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Sustained intra-articular release of celecoxib from *in situ* forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses

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ABSTRACT

In this study, the intra-articular tolerability and suitability for local and sustained release of an *in situ* forming gel composed of an acetyl-capped poly(ε-caprolactone-*co*-lactide)-*b*-poly(ethylene glycol)-*b*-poly(ε-caprolactone-*co*-lactide) (PCLA-PEG-PCLA) copolymer loaded with celecoxib was investigated in horse joints.

The systems were loaded with two dosages of celecoxib, 50 mg/g ('low CLB gel') and 260 mg/g ('high CLB gel'). Subsequently, they were injected into the joints of five healthy horses. For 72 h after intra-articular injection, they induced a transient inflammatory response, which was also observed after application of Hyonate®, a commercial formulation containing hyaluronic acid for the intra-articular treatment of synovitis in horses. However, only after administration of the 'high CLB gel' the horses showed signs of discomfort (lameness score: 1.6 ± 1.3 on a 5-point scale) 1 day after injection, which completely disappeared 3 days after injection. Importantly, there was no indication of cartilage damage. Celecoxib C_{max} in the joints was reached at 8 h and 24 h after administration of the 'low CLB gel' and 'high CLB gel', respectively. In the joints, concentrations of celecoxib were detected 4 weeks post administration. Celecoxib was also detected in plasma at concentrations of 150 ng/ml at day 3 post administration and thereafter its concentration dropped below the detection limit.

These results show that the systems were well tolerated after intra-articular administration and showed local and sustained release of celecoxib for 4 weeks with low and short systemic exposure to the drug, demonstrating that these injectable *in situ* forming hydrogels are promising vehicles for intra-articular drug delivery.

1. Introduction

Osteoarthritis (OA) is a major cause of disability in the elderly [1]. OA is, among others, characterized by progressive loss of cartilage in the joint, which disrupts its functional integrity, and is accompanied by chronic pain [2]. The current treatment options for OA are merely symptomatic, focusing on attenuation of inflammation and pain relief [3]. The most commonly used approach to treat inflammation and pain associated with OA is administration of non-steroidal anti-inflammatory drugs (NSAIDs) [4]. These drugs exert their effect by inhibiting cyclo-oxygenase (COX), a key enzyme involved in the conversion of arachidonic acid to prostaglandins, mediators of pain and inflammation. At present, three COX isoforms are known: COX-1, COX-2 and COX-3. The first-line drug for the treatment of OA is the COX-2 inhibitor celecoxib [5,6]. Although celecoxib induces fewer side effects than less specific NSAIDs (e.g., naproxen and ibuprofen), prolonged use and high dosages increase the risk for cardiovascular events [7]. Hence, local drug delivery might be suitable to avoid drug-related systemic side effects [8–14].

One of the strategies to reduce drug disposition to other organs than the target tissue is the use of drug-loaded *in situ* forming

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systems [15–17]. In particular, systems composed of ABA triblock copolymers (a PEG middle block flanked by polyester blocks of diverse compositions) meet the basic requirements for drug delivery systems (DDSs), e.g., encapsulation efficiency close to 100%, burst < 10%, sustained and controlled release for prolonged duration and complete drug recovery [16,18–21], while being easy to inject and having the capacity of forming gels at body temperature. Indeed, these systems have been shown to be suitable for loading and release of hydrophobic drugs [22–24]. A good example is the paclitaxel-loaded system based on poly(lactide-*co*-glycolide)-*b*-poly(ethylene glycol)-*b*-poly(lactide-*co*-glycolide) (PLGA-PEG-PLGA) [25]. The release of paclitaxel from this system is mediated by a combination of diffusion and chemical polymer degradation, which takes around six weeks in vitro as well as in vivo [22–24].

For some applications however, longer release times are required. Therefore, to slow down hydrolysis and increase the degradation time, the PLGA blocks have been replaced by poly(ε-caprolactone-*co*-lactide) (PCLA) blocks. Systems based on PCLA-PEG-PCLA are indeed stable for longer times depending on the ratio of caproyl units (CL) to lactoyl units (LA). For instance, systems with PCLA blocks containing 70 mol% CL showed complete degradation in about six months [26,27]. Moreover, modification of the terminal hydroxyl groups of these triblock copolymers allows modulating the rheological and degradation properties of the *in situ* forming systems made of these copolymers [28,29]. These systems showed sustained release of celecoxib after subcutaneous injection and are well tolerated after intra-articular (i.a.) injection in rats [21,30].

The rat is an inappropriate animal model for frequent sampling of synovial fluid for local drug concentration determination. Therefore in the present study we used the horse, which is a much more suitable animal model to further investigate whether these systems are suitable for i.a. and sustained release of drugs. Also, horses have been shown to be a good translational animal model for OA, with treatment outcomes relevant for human OA [31–33]. Further, when developing new gels for i.a. administration, it is interesting to compare their tolerability with that of a registered hyaluronic acid gel (Hyonate®), clinically used for the treatment of horses suffering from lameness due to non-septic joint disease, which is usually accompanied by some form of synovitis [34]. Hyonate® is an aqueous solution of sodium hyaluronate, which due to polymer entanglements has high viscosity. Even though its efficacy is controversial, Hyonate® is used as a lubricant and has shock-absorbing as well as anti-inflammatory properties when injected into a joint. It is clinically used to replenish the hyaluronic acid concentration in the joint, which is known to be decreased in the synovial fluid of both human and equine OA patients [35–38].

In the present study, we investigated the tolerability of hydrogels composed of an acetyl-capped poly(ε-caprolactone-*co*-lactide)-*b*-poly(ethylene glycol)-*b*-poly(ε-caprolactone-*co*-lactide) (PCLA-PEG-PCLA) triblock copolymer for i.a. administration and performed a comparative study with Hyonate®. Also, we investigated the suitability of these systems to release locally celecoxib in a sustained and prolonged manner. We administered our systems in the joints of healthy horses.

2. Experiments and protocols

2.1. Materials

L-lactide (purity > 98%) was obtained from Purac Biochem, The Netherlands. Celecoxib (USP Pharm.) was obtained from LC Laboratories, USA. Hyonate® was purchased from Bayer Animal Health. Sterile water for injection was purchased from B. Braun Medical. Dihydroxyl-PEG_{1500} and all other chemicals were obtained from Aldrich and used as received.

2.2. Synthesis of fully acetyl-capped PCLA-PEG-PCLA

The synthesis of acetylated PCLA-PEG-PCLA was performed essentially as previously described [28–30,39]. Briefly, in a three-neck round-bottom flask equipped with a Dean Stark trap and a condenser, 50 g PEG_{1500} (1 mol eq.), 22 g L-lactide (4.6 mol eq.), 88 g ε-caprolactone (23 mol eq.) and 600 ml toluene were introduced and, while stirring, heated to reflux under nitrogen atmosphere. The solution was azeotropically dried by distilling off toluene/water (~100 ml). Next, the solution was cooled to ~90 °C and 0.214 g tin(II) 2-ethylhexanoate (0.016 mol eq. relative to PEG hydroxyl groups) was added. Ring opening polymerization was performed at reflux for 16 h (i.e. 110–120 °C, the boiling temperature of the solution of toluene, PEG, caprolactone and lactide) under nitrogen atmosphere. Subsequently, the solution was cooled to ~90 °C, followed by addition of 20 g triethylamine (6 mol eq.) and 11 g acetic anhydride (3.3 mol eq.). The reaction mixture was allowed to reflux for ~4 h.

The solution was slowly dropped into 2 l of a 1:1 mixture of pentane and diethyl ether to precipitate the polymer. Upon storage at −20 °C, the polymer separated as waxy solid from which non-solvents containing unreacted monomers, unreacted acetic anhydride and formed acetic acid could be decanted easily. The precipitated polymer was dried in vacuo.

2.3. ^1H NMR analysis

^1H NMR analysis of the polymers dissolved in $CDCl_3$ was performed using a Varian Oxford, operating at 300 MHz. ^1H NMR spectra were referenced to the signal of chloroform at 7.26 ppm.

PCLA-PEG-PCLA backbone: 5.25–4.95 ($I_{5.1}$, 1H,–CO–C**H**(CH_3)–O-); 4.35–4.23 (2H,-PEG-O–CH_2–C**H**$_2$–O–C(O)–CH(CH_3)–O- + 1H,–CO–C**H**(CH_3)–OH); –4.15 (2H, PEG-O–CH_2–C**H**$_2$–O–C(O)–$(CH_2)_4$-); 4.15–4.05 (2H, –CO$(CH_2)_4$–C**H**$_2$–O–CO–CH(CH_3)–O–); 4.05–3.95 (2H, –CO$(CH_2)_4$)–C**H**$_2$–O–C(O)-$(CH_2)_4$)–); 3.85–3.25 ($I_{3.6}$, 132H, –O–C**H**$_2$–C**H**$_2$–O– + 2H, CO$(CH_2)_4$)–C**H**$_2$–OH); 2.50–2.35($I_{2.3,}$ 2H, –O–CO–CH(CH_3)–O–CO–C**H**$_2$–$(CH_2)_4$); 2.35–2.20 ($I_{2.2,}$ 2H, –O–CO–CH_2-$(CH_2)_4$–O–CO–C**H**$_2$–$(CH_2)_4$); 1.80–1.55 (2H, –CO–CH_2–C**H**$_2$–CH_2–C**H**$_2$–CH_2–O–); 1.58–1.44 (3H, –CO–CH(C**H**$_3$)–O–); 1.45–1.25 (2H, –CO–CH_2–CH_2–C**H**$_2$–CH_2–CH_2–) [28,30].

Acetyl-end group: 2.14–2.12 ppm ($I_{2.13}$, C**H**$_3$–CO–O–CH(CH_3)-); 2.03–2.05 ppm ($I_{2.04}$, C**H**$_3$–CO–O–C$(H_2)_5$-) and 2.10–2.08 ppm ($I_{2.09}$, C**H**$_3$–CO–O–C$(H_2)_2$–O-) [28,30].

2.4. Preparation of celecoxib-loaded PCLA-PEG-PCLA mixtures

Before preparing the systems, the glassware was depyrogenized at 260 °C for 2 h. Use was made of the solubility of celecoxib (~300 mg/ml) and acetylated PCLA-PEG-PCLA triblock copolymers (>300 mg/ml) in ethyl acetate to prepare celecoxib/polymer solutions with celecoxib/polymer weight ratios between 14/86 and 54/46 w/w. The solutions were filtered with 0.2 μm PTFE filters, aseptically transferred into 6-cm Petri dishes and ethyl acetate was evaporated under nitrogen flow for 48 h.

2.5. Preparation of gels of the copolymer with and without celecoxib

Before preparing the systems, the glassware was depyrogenized at 260 °C for 2 h. For the unloaded systems ('placebo'), 3.1 g of polymer was added to 6.9 g of phosphate buffer pH 7.4 (20 mM

Na_2HPO_4, 5 mM NaH_2PO_4, 120 mM NaCl prepared using sterile water for injection). For the systems loaded with 50 ('low CLB gel') and 260 mg/g celecoxib ('high CLB gel'), 3.6 g and 5.7 g of celecoxib/polymer mixture (containing 3.1 g polymer mixed with 0.5 and 2.6 g celecoxib, respectively) was added to 6.4 and 4.3 g of the same buffer, respectively. Subsequently, the samples were autoclaved for 15 min at 120 °C. In addition, the effect of the autoclaving on the properties of 25 wt% unloaded system was measured by GPC and rheometry as previously described [28–30]. Also the pH of the mixture after autoclaving was measured.

After autoclaving, the samples were vortexed for 1 min and subsequently incubated at 4 °C for 48 h to yield macroscopically homogeneous systems, i.e. a gel with fully dissolved celecoxib for the 'low CLB gel' and a paste containing undissolved celecoxib for the 'high CLB gel'. All handling steps were performed aseptically and resulted in samples containing low endotoxin (<0.1 EU/ml as assessed by a LAL test (PyroGene™, Lonza). To check for the presence of celecoxib crystals, samples were observed under a microscope (Nikon Eclipse TE2000U).

2.6. X-ray diffraction (XRD) analysis

X-ray diffraction patterns were recorded for celecoxib, the acetylated PCLA-PEG-PCLA triblock copolymer, the celecoxib-loaded acetylated PCLA-PEG-PCLA block copolymers and the celecoxib-loaded acetylated PCLA-PEG-PCLA block copolymer gel (31 wt% in phosphate buffer pH 7.4, as described above) using a Bruker-AXS D8 Advance powder X-ray diffractometer, in Bragg-Brentano mode, equipped with automatic divergence slit and a PSD Våntec-1 detector. The radiation used was cobalt Kα1,2, $\lambda = 1.79026$ Å, operated at 30 kV, 45 mA. The patterns were recorded at a sample-to-detector distance of 435 mm. Separate blank patterns were also recorded to allow subtraction of air- and capillary wall-scattering.

2.7. In vivo experimental set up

The study design was approved by the institutional Ethics Committee on the Care and Use of Experimental Animals in compliance with Dutch legislation on animal experimentation. 2 ml of unloaded gel formulation of acetylated PCLA-PEG-PCLA triblock copolymer ('placebo') was administered into the right middle carpal joint of five healthy geldings with clinically normal carpal and talocrural joints, as determined by radiographic evaluation. 2 ml Hyonate® was injected into the contra-lateral middle carpal joint. 2 ml of 50 mg/g celecoxib-loaded gel formulation ('low CLB gel') was injected into the left talocrural joint, while 2 ml of 260 mg/g celecoxib loaded formulation ('high CLB gel') was injected into the right talocrural joint 4 weeks later to allow quantification of the systemic exposure to celecoxib for both gels separately.

2.8. Evaluation of clinical response to the treatment

Lameness was semi-quantitatively evaluated by an experienced clinician using the 0 to 5 scale as established by the American Association of Equine Practitioners [31]. In this scale 0 means no lameness, 1 is a lameness that is inconsistently apparent under special circumstances (such as on the incline or on a hard surface), 2 is a subtle lameness that is consistently apparent under special circumstances, and 3–5 are cases of obvious lameness that is consistently present under all circumstances and of increasing severity. Lameness examinations were conducted at 0, 8, 24, 72 h and 1, 2, 3, 4 weeks (i.e. post injection of Hyonate®, 'placebo' and 'low CLB gel'). At 4 weeks, the right talocrural joint was injected with 'high CLB gel' and lameness examinations were performed again at the same time points (i.e. 0, 8, 24, 72 h and 1, 2, 3, 4 weeks post injection). Horses were monitored throughout the study for signs of discomfort.

2.9. Collection of synovial fluid and plasma

Synovial fluid samples (2 ml) from the treated joints were aspirated at 0, 8, 24, 72 h and 1, 2, 3, 4 weeks after the first series of injections (Hyonate®, 'placebo' and 'low CLB gel') as well as 4 weeks later with the same schedule after injection of 'high CLB gel'. A portion of the fluid was processed for white blood cell (WBC) count and total protein content. The remaining volume was centrifuged, and the supernatant was stored at −80 °C until celecoxib content determination. Blood was collected according to the same time scheme from the left jugular vein of the horses in heparinized vials and spun down for 3 min at 1,500 g to produce plasma. The plasma samples were used for celecoxib content determination.

2.10. Synovial fluid analysis

The synovial fluid WBC and total protein concentrations were determined using a Coulter Counter® Z1 (Beckman Coulter, Inc.) and refractometer [31,40], respectively. Protein concentrations were measured using a validated method based on refractometry [31,40] that is used in the clinic. Synovial fluid samples were also evaluated for glycosaminoglycan (GAG) concentrations using a modified 1,9-dimethylmethylene blue dye-binding assay as previously described [41]. To check for possible damage to the collagen network of the cartilage, we also measured the concentration of the C2C epitope of collagen in synovial fluid, as previously reported [42].

2.11. Celecoxib concentrations in the synovial fluid and serum

Celecoxib was extracted from the synovial fluid as well as from serum samples as follows. To 100 μl synovial fluid or serum sample, 100 μl internal standard (200 ng paracoxib (Dynastat, Pfizer) in 5% BSA) was added. Then, 200 μl 0.1 M sodium acetate buffer (pH 5) and 1 ml ethyl acetate were added and the samples were mixed thoroughly for 10 min. After that, the samples were centrifuged at 8000 g for 10 min and stored at −80 °C for at least 30 min. The upper ethyl acetate phase was transferred into an HPLC glass. The extraction was repeated on the remaining aqueous phase with 1 ml hexane which was pooled with the ethyl acetate fraction. After evaporation of the pooled solvents under a nitrogen flow, the residue was dissolved in 100 μl of a 3:1 vol:vol methanol:acetate buffer (0.1 M, pH 5), of which 10 μl was injected onto a Kinetex® C_{18} (150 × 3.0 mm, particle size of 2.6 μm) analytical column (Phenomenex, Utrecht, NL). Separation was performed at a flow rate of 250 μl/min, with a total runtime of 13 min. The mobile phases consisted of acetonitrile:water (1:1 vol:vol) (A), and acetonitrile:methanol (1:1 vol:vol) (B). Samples were separated using the following gradient A/B vol/vol: 0–2 min, 100/0; 2–8.5 min 25/75 to 0/100; 8.5–11 min, 0/100; 11–12 min 0/100 to 100/0; 12–13 min 100/0. The column effluent was introduced into an API3000 mass spectrometer by an atmospheric pressure chemical ionization (APCI) interface (Sciex, Toronto). 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 times and mass spectrum of celeoxib and paracoxib. For each component two ion transitions were monitored, celecoxib: 380.3 → 316.3 and 380.3 → 276.3 (collision energy: −50 V, both), and paracoxib (Dynastat, Pfizer): 369.3 → 250.2 and 369.3 → 234.2 (collision energy: −30 V, both). The following MS parameters were used: nebulizer gas: 10 psi; curtain gas: 10 psi; ion current: −2 μA; source

temperature: 500 °C; gas flow 1: 30 psi; gas flow 2: 20 psi: decluster potential: −70 V and entrance potential: −10 V. Data were analyzed with Analyst software version 1.4.2 (Applied Biosystems, The Netherlands). Celecoxib peak areas were corrected for the recovery of paracoxib, and concentrations were calculated using celecoxib standards with concentrations ranging from 5 pg to 20 ng/ml. The celecoxib standards were prepared by 1:2 (vol:vol) serial dilutions starting with 20 ng/ml celecoxib, all in normal equine serum or synovial fluid. To 100 μl of each of the resulting celecoxib standards, ranging from 20 ng/ml to 5 pg/ml, 100 μl internal standard (200 ng paracoxib) was added, and the celecoxib was extracted and analysed as described before. The resulting standard curve, after correction of the paracoxib extraction efficacy, was linear within the range of the used standards ($r = 0.998$). The average extraction efficiency was 68%. Further calculations on the pharmacokinetics were done using the freeware PK solver [43]. AUC was calculated using the "linear trapezoidal method".

2.12. Solubility of celecoxib in 10% fetal bovine serum

Celecoxib has a very low aqueous solubility (<1 μg/ml) with a large apparent volume of distribution (>~1 l/kg) due to its high plasma protein binding (~97%) [44]. As the protein content of equine synovial fluid is 1–2 g/dl [41,42,45], we investigated the effect of plasma protein on the solubility of celecoxib in cell medium (DMEM 31965 + pen/strep) with 10% fetal bovine serum (FBS, 30 g/dl [46]). Therefore, an excess of celecoxib (~100 mg) was added to 1 ml of cell medium with FBS, which was incubated for 24 h at room temperature and after centrifugation for 5 min at 3500 rpm, the sample was filtered using a 0.2 μm filter. Subsequently, the proteins present in the sample were precipitated by adding 1 vol of acetonitrile. The celecoxib concentrations in the supernatant were determined by UPLC using a Waters UPLC system equipped with a Waters column (BEH C18 1.7 μm, size: 2.1 × 100 nm). Standards were prepared by dissolving celecoxib in DMSO at 5 mg/ml. This celecoxib solution was diluted 10 times with DMSO and subsequently with buffer containing Tween® 80 to prepare celecoxib standards used for calibration (final celecoxib concentration ranged from 0.5 to 100 μg/ml). Two eluents containing 0.1 vol% trifluoroacetic acid (TFA) were used: 95:5 vol:vol acetonitrile/water (Eluent A) and 45:45:10 vol:vol:vol methanol/acetonitrile/water (Eluent B); the elution rate was 0.08 ml/min, and the column temperature was 50 °C. A gradient was run from 100% Eluent A to 100% Eluent B in 2 min and kept at 100% B for 10 min before returning to 100% Eluent A. Detection was performed with a UV detector at 254 nm and the injection volume was 10 μl. The retention time of celecoxib was 10.5 min with a total run time of 16 min. The autosampler temperature was 20 °C.

2.13. Histological analysis of articular cartilage

8 weeks after the start of the study, i.e. the administration of Hyonate®, 'placebo' and 'low CLB gel', and thus 4 weeks after the injection of 'high CLB gel', the animals were sacrificed. Full thickness cartilage was harvested from the injected joints and fixed in buffered formaldehyde 4% solution (Klinipath, The Netherlands) and, after embedding in paraffin, sections (5 μm) were cut. The sections were deparaffinized and stained as previously described by Gawlitta et al. [47].

2.14. Statistical analysis

Synovial fluid data (WBC, total protein, GAG content, C2C epitope) were compared using two-way ANOVA for repeated measures (GraphPad Prism 5). Significance level was set at $p \leq 0.05$; differences were further analyzed using a Bonferroni post-hoc test. The data are presented as mean ± SEM.

3. Results and discussion

3.1. Synthesis and characterization of fully acetylated PCLA-PEG-PCLA

The synthesis of PCLA-PEG_{1500}-PCLA was performed by ring opening polymerization of L-lactide and ε-caprolactone in solution with PEG_{1500}-diol as macroinitiator in the presence of tin(II) 2-ethylhexanoate as catalyst. Subsequently, acylation of hydroxyl-end groups using an excess of acetic anhydride resulted in the formation of acetyl-capped PCLA-PEG-PCLA with a yield of 81%. The ^{1}H NMR spectrum of the polymer is given in Fig. 1 and shows the characteristic peaks at 5.25–4.95, 3.65–3.55 and 2.50–2.10 ppm corresponding to the methine protons of LA, the methylene protons of PEG and the methylene protons of caproyl units [28–30]. Also, ^{1}H NMR analysis shows that the acetyl-capped triblock copolymers have peaks belonging to the methyl protons of acetyl-end groups at 2.14–2.03 ppm [28]. The composition of the polymers was determined according to Equations A.1 to A.13 as described previously [28–30]. The molar ratio of CL to LA (as defined in Equation (A.7)), k, was calculated from the ^{1}H NMR spectrum and found to be 2.7 mol/mol, in accordance with the CL/LA feed of 2.5 mol/mol. The average length of CL sequences was calculated according to Equation (A.13) and found to be 4.3. This indicates an incomplete random distribution of the monomers in PCLA as previously reported for similar polymers [28–30]. The number molecular weight (M_n) of the synthesized polymer as determined by ^{1}H NMR analysis (Equation (A.12)) was 4500 g/mol, which is slightly below the M_n of 4900 g/mol expected from the feed. The extent of acetylation, calculated by comparing the methyl peaks of end groups to the methylene peak of PEG measured by ^{1}H NMR, was found to be ~100% indicating that the polymer was fully capped with acetyl end-groups. Table 1 summarizes the characteristics of the polymer.

3.2. Characteristics of celecoxib-loaded acetylated PCLA-PEG-PCLA gels

Fig. 2A shows a microscopic photograph of a 'high CLB gel' (a celecoxib-loaded gel made of acetylated PCLA-PEG-PCLA triblock copolymers in buffer containing celecoxib at 260 mg/g (i.e. 26 wt%) and 31 wt% polymer). Needle-shaped crystals (~500 × 1 μm) were clearly visible which were neither present in the unloaded systems ('placebo') nor in the 50 mg/g celecoxib ('low CLB gel'), as reported previously for similar gels of 25 wt% polymer in buffer [21]. X-ray analysis convincingly showed that celecoxib crystals were present (Fig. 2B). Thus, as previously reported [21], celecoxib up to 50 mg/g was fully dissolved in the gel, but present in the form of crystals at 260 mg/ml.

The formulation without celecoxib as well as that loaded with 50 mg celecoxib/g ('low CLB gel') were injectable sols at room temperature [21] and gels at 37 °C. However, 'high CLB gel' was a paste at room temperature and above, but could still be injected through an 18G needle, which is the size mostly used to inject medications into horse joints.

The main reason why gels with these low and high doses (50 and 260 mg/g) were selected for the in vivo studies was because celecoxib is homogenously dispersed in the gel matrix at the low dose whereas the high dose (in which celecoxib is released from the crystallized form) was similar to the dose in previously reported depot systems [48]. We anticipated that these different formulations would result in different PK profiles which indeed turned out to be the case.

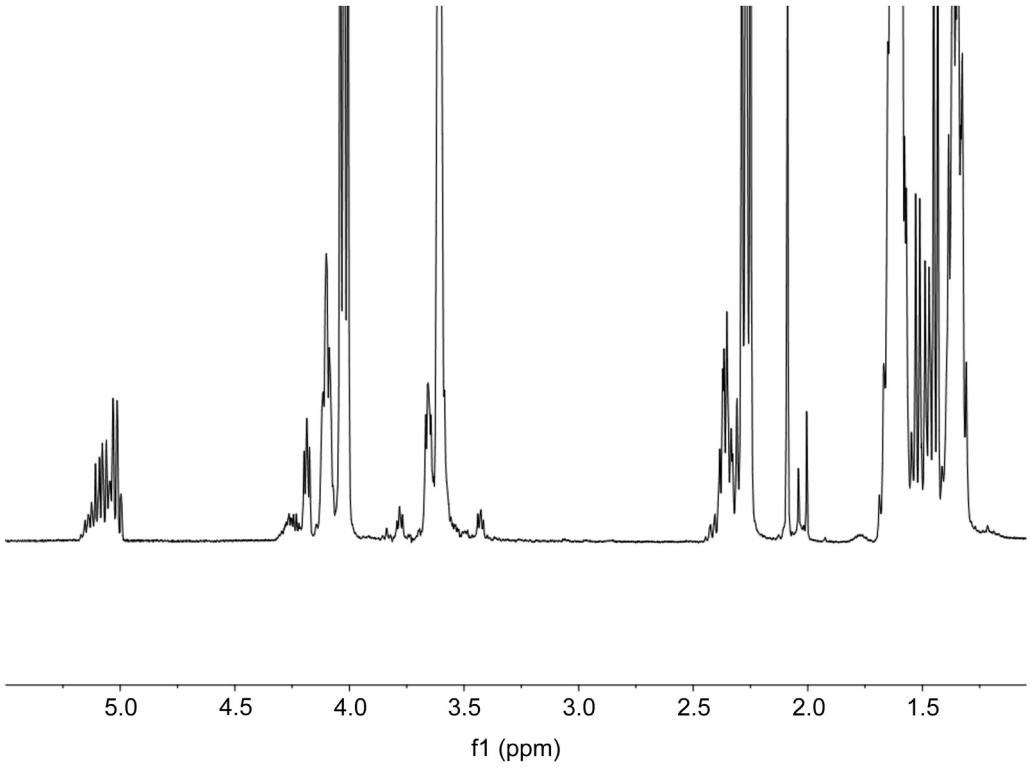

Fig. 1. ^{1}H NMR spectrum of acetyl-capped PCLA-PEG-PCLA in $CDCl_3$.

Table 2 shows that no significant effects of the autoclaving of unloaded systems of 25 wt% in buffer compared to control were observed as measured by GPC and oscillatory rheology measurements, even though a slight decrease of ~40 Pa was observed for G'_{max} (i.e. the maximum value of G′ in the temperature range 4–50 °C). Table 2 shows a slight drop in pH from 7.1 to 6.9 after autoclaving, which might indicate formation of traces of acidic degradation products. Sterilization of polyester-based drug delivery systems by autoclaving has not been reported, likely because significant hydrolysis of the polymers will occur. We hypothesized that autoclaving of our systems with only minimal hydrolysis of the polyester chains is possible because of the presence of end-capping groups, which slows down back biting reactions and thus in turn degradation [21,28–30].

Table 1
Characteristics of the acetyl-capped PCLA-PEG-PCLA triblock copolymer used in this study.

Polymer	Acetyl-PCLA-PEG-PCLA-acetyl
PEG feed [g]	50
ε-caprolactone feed [g]	88
Lactide feed [g]	22
Acetic anhydride feed [g]	11.1
Aimed M_n	4900
Yield [%]	81
PCLA/PEG[a]	1.9
CL/LA (mol/mol)[b]	2.7
DM[c]	~2
M_n[d] [g/mol]	4300

[a] PCLA/PEG: the weight ratio of PCLA to PEG determined by ^{1}H NMR.
[b] CL/LA: the weight ratio of ε-caprolactone to L-lactide determined by 1H NMR.
[c] DM: the degree of modification represents the number of end groups per triblock copolymer determined by ^{1}H NMR.
[d] Mn: number average molecular weight determined by ^{1}H NMR.

3.3. Clinical response to intra-articular injection of the gels

All horses were free of lameness prior to injection and none of the limbs injected with Hyonate®, 'placebo' or 'low CLB gel' showed signs of lameness throughout the study. However, four of the five horses, which received an injection of 'high CLB gel' in their left tarsus showed signs of lameness 24 h post injection (average lameness score was 1.6 ± 1.3 on a 5-point scale). The lameness was only temporary as at 72 h post injection it had disappeared. These temporary adverse clinical symptoms can be ascribed to the acute inflammatory response to 'high CLB gel', likely triggered by the presence of needles of crystalline celecoxib (Fig. 2A). Any cytotoxic effect of the high dose of CLB is unlikely (see 3.4).

3.4. Synovial fluid analysis

Fig. 3 shows the WBC count, total protein count, GAG content and C2C content in synovial fluid prior and post injection of Hyonate®, 'placebo' and celecoxib-loaded gels. At 8 h post injection, the synovial fluid from joints injected with Hyonate®, 'low CLB gel' or 'high CLB gel' all had an increased WBC count (>10 × 10^9 cells/l) compared to synovial fluid from joints injected with 'placebo'. At 24 h post injection, only WBC in joints treated with Hyonate and 'high CLB gel' remained significantly increased (~30 and ~20 × 10^9 cells/l, respectively). At 72 h post injection, the WBC count of all injected joints returned to control levels (~2 × 10^9 cells/l) and remained at this level until the end of the study (Fig. 3A), indicating that the inflammatory response to the gels was transient and of a similar magnitude as after Hyonate® injection.

Concentrations of synovial fluid proteins are known to vary with the extent of joint inflammation [31,40,42]. At 8 h post injection, the synovial fluid from joints injected with 'placebo' or 'high CLB gel' had an increased protein content (~4 g/dl) compared to

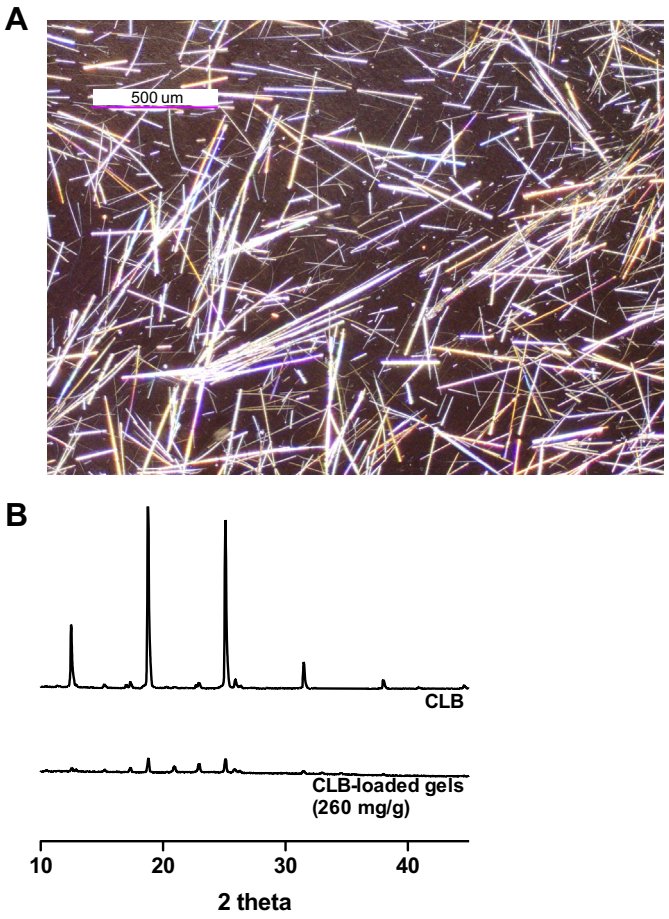

Fig. 2. Samples of 'high CLB gel' (260 mg celecoxib per g gel). (A) Microphotograph (magnification = 10x). (B) X-ray diffraction pattern of celecoxib and the gel.

synovial fluid from joints injected with Hyonate or 'low CLB gel' (1–2 g/dl). At 24 h post injection, the synovial fluid from joints injected with 'placebo' or 'high CLB gel' had still an increased protein content of ~4 g/dl, whereas the synovial fluid from joints injected with 'low CLB gel' remained close to baseline, and that of Hyonate® had an increased protein content of ~6 g/dl. Just like WBC, the total protein concentrations returned to control levels 72 h post injection (Fig. 3B), again indicating that the inflammatory response due to the injected gel was transient and in the same range as after Hyonate® injection.

To determine whether the inflammatory response had a detrimental effect on the cartilage, the GAG content as well as the concentration of the C2C epitope of collagen in the synovial fluid samples was measured. Fig. 3C shows the total GAG content in the synovial fluid samples. At 8 h post injection, the synovial fluid from all injected joints showed a GAG content in the range of the baseline level (50 μg/ml). At 24 h post injection, the synovial fluid from joints injected with Hyonate® or 'high CLB gel' had an increased GAG content (271 and 197 μg/ml, respectively). Just like WBC and protein content, the GAG concentrations returned to control levels 72 h post injection, again indicating that the inflammatory response due to the injected gel was transient and in the same range as after Hyonate® injection.

Fig. 3D shows the concentration of the C2C epitope of collagen type II, a biomarker for cartilage damage [31,42]. There were no significant differences in level of C2C epitope of collagen between the Hyonate®, 'placebo' and the celecoxib-loaded gels indicating that the treatments did not have adverse effects on the collagen component of the cartilage.

These findings are in line with data from a separate in vitro study (JC de Grauw et al., in preparation), in which the effect of celecoxib on healthy and re-IL-1beta stimulated equine articular cartilage explants was studied. Data for glycosaminoglycan (GAG) release (a very sensitive parameter for early cartilage damage) at 24 and 48 h showed that a dose of 100 μM (38 μg/mL) CLB did not have an effect on GAG release (Fig. 4A,B). The maximum concentration measured in the in vivo experiment was about 36 μg/mL (see 3.5), which is practically equivalent with the 100 μM concentration in the in vitro study.

To further confirm the harmlessness of the injected materials, histological staining of cartilage harvested from the injected joints was performed. Fig. 5A–D shows representative histological sections of cartilage from the joints injected with Hyonate®, 'placebo' and the celecoxib-loaded gels. The surface of the cartilage was smooth, indicating that the treatments had not damaged the structural components of the cartilage, even though one may have hypothesized that the presence of celecoxib crystals in the 'high CLB gel' could have hindered tolerability. Moreover, the intact cartilage stained deeply reddish with safranin-O for glycosaminoglycans, which further demonstrates that no lasting cartilage damage occurred, as there were no differences between Hyonate®, 'placebo', 'low CLB gel' and 'high CLB gel'. Any GAG loss from the extracellular matrix of the cartilage, as may have occurred shortly after the injection given the temporary rise in synovial fluid GAG content, was hence repaired in the following period. It is known that in articular cartilage damage GAG loss is reversible, as long as the collagen network remains intact [49]. The fact that no increase occurred in C2C content demonstrates there was no significant damage to the collagen network in our study. Thus, the gels were well tolerated. Larsen et al. [48] reported the injection into equine carpal joints of 2 ml *in situ* forming depot composed of PEG_{400} and celecoxib loaded at 290 mg celecoxib per g solution, which is similar to the dose of the 'high CLB gel' (2 ml of 260 mg celecoxib per g gel). They did however report the presence of granulomatous synovitis 10 days after i.a. injection of the PEG_{400} solution of celecoxib, and the synovial membrane being hyperaemic, which indicate tolerability issues of their system. Histological examination of the synovial membrane of the joints from this study did not show signs of granulomatous synovitis, or any other form of overt inflammation (Fig. 6). The difference in degree of synovial membrane inflammation cannot be explained unambiguously explained, as no direct comparison was made between the two approaches. Given the similar celecoxib concentrations, the most likely explanations are either an influence of the carrier, or carrier–celecoxib combination, or the differences in follow-up time. In the study by Larsen et al. horses were euthanized at 10 days post injection, in the present study not before day 28 when all inflammatory effects in the synovial fluid (Fig. 3) had subsided since long. We cannot exclude that at day 10 after injection more signs of synovitis were present/visible than at four weeks after injection.

Table 2
Effect of autoclaving on acetyl-capped PCLA-PEG-PCLA of 25 wt% in buffer as measured by ^{1}H NMR, GPC, oscillatory rheometry and pH.

Treatment	$M_{n,NMR}$ [g/mol]	GPC		G'_{max} [Pa]	pH
		M_n [g/mol]	PDI		
None	4600	4900	1.40	192	7.1
121 °C for 15 min	4600	5000	1.42	154	6.9

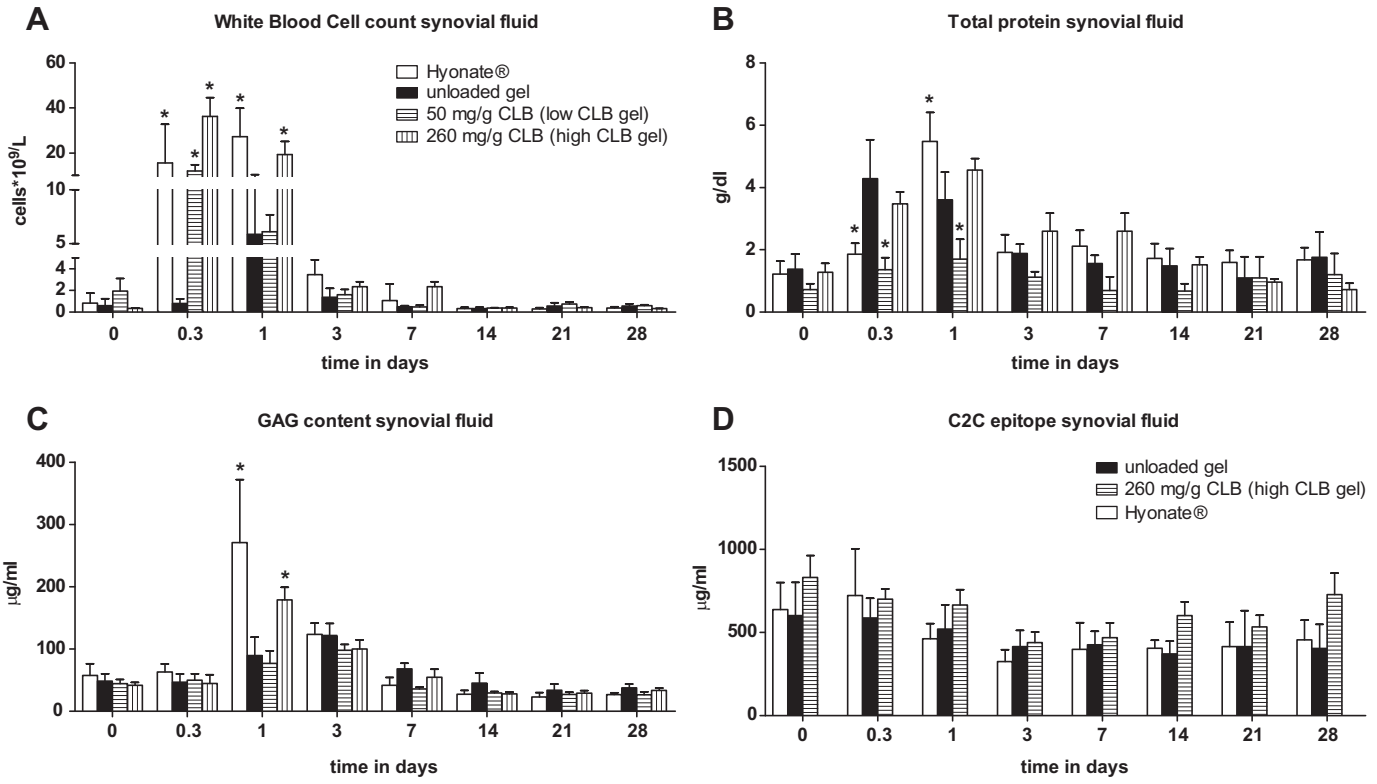

Fig. 3. Synovial fluid analysis. (A) White Blood Cell count. (B) Total protein levels. (C) GAG content. (D) C2C content. Values are depicted as mean ± sem (n = 5); *: $p < 0.05$ vs. 'placebo'.

3.5. *In vivo release of celecoxib from gels administered in the horse joints*

After administration of the 'low CLB gel' and 'high CLB gel', the celecoxib concentrations in synovial fluid as well as in plasma were measured over time. Fig. 7 shows the celecoxib concentrations in synovial fluid. Celecoxib concentration in synovial fluid 8 h after i.a. administration was 20–25 µg/ml and independent of the type of gel administered. For the 'low CLB gel', the C_{max} of celecoxib in synovial fluid was reached at 8 h (four animals) and 24 h (one animal) post injection (23.6 ± 6.6 µg/ml). For the 'high CLB gel', the C_{max} of celecoxib in synovial fluid was reached at 8 h (three animals) and 72 h (one animal) with a t_{max} at 24 h for one animal and was 36 ± 14 µg/ml. The high values of the C_{max} are in line with celecoxib solubility in 10% FSB solution, which was found to be 10–20 µg/ml likely due to celecoxib solubilization by proteins. After t_{max}, the celecoxib concentration in synovial fluid of the joints treated with 'low CLB gel' rapidly decreased in time reaching a concentration of 0.034 ± 0.041 µg/ml 7 days post injection, whereas the celecoxib concentration in synovial fluid taken from the joints that received 'high CLB gel' decreased more slowly to reach 1.6 ± 1.4 µg/ml at day 7 and finally dropped at day 28 below 0.1 µg/ml. The calculated AUC in synovial fluid was 30.8 ± 8.9 and 113 ± 85 µg × d/ml for the joints that received the 'low CLB gel' and

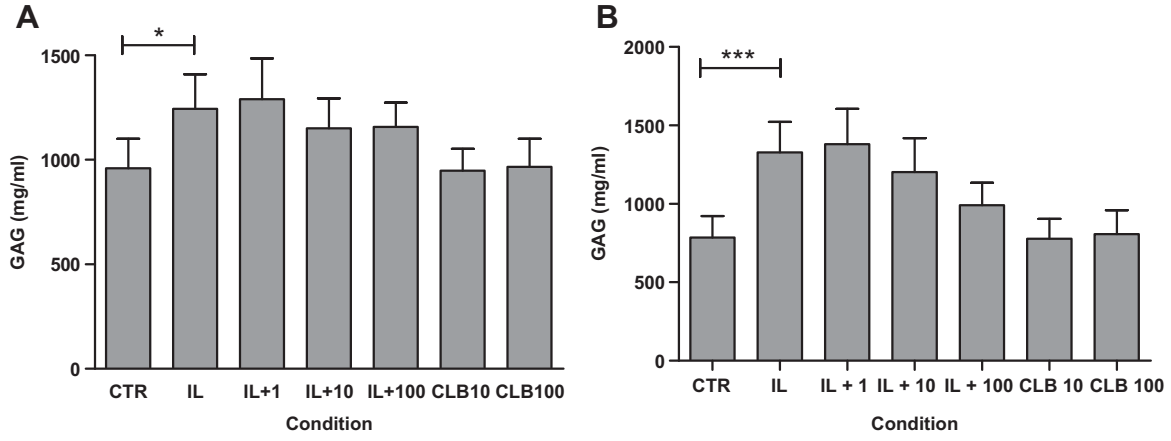

Fig. 4. GAG release into culture medium after 24 (A) and 48 (B) hrs of culture of explants of equine articular cartilage with or without IL-1b (10 ng/ml) and with 0, 1, 10 or 100 µM celecoxib (CLB).

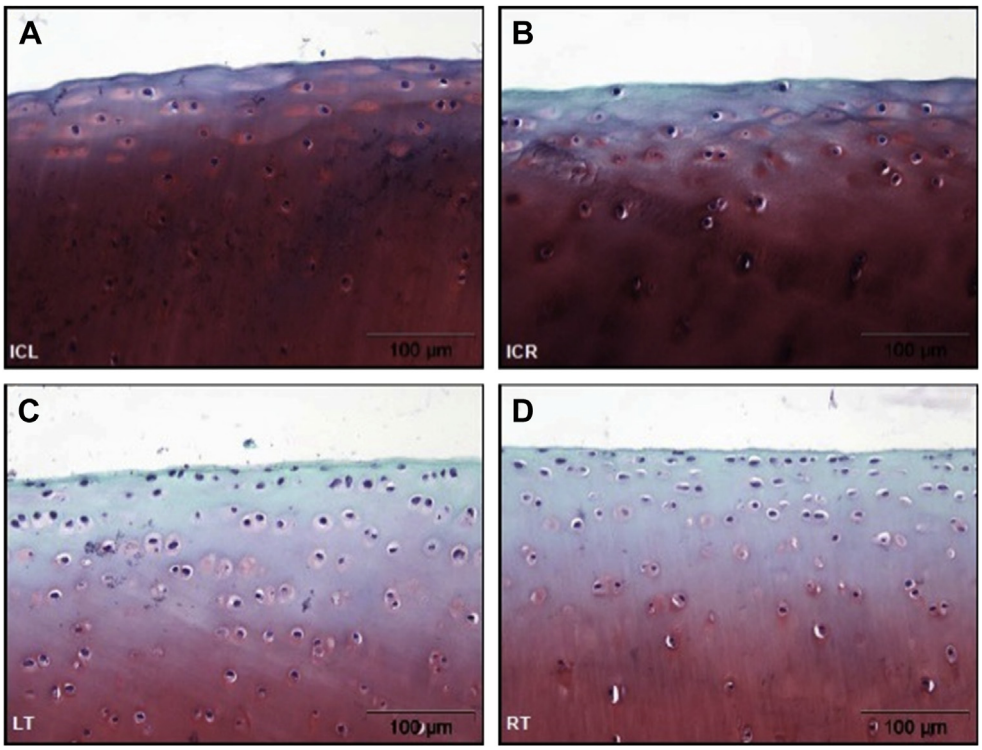

Fig. 5. Representative histological sections of cartilage harvested from injected joints stained with Safranin O/Fast green. (A) Full thickness cartilage harvested from a joint injected with Hyonate® 8 weeks post injection. (B) Full thickness cartilage harvested from a joint injected with 'placebo' 8 weeks post injection. (C) Full thickness cartilage harvested from a joint injected with 'high CLB gel' 4 weeks post injection. (D) Full thickness cartilage harvested from a joint injected with 'low CLB gel' 8 weeks post injection.

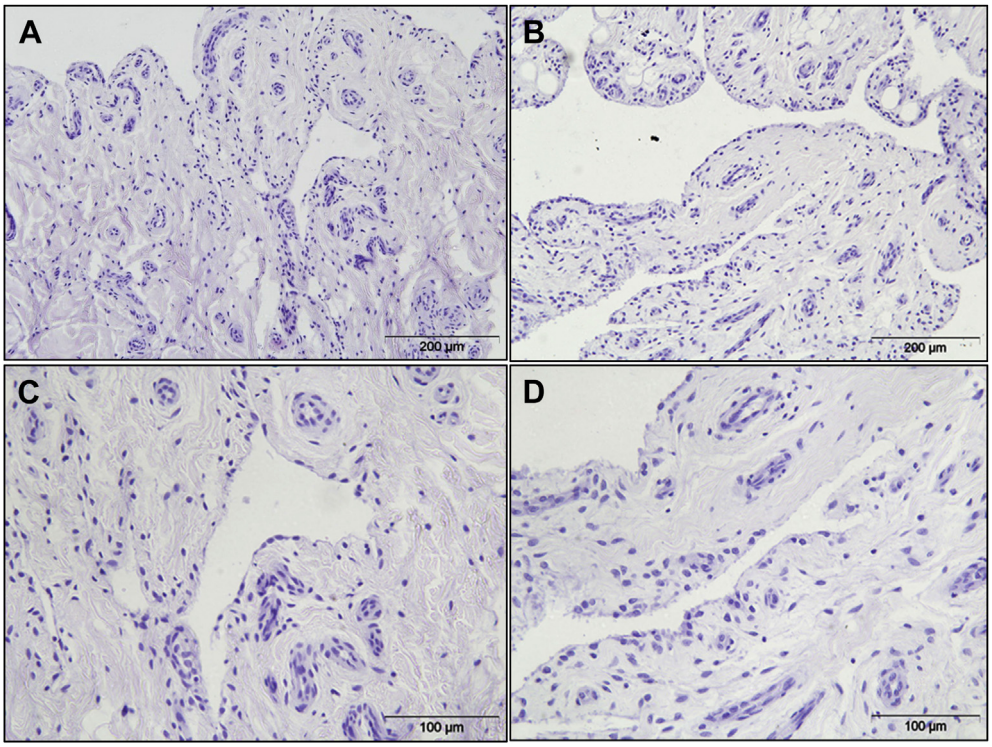

Fig. 6. Representative histological sections of synovial membrane harvested from injected joints stained with haematoxylin and eosin. A,C: synovial membrane from joint injected with low-dose celecoxib-laden hydrogels 8 weeks post injection (magnifications x10 and X20). B,D: synovial membrane harvested from joint injected with high-dose celecoxib-laden hydrogels 4 weeks post injection (magnifications x10 and x20).

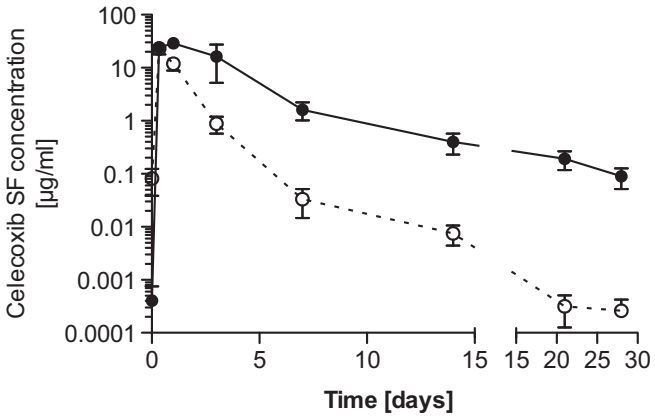

Fig. 7. Celecoxib concentrations in synovial fluid after i.a. administration of 'low CLB gel' (open circles) and 'high CLB gel' (closed circles) loaded with 50 and 260 mg celecoxib per g gel, respectively. The celecoxib concentration in one of the samples of synovial fluid at 2 weeks after administration of the 'low CLB gel' was abnormally high and was excluded.

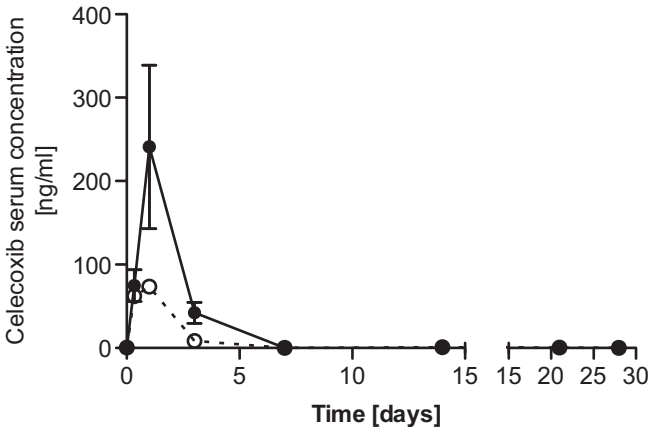

Fig. 8. Celecoxib concentrations in serum after i.a. administration of 'low CLB gel' (open circles) and 'high CLB gel' (closed circles) loaded at 50 and 260 mg celecoxib per g gel, respectively.

'high CLB gel', respectively. Thus, there is a factor 3-4 difference in the AUCs between the joints that received the 'low CLB gels' and 'high CLB gels', which is in the range of the factor ~5 difference in loading between the two administered gels (50 and 260 mg/g, respectively). Table 3 summarizes the pharmacokinetics values.

The celecoxib concentration in the synovial fluid depends on the release kinetics of the drug from the gel and of the elimination rate of the drug from the synovial fluid. The latter is likely not dependent on the concentration of the drug. So, the measured concentration therefore reflects the release of celecoxib from the gel formulations. The release of drug that is homogeneously dispersed in a polymer matrix follows the Higuchi equation, meaning that the amount of drug that is released is proportional to the square root of time ('first order release'); the amount of drug released per time unit therefore decreases in time. This was recently reviewed [50]. This Higuchi equation is valid for a drug that is dispersed in the matrix (high celecoxib gel) and a decrease in amount of drug released per time unit is expected. If we further assume that elimination rate is not dependent on the drug concentration, a decrease in celecoxib levels in the synovial fluid can be expected. However, this equation is valid for a stable matrix. Therefore, since the gel systems swell and also degrade (erode) in time, the release kinetics likely follow different kinetics than first order. Additionally, in a previous study we found that in vitro, the release of celecoxib from celecoxib-loaded acetyl-capped PCLA-PEG-PCLA thermogels was governed by erosion of the gels [21]. However, in vivo a more rapid release was observed indicating that e.g. solubilisation of the drug by proteins contributed to its in vivo release.

Fig. 8 shows that plasma celecoxib concentrations in horses that received the celecoxib-loaded gels were detected up to 72 h. The C_{max} of celecoxib in plasma was reached at 8 h (one animal) and 24 h (four animals) post injection (81 ± 72 and 230 ± 220 ng/ml for 'low CLB gel' and 'high CLB gel', respectively). The plasma C_{max} values were thus 150 and 330 times lower than in synovial fluid for 'low CLB gel' and 'high CLB gel', respectively. It is remarkable that the serum C_{max} coincided with the maximum of the inflammatory response (Fig. 3A–B), which indicates that celecoxib leaked from the joint into the periphery most likely because of the increased vascular permeability due to the inflammatory response in the joints [51,52]. In line with the biochemical analysis of the synovial fluid (WBC, protein count) showing inflammation for 3–7 days, no celecoxib could be detected in plasma at 7 days. The AUC in serum was 0.177 ± 0.075 and 0.38 ± 0.33 µg × d/ml for horses that received 'low CLB gel' and 'high CLB gel', respectively. Thus, there is a factor 2-3 difference between the serum AUC of animals that received the 'low CLB gels' and 'high CLB gels', which is in line with that of synovial fluid.

Table 3
PK parameters of celecoxib in synovial fluid and in serum as calculated by the PKsolver after injection of 'low CLB gel' and 'high CLB gel'.

	'Low CLB gel'		'High CLB gel'	
	Synovial fluid	Serum	Synovial fluid	Serum
T_{max} [h]	8–24	8–24	8–72	8–24
C_{max} [µg/ml]	23.6 ± 6.6	0.081 ± 0.072	36 ± 13	0.23 ± 0.22
AUC [µg*d/ml]	30.8 ± 8.9	0.18 ± 0.08	113 ± 85	0.38 ± 0.33

In the earlier referred study by Larsen et al. [49], celecoxib was detected in serum samples for ten days after intra-articular administration of a formulation of celecoxib in PEG_{400}, but they could not quantify these low concentrations (~1 ng/ml) and no data regarding celecoxib concentrations in synovial fluid were reported. Although we show a higher systemic exposure to celecoxib in the present study than reported by Larsen et al., we found a shorter systemic exposure time.

A study by Gika et al. [53] showed that in only six out of seventeen patients, treated with 200 mg celecoxib/day for 5 days orally, celecoxib could be detected in synovial fluid (0.33–0.79 µg/ml), while the plasma concentrations after oral administration (200 mg/day for 5 days) ranged from 350 to 1850 ng/ml. In the present study, 8 h after administration of the celecoxib-loaded gels, a celecoxib concentration in the synovial fluid of 20–25 µg/ml was reached (Fig. 7), thus ~50 times higher than that after oral administration [53]. Importantly, 24 h after a single injection of the celecoxib-loaded gels, the peak serum concentration was 75–145 ng/ml (Fig. 8), thus 5–25 times lower than that after oral administration [53]. Hence, by using *in situ* forming gels based on acetyl-capped PCLA-PEG-PCLA triblock copolymers loaded with celecoxib, we greatly improved the celecoxib concentrations in synovial fluid while drastically reducing the exposure to systemic concentrations of the drug. It can be assumed that, by reducing the systemic celecoxib concentrations through local delivery, the occurrence of side effects will also be reduced [54].

4. Conclusions

This study demonstrates that gels based on acetyl-capped PCLA-PEG-PCLA triblock copolymer loaded with celecoxib have potential

for successful local drug delivery for the treatment of osteoarthritis. It is shown that injection in the joints of horses results in high local celecoxib concentrations in the joint for a prolonged time, while significantly limiting systemic exposure. Histological and synovial fluid analyses showed that the gels had a good tolerability and did not result in damage of the cartilage. These gels are therefore attractive vehicles for local drug administration which generate high local doses without the risk of side effects associated with oral administration and have therefore potential for clinical applications.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.02.109.

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