzylidene protected alkyne-MG (2-phenyl-1,3-dioxane-5yl hex-5-yne) as a yellow oil (2.10 g). To remove the benzylidene protecting group, the intermediate (2.10 g, 7.66 mmol) was dissolved in 80% acetic acid at 45 °C for 2 h and then filtered to give a clear, very pale yellow oil (1.15 g). See Supporting Information for characterization.

### PEG3000 Diazone (Scheme S4, Supporting Information).

Methanesulfonyl chloride (5 eq., 1.15 g, 10.0 mmol) in 20 mL of DCM was added dropwise into a solution containing triethylamine (5 eq., 1.01 g, 10.0 mmol) and PEG3000 (1 eq, 6.00 g, 2.00 mmol) in 40 mL of DCM. The mixture was stirred at room temperature under \(N_2\) for 5 days, when more triethylamine (5 eq., 1.01 g, 10.0 mmol) and methanesulfonylic chloride (5 eq., 1.15 g, 10.0 mmol) were added. The reaction was stirred for another 9 days at room temperature and then concentrated in \textit{vacuo} to approximately 25 mL. The resulting precipitate was removed via filtration, the volume was further reduced to 10 mL, and additional precipitate was removed. The solution was added to diethyl ether (200 mL) to yield the intermediate PEG3000 bis mesylate as an off-white solid (7.29 g). (ii) PEG3000 bis mesylate (5.00 g, 1.57 mmol) was dissolved in DMF (50 mL) and stirred vigorously at 55 °C. Sodium azide (1.02 g, 15.7 mmol) was added in small portions, and the reaction was stirred in the dark for 72 h. The solid was removed by filtration, and the organic solvent was removed under a stream of air to yield a yellowish solid. This was suspended in

\[ \text{DOI: 10.1021/acsabm.9b00232} \]
ethyl acetate (50 mL), the solid was removed by centrifugation, and the supernatant was concentrated to approximately 20 mL before precipitation into 400 mL of diethyl ether to yield a white solid after isolation via centrifugation. MALDI TOF confirmed quantitative azidation and the molecular weight was $M_a = 3156$ Da, $D = 1.01$.

**Synthesis of Linear DCF Polyurethane (L-DCF-RH-PU) (Scheme 1i).** DCF-PHB-MG (1 g, 2.04 mmol), PEG3000 (1.57 g, 0.523 mmol), alkyne-MG (0.487 g, 2.62 mmol), and rhodamine-MG (0.029 g, 0.0523 mmol) were placed in a 100 mL round-bottom flask and dissolved in 30 mL of anhydrous THF. Subsequently, ELDI (1.18 g, 5.23 mmol) and DBTDL (0.0644 g, 0.102 mmol) were added into the reaction mixture, the flask was sealed, and the reaction was stirred at 50 °C for 24 h. The reaction mixture was concentrated in vacuo to a volume of approximately 15 mL, and the polymer was precipitated by addition of at least a 20-fold excess of diethyl ether and washed several times with 50 mL of diethyl ether to yield a soft, magenta colored solid (3.82 g). GPC: $M_a = 16.1$ kDa, $M_d = 53.0$ kDa, $D = 3.30$. $^1$H NMR (400 MHz, CDCl$_3$); refer to Results section.

Another linear construct without the rhodamine (L-DCF-P) was also synthesized following the same procedure. Yield = 3.8 g of off-white solid. GPC: $M_a = 25.2$ kDa, $M_d = 42.5$ kDa, $D = 1.70$. $^1$H NMR (400 MHz, CDCl$_3$); refer to Figure S1.

**Synthesis of Cross-Linked DCF-Hydrogel (C-DCF-RH-PU) (Scheme 1ii).** L-DCF-RH-PU (0.45 g) was dissolved in water/tert-butanol (50/50, 14 mL) by gentle stirring in a round-bottom flask. Once dissolved, PEG3000 diazide (0.927 g, 0.293 mmol) was added as the cross-linker into the solution to give an overall azide to alkyne ratio of 0.94 to 1. The solution was purged with N$_2$ for 15 min before addition of CuBr$_2$ (9.48 mg, 0.0425 mmol) and PMDETA (7.36 mg, 0.0425 mmol) dissolved in water/tert-butanol (50/50, 0.1 mL) under N$_2$. After 10 min of stirring, sodium ascorbate (45.9 mg) in water/tert-butanol (50/50, 0.1 mL) was added, and the reaction was stirred under N$_2$ for 16 h. The resulting gel was washed with water (~50 mL × 4) until the wash solution was clear and then dried in vacuo to yield a purple solid (1.06 g).

**Formulation of C-DCF-RH-PU Injectable Suspension.** Dried C-DCF-RH-PU (150 mg) was placed into a mortar, and initially approximately 100 mg of PEG400 was added. The wetted polymer was then ground manually using a pestle to form smaller particulates. More PEG400 was added in small portions (~100 mg increments) while the grinding process continued until a uniform suspension with a concentration of 10% or 15% w/w that could be injected via 25G needle was formed.

**In Vitro DCF Release Studies.** Accurately weighed samples (10 mg) of each polymer were placed into 20 mL vials with PBS pH 7.4 (15 mL), and the samples were stirred at 300 rpm at 37 °C to determine diclofenac release under sink conditions. At predetermined time points, an aliquot (100 μL) was collected, replaced with an equal volume of fresh PBS pH 7.4, and the sample was analyzed for DCF content by HPLC using an established method.

**In Vivo Polymer Retention Studies.** In vivo polymer retention studies were approved by the Royal North Shore Animal Care and Ethics Committee (protocol 1309–001A). Female Lewis rats weighing approximately 200 g (10–11 weeks of age) were purchased from the Animal Resources Centre (ARC, Perth) and were housed 2–3 animals per cage with ad libitum food and water. General animal health and weight were assessed regularly. Animals were allowed to acclimatize for 7 days prior to start of the procedures. Treatments were administered on day –1 by intra-articular injection of treatment solutions according to Table 1 into the right knee, with the left knee serving as control. The C-DCF-RH-PU suspension was prepared by manual grinding in PEG400 as described in Formulation of C-DCF-RH-PU Injectable Suspension. Inflammation and pain were assessed at regular time points (Figure 1) until termination of the experiment on day 11. Animals were euthanized by

---

Table 1. Experimental Groups for Assessment of Efficacy

<table>
<thead>
<tr>
<th>group</th>
<th>no. of animals</th>
<th>priming (i.a.)</th>
<th>reactivation (i.v.)</th>
<th>treatment (i.a.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>50 μL PGPS (0.125 mg/mL)</td>
<td>no reactivation</td>
<td>50 μL PEG400 (vehicle)</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>50 μL PGPS (0.125 mg/mL)</td>
<td>0.05 mL PGPS (0.4 mg/mL)</td>
<td>50 μL PEG400 (vehicle)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>50 μL PGPS (0.125 mg/mL)</td>
<td>0.05 mL PGPS (0.4 mg/mL)</td>
<td>50 μL C-DCF-RH-PU (150 mg/mL, 22.5 μg DCF/day)</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>50 μL PGPS (0.125 mg/mL)</td>
<td>0.05 mL PGPS (0.4 mg/mL)</td>
<td>50 μL trimcinolone (1.2 mg/mL, 60 μg)</td>
</tr>
</tbody>
</table>

*Estimate based on in vitro drug release.
CO₂ inhalation, and right knees were collected into 10% neutral buffered formalin for histopathology.

Assessment of Inflammation by Knee Caliper Measurements. Knee thickness of both left knee (untreated) and right knee (treated) was measured using calipers on days −14, −13, −1, 0, 2, 4, 7, 9, and 11. The difference between right knee and left knee on individual days was calculated, and from this plot, the area under the curve (AUC) was determined for the first (days 0–4) and second (days 7–11) reactivation periods as well as the full experiment (days 0–11).

Assessment of Pain by Gait Analysis. Gait analysis was performed on days −14 (baseline), 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11 by applying ink to the ventral surface of rear feet of the rats and documenting weight bearing during movement by allowing them to walk the length of an A4 sheet of paper. This process was repeated as necessary to generate four clear, evenly inked footprint pairs representing the overall pattern of gait. Gait was scored visually, and the prints were analyzed digitally using Fiji ImageJ to measure the area of the ink on a 300 dpi black and white scan. To achieve the latter, the image was smoothed, thresholds set at 0 (low) and 254 (high), and the built-in Analyze Particles plugin was used for the actual measurement. Area of the right footprint was divided by the average of both footprints to determine gait deficiency for each pair of prints.

Scoring schemes can be found in the Supporting Information.

Statistical Analysis. Knee caliper AUC, gait AUC scores, and histopathology scores were compared using two-way ANOVA with post hoc Tukey test in GraphPad Prism (version 7.02).

RESULTS AND DISCUSSION

Synthesis of Linear (L-DCF-RH-PU) and Cross-Linked (C-DCF-RH-PU) Diclofenac Polyurethane Polymers. The linear diclofenac polyurethane polymer L-DCF-RH-PU was synthesized in a one-pot synthesis by reacting ethyl L-lysine diisocyanate (ELDI) (50 mol eq) with prodrug monomer DCF-PHB-MG (19.5 mol eq), PEG3000 (5 mol eq), alkyne-MG (25 mol eq), and rhodamine-MG (0.5 mol eq) (Scheme 1).

Scheme 1. Synthesis of Linear Diclofenac Polyurethane Conjugate (L-DCF-RH-PU) via (i) Step-Growth Polymerization Reaction and (ii) Subsequent Cross-Linking Reaction with PEG3000 Diazide To Form Cross-Linked (C-DCF-RH-PU) Hydrogel

Statistical Analysis. Knee caliper AUC, gait AUC scores, and histopathology scores were compared using two-way ANOVA with post hoc Tukey test in GraphPad Prism (version 7.02).

RESULTS AND DISCUSSION

Synthesis of Linear (L-DCF-RH-PU) and Cross-Linked (C-DCF-RH-PU) Diclofenac Polyurethane Polymers. The linear diclofenac polyurethane polymer L-DCF-RH-PU was synthesized in a one-pot synthesis by reacting ethyl L-lysine diisocyanate (ELDI) (50 mol eq) with prodrug monomer DCF-PHB-MG (19.5 mol eq), PEG3000 (5 mol eq), alkyne-MG (25 mol eq), and rhodamine-MG (0.5 mol eq) (Scheme 1). The feed ratio of monomers was adjusted to result in equimolar ratio of isocyanates and the total number of hydroxyl groups to achieve the highest molecular weight possible within the thermodynamic and kinetic constraints of polyurethane synthesis. The DCF-PHB-MG contains a labile para-hydroxy benzoic ester (PHB), which hydrolyses at physiological pH to release the active diclofenac drug. On the basis of our previous results, a certain amount of PEG needs to be incorporated covalently into the polymer backbone.

Statistical Analysis. Knee caliper AUC, gait AUC scores, and histopathology scores were compared using two-way ANOVA with post hoc Tukey test in GraphPad Prism (version 7.02).
to facilitate immediate hydrolysis of the pendent DCF and eliminate any lag phase. Therefore, PEG3000 was incorporated covalently in the backbone to improve water penetration and promote hydrolysis of the DCF drug.

The L-DCF-RH-PU contains pendent alkyne groups from alkyne-MG monomer along the polymer chain, which can be used for cross-linking reaction via orthogonal copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC) to form the cross-linked C-DCF-RH-PU (Scheme 1ii). In addition, to aid the visualization of the polymer distribution in vivo studies via fluorescence imaging, a small amount rhodamine-MG was incorporated in the polymer chain (Scheme 1i), which gave the isolated polymer a pink color. Both alkyne-MG and rhodamine-MG were conjugated directly to the monoglyceride to form much more stable alkyl esters than the aryl-PHB linker to ensure that the DCF will be hydrolyzed first (i.e., before the other esters start to hydrolyze).

Gel permeation chromatography (GPC) revealed successful polymer formation with $M_n = 16.1$ kDa, $M_w = 53.0$ kDa, $D = 3.30$. Analysis of L-DCF-RH-PU by $^1$H NMR spectroscopy confirmed the incorporation of each starting material into the final polymer, as shown by proton peaks that are consistent with the structure of the polymer (Figure 2).

The composition of L-DCF-RH-PU was calculated from its $^1$H NMR spectrum by comparing the integration ratios of proton peaks corresponding to each monomer (Table 2) and showed good agreement with the molar feed ratio of starting materials. The $^1$H NMR also revealed the presence of a negligible amount of free PHB (Figure 2 inset) due to hydrolysis during the polymerization or work up (Table 2). By taking this into account, the amount of DCF available for release from the polymer was calculated to be approximately 13.1% w/w.

As it has been reported that even larger molecules can be cleared from the synovial cavity rapidly, L-DCF-RH-PU was cross-linked with PEG3000 diazide via CuAAC to form the high molecular weight hydrogel C-DCF-RH-PU (Scheme 1ii). Although the incorporation of additional PEG chains into the system further diluted the amount of DCF in the C-DCF-RH-PU polymer to 3.3% w/w, the cross-linked hydrogel will have a

![Figure 2](https://example.com/figure2.png)  
**Figure 2.** $^1$H NMR spectrum (CDCl$_3$) and the assigned structure of the linear DCF polyurethane polymer L-DCF-RH-PU. Inset: Zoomed aromatic region showing proton peaks of the pendent rhodamine and $p$-hydroxy benzoate (PHB) group, the latter resulting from minor hydrolysis of DCF during polymer synthesis/work up (see Table 2).

<table>
<thead>
<tr>
<th>composition</th>
<th>feed ratio (mol %)</th>
<th>$^1$H NMR (mol %)</th>
<th>$^1$H NMR peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELDI</td>
<td>50.0</td>
<td>49.6</td>
<td>peak 9, $\delta$ 3.13 ppm</td>
</tr>
<tr>
<td>DCF-PHB-MG</td>
<td>19.5</td>
<td>19.0</td>
<td>peak 21, $\delta$ 6.57 ppm</td>
</tr>
<tr>
<td>Alkyne-MG</td>
<td>25.0</td>
<td>24.8</td>
<td>peak 34, $\delta$ 2.00 ppm</td>
</tr>
<tr>
<td>PEG3000</td>
<td>5.0</td>
<td>5.8</td>
<td>peak 1–4, $\delta$ 3.55 - 3.75 ppm</td>
</tr>
<tr>
<td>Rhodamine-MG</td>
<td>0.5</td>
<td>0.5</td>
<td>Figure 2 inset red circles, $\delta$ 8.20–8.40 ppm</td>
</tr>
<tr>
<td>pendent PHB</td>
<td>N/A</td>
<td>0.3</td>
<td>peaks 15' and 16', $\delta$ 7.90–8.00 ppm</td>
</tr>
</tbody>
</table>

*See Figure 2 for assigned peaks.*
prolonged residence time in the joint to allow for a longer treatment time.

**In Vitro Drug Release Studies.** Release of free DCF from L-DCF-RH-PU and C-DCF-RH-PU was tested *in vitro* by immersing the polymers in PBS pH 7.4 at 37 °C under gentle stirring. The amount of drug released at different time points was plotted as cumulative DCF release and the rate of drug release was also calculated (Figure 3). To investigate whether the pendent rhodamine would alter the DCF release rate due to its charge, another linear DCF polymer without pendent rhodamine (L-DCF-PU) was synthesized. It has $M_n = 25,2$ kDa, $M_w = 42.5$ kDa, $D = 1.70$ and a very similar composition to the L-DCF-RH-PU (Table S1, Supporting Information) and was subsequently cross-linked to form C-DCF-PU. The release rates of both nonfluorescent linear and cross-linked versions were measured and used as a comparison.

Figure 3 shows that the pendent rhodamine did not have a major influence on the release profile of the polymers. Both L-DCF-PU and L-DCF-RH-PU released their drug load over 30 days, with DCF release kinetics resembling first order drug release. While most of the loaded DCF was released from the cross-linked polymer hydrogel within 8 days, this material did...
Figure 5. Representative in vivo fluorescence images recorded at (A) 1, (B) 2, (C) 3, (D) 8, and (E) 14 days post injection, each image also showing the same animal on day 0 immediately after administration of the two compounds for comparison. Left knee joints were injected with C-DCF-RH-PU suspensions; right knee joints were injected with equivalent concentrations of free rhodamine as a control. (F) Fluorescence intensity in the region of interest (ROI, blue oval on knee joint) for each animal was used in the calculation of mean fluorescence intensity over time (n = 4).

approximate the preferred zero-order drug release kinetics over the first 4 days before dropping off to complete depletion by day 15 (Figure 3). Zero-order drug release kinetics result in stable drug concentration, so-called steady-state conditions where the drug concentration remains constant as the rate of drug release equals that of drug clearance. The shorter time frame for drug depletion in the hydrogels cross-linked with PEG3000 diazide is consistent with the fact that drug release rates from our polymers are known to increase with increasing PEG incorporation into the polymer.\(^\text{16}\) For future long-term applications, it will be necessary to extend the drug release period by reducing the total amount of PEG incorporated into the hydrogel. However, the time frame for drug release from the cross-linked C-DCF-RH-PU is sufficient to proceed with proof-of-concept studies for this type of delivery system, particularly as this is a longer time frame than that of other reported i.a. drug delivery systems,\(^\text{10,12}\) and it is encouraging that C-DCF-RH-PU is capable of maintaining a constant drug concentration for a few days.

**Formulation of C-DCF-RH-PU Injectable Suspension.** The C-DCF-RH-PU hydrogel needed to be formulated into an injectable form to allow for i.a. administration. Therefore, the dried C-DCF-RH-PU hydrogel was soaked in a small amount of PEG400 overnight, and the swollen gel was ground manually using mortar and pestle to form a microgel suspension with a final polymer concentration of 10% w/w, which could be injected through a 25G needle. Laser diffraction analysis showed a median particle diameter (\(d_{(0.5)}\)) of 59.4 \(\mu\)m with a wide distribution of particle sizes (Figure 4A). This was consistent with the observation from optical microscopy and SEM, which also revealed the irregular shapes and wide size distribution of the microparticles that ranged from \(\leq 20 - 200 \ \mu\)m (Figure 4B, C).

The effect of microparticle particle size on the biocompatibility of i.a. drug delivery systems has been investigated by several researchers. For example, Horisawa et al. demonstrated that PLGA nanospheres (110–670 nm) and microspheres (3–60 \(\mu\)m) were well tolerated in rat joints; however, the nanospheres were phagocytosed by macrophages in the synovium and produced slight inflammation of the synovial membrane, whereas the microspheres adhered to the surface of the cartilage and synovium with no observed inflammation.\(^\text{22}\) This is consistent with other similar studies that proposed upper size limits of 5–20 \(\mu\)m for phagocytosis-induced inflammatory responses by synovial macrophages\(^\text{22,24}\) and showed that larger particles (20–200 \(\mu\)m) exhibited no adverse effects on the joint.\(^\text{24–26}\) As the majority of C-DCF-RH-PU particles produced from the grinding process fall into the latter category, they should be well-tolerated on i.a. administration. However, it should be noted that some of the particles in our formulation are bigger than 200 \(\mu\)m and their shape is different from the spherical microparticles tested previously. Moreover, it is known that particle shape also influences cellular uptake.\(^\text{27}\) Therefore, the biocompatibility and residence time of the C-DCF-RH-PU formulation in the synovium were subsequently assessed and the results are presented below.

**In Vivo Polymer Retention Studies.** The C-DCF-RH-PU microgel suspension needs to be retained in the knee joint until its drug load has been depleted to achieve optimum efficacy. To test this, a C-DCF-RH-PU suspension (10% w/w in PEG400, 100 \(\mu\)L) was injected into the left knee joints of healthy rats, and a solution of free rhodamine with a concentration equivalent to that present in the administered C-DCF-RH-PU suspension was injected into the right knee as a control. In vivo fluorescence images were recorded immediately following administration of both agents and at 1, 2, 3, 8, and 14 days post injection to compare residence time of the C-DCF-RH-PU microgel and that of the small molecule rhodamine. Representative images comparing the same animal
at time of injection and at each time point (Figure 5A–E) showed a strong fluorescence signal in C-DCF-RH-PU treated right knee joints at all time points and a signal similar to background in rhodamine-treated control knee joints from day 1 onward. In some cases, the C-DCF-RH-PU-treated side exhibited a strong fluorescence signal in the soft tissues surrounding the joint and qualitative post-mortem examination revealed rhodamine staining in the muscle on the tibia (tibialis anterior, data not shown). This may be caused by slow leakage from the intra-articular injection site, or may be natural diffusion, as in quadrupeds a synovial fossa is situated at the anterior lateral aspect of the knee through which the long digital extensor tendon passes. Nonetheless, a substantial amount of C-DCF-RH-PU polymer was retained in the joint for 14 days.

To quantify retention of C-DCF-RH-PU within the joint cavity and exclude any leaked polymer, only the fluorescence intensity within a region of interest (ROI) drawn around the knee joint (blue ovals in Figure 5A–E) was used in the calculation of mean fluorescence intensity for both knee joints at each time point (Figure 5F). No significant difference was found in mean fluorescence intensity in C-DCF-RH-PU (646 ± 175 A.U.) and free rhodamine-treated (475 ± 110 A.U.) joints immediately after injection. However, within 1 day, the mean fluorescence intensity in right knee joints injected with free rhodamine control had dropped by over 90% and from day 3 onward was indistinguishable from background (Figure 5F). This is consistent with poor retention of small molecules administered to the synovium.8 In contrast, the mean fluorescence intensity in the left knee joints injected with C-DCF-RH-PU remained stable for 2 days, increased slightly on day 3, and then tapered off to approximately 30% of initial mean fluorescence intensity by day 14 (Figure 5F).

The intriguing increase in mean fluorescence intensity on day 3 was investigated in vitro using L-DCF-RH-PU solutions, as, unlike the cross-linked polymer, it is soluble in PEG400 and can therefore be diluted accurately to generate a fluorescence calibration plot. The fluorescence intensity of L-DCF-RH-PU solutions of different concentrations was measured with the same technique used for imaging the animals (Figure 6A). At the lower concentrations of L-DCF-RH-PU (up to 25 mg/mL), a logarithmic relationship between concentration and fluorescence intensity was observed. However, at 50 mg/mL L-DCF-RH-PU, the increase in fluorescence intensity was no longer proportional to the logarithmic increase in concentration and at 100 mg/mL dropped off to below that of the more dilute solution (Figure 6). This behavior can be explained by quenching of rhodamine fluorescence at high polymer concentrations,28 and the same rationale can be applied to the observation that in vivo mean fluorescence intensity increased on day 3. Slow diffusion of polymer from the rat knee joint caused a gradual decrease in i.a. C-DCF-RH-PU concentration and hence rhodamine, which ultimately resolved rhodamine quenching and led to an initial increase in mean fluorescence intensity on day 3, before decreasing at later time points with further reduction in i.a. C-DCF-RH-PU concentration.

This initial in vitro study demonstrated that the C-DCF-RH-PU microgel suspension can be injected successfully into rat knee joints, and while not evaluated quantitatively, there was no apparent reaction to the injection of polymer or rhodamine: all animals continued to use their hind limbs and showed normal spontaneous activity (including climbing and standing on hind limbs). There was no marked swelling of the knee joints and no pain on palpation of injected joints. All animals maintained or gained weight over the course of the experiment. Furthermore, in comparison with a small molecule like rhodamine, the data showed prolonged retention of the C-DCF-RH-PU polymer within the knee joint.

In Vivo Polymer Efficacy Studies. To assess the efficacy of C-DCF-RH-PU in vivo, we tested its performance in a reactivation arthritis model induced by injection of peptidoglycan polysaccharide (PGPS) fragments isolated from Streptococcus pyogenes into the knee joint of male Lewis Rats.17 This priming injection (on day −14) resulted in a local inflammatory response, which gradually subsided over 2 weeks, and could be reactivated multiple times by intravenous injection of a subarthritogenic dose of PGPS. On day −1, after knee caliper measurements determined a significant reduction in inflammation (Figure 7A), treatments were administered. The injection volume was reduced to 50 μL due to leakage of the polymer suspension from the synovium observed during the retention studies. To partially compensate for the reduced volume, the concentration of the C-DCF-RH-PU polymer suspension was increased to 15% w/w in PEG400. Two treatment groups were injected with PEG400 vehicle (no reactivation, and vehicle control with reactivation), while the other two groups were treated with either triamcinolone (0.06 mg) or C-DCF-RH-PU (7.5 mg polymer, estimated to release approximately 22.5 μg DCF/day). Although triamcinolone is a corticosteroid rather than an NSAID like DCF, it was chosen as a clinically relevant positive control due to the high efficacy of i.a. corticosteroids in short-term pain relief of knee osteoarthritis.29,30 Free i.a. diclofenac is not used as a treatment option in a clinical setting due its short half-life of only 5 h in synovial fluid,9 and it has been shown that i.a. injection of free NSAIDs did not influence the gait of rats in the short or long-term.31

Reactivation of the inflammatory response was carried out on days 0 and 7, and knee caliper measurements and gait analysis were performed throughout the course of the
experiment to assess the efficacy of the administered treatments. Knee caliper measurements estimated the degree of inflammation by determining swelling of the arthritic right knee joint compared to the normal left knee joint (Figure 7). Priming induced significant knee joint swelling in all animals, and this had reduced to near-baseline levels after 2 weeks and remained stable over the course of the experiment in the group that was not reactivated (Figure 7A). Reactivation caused a spike in swelling on day 2 and day 9 in the vehicle control group, whereas the group treated with C-DCF-RH-PU experienced a spike in swelling only after the first reactivation, with minimal swelling after the second reactivation. The triamcinolone positive control was effective throughout the course of the experiment compared to vehicle control, although the well-documented time limitation of this treatment due to its rapid clearance from the joint8 emerged in the second reactivation, when some swelling was observed, and there was no significant difference in caliper measurements between triamcinolone and C-DCF-RH-PU treated animals (Figure 7).

This finding correlates with a comparison of the effect of liposomal formulations of diclofenac and the corticosteroid dexamethasone, which is the same class of drug as triamcinolone, on joint swelling.11 An i.a. corticosteroid was found to be faster acting, with a decrease in inflammation observed within 3 days of administration of treatment, whereas the effect of i.a. liposomal diclofenac was only observable after 10 days.11 This difference can be attributed to the differences in the mechanism of action between NSAIDs and corticosteroids as well as the fact that corticosteroids generally show stronger anti-inflammatory properties.12 It has also been shown that other i.a. DCF-releasing depot formulations exhibit a similar time lag in exerting an effect on joint swelling, while i.a. free DCF is faster acting, albeit with a very limited time span of action.10,12 Therefore, for future applications, it may be beneficial to combine these effects in a single formulation by including unconjugated DCF in the microgel formulation to take advantage of the short-term effect of free diclofenac and the long-term effect of conjugated DCF released over time.

The level of pain associated with the inflammation can be assessed by analysis of gait deficiency (Figure 8), where higher scores indicate increasingly uneven use of right and left leg when walking due to pain in the arthritic right knee joint. Gait scores for the group that was not subjected to reactivation were stable throughout the experiment, whereas both reactivations caused a significant increase in gait score in vehicle control animals and to a slightly lesser extent in C-DCF-RH-PU treated animals (Figure 8). The triamcinolone positive control was effective after both reactivations but in agreement with results from the knee caliper measurements a decrease in efficacy was observed following the second reactivation (Figure 8).

A decrease in pain would be expected to coincide with a decrease in joint swelling, and this was the case in the triamcinolone-treated group. Because of the time lag in the

Figure 7. Knee caliper measurement differences between arthritic right knee joint and normal left knee joint to assess inflammation, showing (A) knee caliper differences for each treatment group on individual days and (B) AUC calculated for both reactivation periods as well as the full experiment. Data are displayed as mean ± SEM. Statistical analysis: two-way ANOVA with Tukey post hoc test. *** P ≤ 0.001, **** P ≤ 0.0001.

Figure 8. Gait deficiency analysis to assess pain in arthritic right knee joint during walking, showing (A) gait scores for each treatment group on individual days and (B) AUC calculated for both reactivation periods as well as the full experiment. Data are displayed as mean ± SEM. Statistical analysis: two-way ANOVA with Tukey post hoc test. * P ≤ 0.05, *** P ≤ 0.001, **** P ≤ 0.0001.
reduction in joint swelling observed in the C-DCF-RH-PU-treated group (Figure 7), it is possible that a similar time lag affects the efficacy of pain reduction and that this may be addressed through the addition of free diclofenac to the formulation. Nonetheless, over the course of the full study, a small but statistically significant reduction in gait deficiency was observed in the C-DCF-RH-PU-treated group (Figure 8B). This was achieved with a relatively low dose of polymer-conjugated DCF (equivalent to 0.24 mg free DCF) compared to other studies where i.a. delivery systems carried up to 1 mg of an NSAID.12,13

Histopathology (Figure S3) showed good tolerability of the polymer–drug conjugate over the course of the study and was consistent with observed live-phase symptoms of joint swelling and pain. This correlates with good biocompatibility of polyurethanes in general, established through their applications in the food industry and for medical devices.33,34 However, more detailed studies around biocompatibility and clearance of the C-DCF-RH-PU formulation for long-term treatment of the knee joint will be necessary. Direct comparison of our i.a. DCF-delivery system with others is difficult due to differences in animal models and methodologies used. However, the performance of C-DCF-RH-PU was not inferior to other systems tested that encapsulate drug into delivery vehicles,10−13,35 and as already highlighted, this polymer system allows great flexibility for further modifications such as addition of a bolus dose of free DCF, altering the DCF dose administered, extending the time frame of treatment by adjusting the amount of PEG in the polymer architecture, and introduction of biodegradation points to optimize clearance of the hydrogel once the drug is depleted.16

■ CONCLUSION
A cross-linked hydrogel conjugated with DCF via a substituted phenyl ester linker was synthesized and formulated for i.a. injection. In vitro studies showed zero-order drug release for 4 days and a gradual decline in release rate until drug was depleted from the polymer after 2 weeks. In contrast to a small molecule control, the DCF-conjugated polymer was retained in the knee joints of healthy rats for 2 weeks, although some leakage was observed. After a short lag phase, C-DCF-RH-PU was moderately effective in reducing pain and very effective in treating knee joint swelling in a rat arthritis in vivo model.8B). This was achieved with a relatively low dose of polymer−drug conjugate DCF−delivery system with others is difficult due to differences in animal models and methodologies used. However, the performance of C-DCF-RH-PU was not inferior to other systems tested that encapsulate drug into delivery vehicles,10−13,35 and as already highlighted, this polymer system allows great flexibility for further modifications such as addition of a bolus dose of free DCF, altering the DCF dose administered, extending the time frame of treatment by adjusting the amount of PEG in the polymer architecture, and introduction of biodegradation points to optimize clearance of the hydrogel once the drug is depleted.16

■ ASSOCIATED CONTENT
2 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsabm.9b00232.

Synthetic schemes, NMR characterization of monomers, NMR and composition of L-DCF-PU, in vivo and histopathology scoring schemes, knee joint histological analysis (PDF)

■ AUTHOR INFORMATION
Corresponding Authors
*E-mail: john.f.quinn@monash.edu.
*E-mail: Russell.tait@polyactiva.com.

Friederike M. Mansfeld: 0000-0002-0768-8763
Anton Blencowe: 0000-0002-7630-4874

Greg G. Qiao: 0000-0003-2771-9675
John F. Quinn: 0000-0002-4593-1170
Michael R. Whittaker: 0000-0001-5706-3932
Thomas P. Davis: 0000-0003-2581-4986

Author Contributions
©A.S. and F.M.M. contributed equally to this work.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
This collaborative research was conducted by PolyActivia Pty Ltd, University of Melbourne (ARC Linkage Grant No. LP12020010) and the Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology (Project No. CE140100036) (ARC Linkage Grant No. LP140100284). T.P.D. wishes to acknowledge the award of an Australian Laureate Fellowship.

■ REFERENCES
(13) Zhang, Z.; Huang, G. Intra-articular lornoxicam loaded PLGA microspheres: enhanced therapeutic efficiency and decreased systemic


