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Prolonged inhibition of inflammation in osteoarthritis by triamcinolone acetonide released from a polyester amide microsphere platform

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ABSTRACT

Controlled biomaterial-based corticosteroid release might circumvent multiple injections and the accompanying risks, such as hormone imbalance and muscle weakness, in osteoarthritic (OA) patients. For this purpose, microspheres were prepared from an amino acid-based polyester amide (PEA) platform and loaded with triamcinolone acetonide (TAA). TAA loaded microspheres were shown to release TAA for over 60 days in PBS. Furthermore, the bioactivity lasted at least 28 days, demonstrated by a 80–95% inhibition of PGE_2 production using TNFα-stimulated chondrocyte culture, indicating inhibition of inflammation. Microspheres loaded with the near infrared marker NIR780-iodide injected in healthy rat joints or joints with mild collagenase-induced OA showed retention of the microspheres up till 70 days after injection. After intra-articular injection of TAA-loaded microspheres, TAA was detectable in the serum until day seven. Synovial inflammation was significantly lower in OA joints injected with TAA-loaded microspheres based on histological Krenn scores. Injection of TAA-loaded nor empty microspheres had no effect on cartilage integrity as determined by Mankin scoring. In conclusion, the PEA platform shows safety and efficacy upon intra-articular injection, and its extended degradation and release profiles compared to the currently used PLGA platforms may render it a good alternative. Even though further *in vivo* studies may need to address dosing and readout parameters such as pain, no effect on cartilage pathology was found and inflammation was effectively lowered in OA joints.

1. Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease, burdening patients worldwide, with the knee being one of the commonly affected joints [1,2]. The burden for OA is expected to increase, given the rapid increase in prevalence of its risk factors, such as age and obesity [2,3]. The aetiology of OA is still not fully understood, but both cartilage and synovial tissue pathogenesis contribute to this joint disease [4]. Synovial inflammation and the inflammation mediators produced have found to be elevated in OA patients and influences OA processes and symptoms [4–6]. Corticosteroids administered *via* intra-articular (IA) injections are being used to relieve pain in OA patients [7]. Their effects are of limited duration since they are cleared out of the synovial space and human body relatively fast [8–10]. Repeated applications are therefore needed to sustain the pain relief over a longer period of time, although these are associated with adverse side effects, such as infection, muscle weakness and hormone imbalance [7,11–13].

Applying CS in controlled release platforms can prevent fast clearance and overcome the disadvantages of repeated injections. Ideally, biomaterials used for these purposes show little or no local inflammatory responses and have a slow degradation profile. Biodegradable polymers have already been widely used in orthopaedic applications, but foreign body reaction to the biomaterial leading to further tissue degeneration are a safety concern [14–16]. Moreover, most of the platforms described give sustained release over a relative short period of days, compared to the clinical need of months to years. One of the FDA-

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approved biomaterial platforms commonly used for controlled release is poly(lactic-co glycolic acid) (PLGA). PGLA is a commonly used biomaterial to achieve controlled release of drugs in OA [17–20]. Typically *in vivo*, release was demonstrated for 4–32 days [17,20–22]. Recently, in a clinical trial with osteoarthritis patients, a PLGA-based controlled release formulation of TAA resulted in a more profound pain reduction than a single intra-articular bolus injection [23]. However, the analgesic effect from the sustained TAA release was only superior to bolus injections between five and ten weeks after injection. A further disadvantage of PGLA is its degradation into lactic and glycolic acid, with a risk of inducing toxic local pH levels [18,24].

Recently, a polyester amide (PEA) biomaterial platform was described that induced a minor foreign body reaction in the eye, knee joint and the intervertebral disc [25–27]. Furthermore, it has been shown to be biocompatible upon injection in those tissues with slow clearance rates due to the avascular environment, with the eye and intervertebral disc known to be immunoprivileged [28]. This platform is built of amino acid moieties and hence degradation will not result in toxic conditions [29–31]. PEA particle erosion is dependent on serine protease activity and hence no erosion occurs *in vitro* in PBS [32]. More importantly, PEA microspheres are a suitable platform for prolonged release of small molecule drugs such as corticosteroids, as shown by their *in vitro* release of celecoxib of 80 days [27]. In addition, PEA microspheres were injected into a rat degenerative articular joint, a location that is readily accessible to cells from the acquired and the innate immune system and hence more likely to show inflammatory responses to biomaterials. Whether the clinically used corticoid TAA, applied in sustained release, is safe and effective in reducing inflammatory processes still has to be determined. Hence, in the current study, PEA microspheres loaded with TAA were characterized in terms of loading and release *in vitro*, using a cell-based model of induced inflammation [19]. Furthermore, the release of TAA into the systemic circulation and its effects on articular cartilage and synovial lining inflammation in a rat model of osteoarthritis was compared to a bolus of TAA.

2. Materials and methods

2.1. Preparation and loading of polyester amide microspheres

PEA was synthesized in accordance to procedures reported previously [25,26,29]. For the preparation of microspheres, PEA was dissolved in dichloromethane (Merck Millipore). 20 wt% triamcinolone acetonide was added to the solution and homogenized by ultrasound. The suspension was added to 20 ml of an aqueous solution containing surfactants for stabilization (1 wt% of poly(vinyl alcohol and 2.5 wt% NaCl, Sigma Aldrich) under high shear, using an ultra-Turrax. After a stable suspension was obtained the particles were allowed to harden in 100 ml of water containing 1 wt% of poly(vinyl alcohol) and 2.5 wt% NaCl for 12 h. Excess of water and surfactant was removed by rinsing and centrifugation. Finally, particles were frozen and dried. Preparation procedures of microspheres loaded with IR-780 iodide are exactly the same, but are loaded with 0.2% w/w of IR 780 iodide instead of TAA and dissolved in DCM. Size distribution of PEA particles was measured by static light scattering and ranged from 8 to 50 μm.

2.2. TAA release by PEA microspheres in vitro

2.2.1. In vitro release of TAA in PBS buffer

Release kinetics of TAA from PEA microspheres in PBS buffer were determined by incubation in a volume of 50 ml at 37 °C, of which 45 ml buffer was renewed. Buffer exchange was performed twice the first day, every day up to day 3 and from there every 3–4 days up to day 24. After that, the buffer was renewed on a weekly basis. Size distribution of TAA-loaded particles was determined with Static Light scattering, using a Malvern Mastersizer 2000. Approximately 10 mg of microparticles were accurately weighted and dissolved in appropriate amount of methanol and shaken until a clear solution was obtained. The samples were analysed for TAA concentration by High performance Liquid chromatography (HPLC), using a Waters e2695 Alliance HPLC with UV detector. The method was obtained from the pharmacopoeia collection.

2.2.2. Chondrocyte isolation and culture

Articular cartilage was harvested from knee joints derived from patients undergoing arthroplasty. Anonymous use of redundant tissue from joint surgery used for scientific purposes is part of the standard treatment agreement with patients from the University Medical Center Utrecht [33]. Chondrocytes were isolated by a 3-hour enzymatic digestion in 0.1% pronase (Roche, Mannheim, Germany), followed by an overnight enzymatic digestion in 0.04% collagenase type 2 (Worthington Biochemical, Lakewood, NJ, USA) at 37 °C. Undigested debris was removed using a 70 μm-cell strainer (Becton Dickson, Franklin Lakes, USA). The resulting suspension of cells was washed in PBS and centrifuged. Afterwards, the cells were re-suspended in expansion medium (3000 cells/cm^2) consisting of DMEM (Gibco® Life Technologies, Carlsbad, CA, USA) containing 4.5 mg/ml glucose, 0.8 mg/ml glycyl-L-glutamine, 10% foetal bovine serum (FBS) (HyClone® Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco® Life Technologies) and 10 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA). The cells were cultured at 37 °C and 5% CO_2. The culture medium was renewed every 3–4 days. At passage two, cells were frozen in aliquots of 1 million per vial in freezing medium, containing 10% DMSO (Merck) and 20% FBS in DMEM. For each release experiment, cells of the same donor were used at each of the successive time intervals.

2.2.3. Release and bioactivity of TAA from microspheres

One day before the experiment, cells were thawed and seeded onto a 24-well culture plate, at a density of 40,000 cells per well. Cells were cultured in medium containing DMEM (including glucose and glycyl-L-glutamine), 10% FBS and antibiotics. The following day, the medium was renewed before starting the experiment. PEA microspheres, loaded with TAA at 20%, were dispersed in a total of 1 ml culture medium and placed in Transwell® baskets at 8.7 or 0.87 μg per well (0.4 μm pore size, polycarbonate membrane) (Corning, Amsterdam, The Netherlands) over the plated cells. Unloaded microspheres were taken as controls. Cells and microspheres were pre-incubated for 4 h at 37 °C, at 5% CO_2 and 95% humidity. Subsequently, tumour necrosis factor alpha (TNFα) (eBioscience, San Diego, CA, USA) was added at a final concentration of 10 ng/ml to the culture medium in all conditions. Cells and microspheres were co-incubated further for another 72 h before the microspheres were transferred to a new 24-well culture plate containing cells seeded according to the procedure described above. This procedure was repeated 9 times accounting to a total release period of 27 days. Cells treated with 10^{-7} M or 10^{-8} M TAA directly added to the culture medium and continuously renewed, were included as positive control in each time interval. Each condition was analysed in 4 replicates ($n = 4$) and experiments were performed for three different donors. At every 72-hour time point medium was collected and cells were lysed in KDalert™ Lysis Buffer (Ambion®, Life Technologies). Samples were stored at −80 °C until further analysis if not used immediately.

2.2.4. PGE_2 release

Cell culture medium was collected at day 3, 6, 9, 12, 15, 18, 21, 24 and 27 and stored at −80 °C; media were brought to room temperature immediately prior to PGE_2 measurement. PGE_2 was measured using the enzyme immunoassay Prostaglandin E_2 Parameter Assay Kit (R&D Systems) following the manufacturer's instructions. Colorimetric intensity was determined using the Benchmark Microplate Reader (Bio-Rad) at 450 nm. The readings were subtracted by those at 540 nm. The concentration of PGE_2 in the samples was determined by using a calibration

curve. PGE_2 amount was normalized to DNA content per well and, as the intensity of the response of OA chondrocytes to TNF varied between donors, for each condition normalized to the average PGE2 content of controls, which received TNFα only. The average values of controls were set to be 100%.

2.3. Study design

Animal experiments were carried out under the approved protocol (DEC# 2013.III.04.036) of the Utrecht University Ethical Committee for Animal Care and Use, following the central commission of animal experiments guidelines for animal research in the Netherlands. 16 female adult Sprague-Dawley rats (Harlan Laboratories, Horst, The Netherlands) were used in this study: 4 to test the degradation rate of microspheres and 12 to determine safety and release of microspheres loaded with triamcinolone acetonide. Animals were allowed to acclimatize for one week and were housed in groups. *Ad libitum* food and water was provided. Intra-articular injections and Near-Infrared fluorescence (NIRF) imaging were performed under isoflurane anaesthesia (4–5% induction, 1–2% for maintenance).

2.3.1. Induction of osteoarthritis in rat knee joints

OA was induced *via* intra-articular injection of 30U type II collagenase through the patellar ligament using a 30G insulin needle (Sigma-Aldrich) in 25 μl, dissolved in NaCl + 1 mM CaCl dihydrate (Sigma-Aldrich). For the retention of microspheres by NIRF imaging, in 4 rats OA was induced unilaterally and for the evaluation of safety and TAA release profiles, OA was induced bilaterally by collagenase injection in 12 rats. Postoperatively, animals received 0.05 mg/kg Buprenofin (Temgesic) subcutaneously as prophylactic analgesia.

One week after bilateral OA induction, 6 rats were intra-articularly injected with 25 μl of PEA microspheres loaded with triamcinolone (50 mg/ml particles containing 18 wt% TAA, total dosage of 250 μg), 6 rats with 25 μl TAA bolus injection (10 mg/ml, Kenacort; Bristol-Myers Squibb, Woerden, The Netherlands) in the experimental joint. Contralateral knees of rats were injected intra-articularly with saline or 50 mg/ml empty microspheres as control. After injection, blood for serum analysis was drawn *via* tail vein. This was repeated at one hour interval on the first day, twice daily on the following 2–5 days, daily on day 6–14 and once at day 16, 18, 20, 22, 25, 28, 35, 42 and 49. Blood samples were cooled and centrifuged, and serum was frozen at −20 °C. After these seven weeks, animals were sacrificed and knees were prepared for histology.

2.4. PEA microsphere retention in vivo

2.4.1. NIRF780-iodide injection and imaging

One week after OA induction, 25 μl microspheres (50 mg/ml) loaded with 0.2% w/w of IR 780 iodide were injected intra-articularly in both knees of 4 rats. Every other week, in both joints the intensity of NIRF780-iodide was determined in sedated rats. Rats were scanned using an Ivis spectrum device (Perkin Elmer). Spectral unmixing excitation 675 nm and emission 740, 760, 780, 800 and 820 nm was used. Data were analysed using Living Image 4.3 software (Perkin Elmer). After ten weeks, rats were euthanized using CO_2/O_2 asphyxiation, subsequently both legs were collected and removed of skin and scanned again.

2.5. Biological efficacy of TAA-loaded PEA microspheres in vivo

2.5.1. LC-MS/MS analysis of serum

Concentrations of triamcinolone acetonide in rat serum were determined by means of a LC-MS/MS method using a Thermo Fisher Scientific (Waltham, MA) triple quadrupole Quantum Acces LC-MS/MS system. Sample preparation involved a liquid– liquid extraction step with methyl tert-butyl ether (TBME) using triamcinolone acetonide-13C3 as internal standard. After centrifugation, the organic supernatant was removed and evaporated to dryness. The residue was reconstituted with 50 μl 0.1% formic acid and injected for LC–MS/MS analysis. The method showed linearity over the range of 0.5–15.0 μg/l with a regression coefficient of 0.996. The analytical accuracy and precision are within the maximum tolerated bias and CV, 10% for LLOQ (0.5 μg/l), 7% for the LOW (2.0 μg/l), 6% for the MED (7.5 μg/l) and 4% for the HIGH (12.0 μg/l).

2.5.2. Tissue processing and histology of synovium and cartilage

All joints were decalcified in 10% formic acid solution in combination with microwaving [34], thereafter embedded in paraffin. 5 μm knee joint sections were made and stained either with Safranin-O/Fast green or haematoxylin and eosin to evaluate cartilage damage and synovial tissue inflammation, respectively, using Mankin and Krenn scoring systems. Krenn score indicates no synovitis with grade 0–1 and high grade synovitis with grade 5–9 [35]. Mankin score is ranging from healthy grade 0 to total joint destruction grade 14 [36]. Scoring was done by two blinded observers (SC and SP).

2.6. Statistical analyses

All data were analysed using IBM® SPSS® Statistics version 21. Data concerning PGE_2 production and serum TAA levels were logarithmically transformed first. Then, assumptions of normality of residuals and homogeneity of variance were met. Linear mixed models was used to analyse repeated measurements, followed by pairwise comparisons with LSD adjustment. Model selection was based on the lowest Akaike Information Criterion. Donor served as random effect factor and treatment, time and their interaction served as fixed effect factors in this model. Regression coefficients were estimated by the maximum likelihood method. Krenn and Mankin data (indicating synovitis and cartilage integrity, respectively) met assumptions of normal distributed residuals and homoscedasticity and were therefore not transformed. ANOVA with Tukey-HSD post-hoc correction was used to observe differences between group means. $p < 0.05$ is found significant.

3. Results

3.1. Characterization of TAA loaded microspheres

SEM analysis of the loaded microspheres showed part of the TAA was incorporated as crystals (Fig. 1A). TAA release in PBS buffer at 37 °C showed an initial burst at day one with a gradual increase of TAA release up to 50% cumulative release after 60 days (Fig. 1B). The average size of the microparticles was 22.4 μm, with a dispersity index of 1.205. Loading was 20 wt%, with 12% located outside of the spheres. Due to the buffer exchange ~8% of particles were lost and 35% of TAA was recovered.

3.2. Bioactivity by released TAA

PGE_2 production levels were negligible in cells not treated with TNFα or only exposed to medium containing empty microspheres (data not shown). When TNFα was added to the cells, sufficient PGE_2 was produced. However, a transient increase in PGE_2 production at day 4 was found in cells treated with TNFα in the presence of empty microspheres compared to cells treated with TNFα in the absence of empty microspheres ($p < 0.05$, Fig. 2a).

When TAA loaded in microspheres (0.435 mg, equivalent to 0.1 μM ("MS-TAA 10^{-7} M") if released immediately) was added to the culture system, PGE_2 production was reduced to <5% of non-treated TNFα-stimulated cells until day 12, after which PGE_2 production levels increased to 20% by the end of the culture period. The lower dosage of TAA released from the microspheres (equivalent to 0.01 μM ("MS-TAA 10^{-8} M") at 1 ml) reduced PGE_2 production to 8% non-treated TNFα-

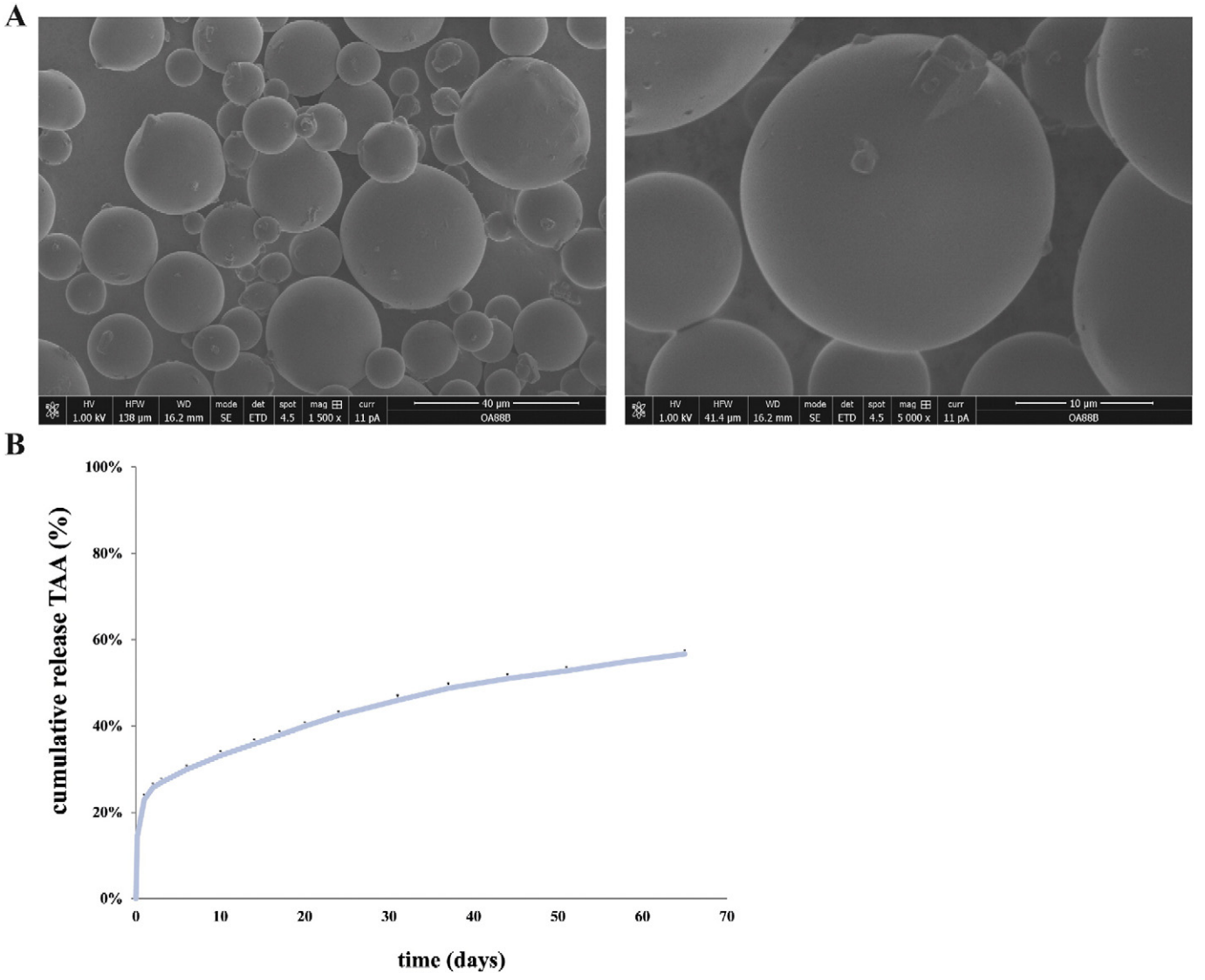

Fig. 1. A) SEM of TAA loaded microspheres. B) TAA release from loaded microspheres in PBS buffer, after 60 days, showed an initial burst release at the first day and a gradual increase up to 50%.

stimulated cells at day 9. The TAA released from microspheres containing the lower dosage thus resulted in a faster loss of PGE_2 inhibition. However, both dosages TAA released from the microspheres still showed an over 80% inhibition over the remainder of the culture period (Fig. 2b). Free TAA in two different concentrations (0.435 mg, equivalent to 0.1 μM ("TAA 10^{-7} M") and 0.0435 mg, equivalent to 0.01 μM ("TAA 10^{-8} M") directly added to the culture medium at each time interval also completely inhibited PGE_2 production to below 5% ($p < 0.05$, Fig. 2).

3.3. Microsphere retention in rat joints

In 7 of the 8 knees, clear signal was visible after intra-articular injection of IR780-loaded microspheres. Injection appeared to have failed in one out of eight rat knees, as no signal was observed at any of the time points. In the 7 knees with signal, a gradual decline of signal over 70 days was observed (Fig. 3), which was still detectable *in vivo* in two out of seven joints. Removal of the skin after euthanasia revealed 6 out of the 7 remaining joints to contain NIRF label (Fig. 3). The loss of signal over time seemed not reliant of the presence of osteoarthritic processes in the joint, despite a trend towards a decreased signal compared to the contralateral healthy knee. The signal obtained around the front paws of the rat, and in some cases over the lower abdomen, was autofluorescence, as verified by spectral unmixing emission analysis of the upper region (front paws) separately from the bottom region (hind paws), where we noted a shift in emission from 800 nm to 760 nm.

3.4. Release kinetics TAA

Serum TAA levels were measured to indirectly determine release kinetics and systemic load. Systemic TAA was detected both in animals receiving intra-articular TAA by bolus or microspheres. Peak serum levels of TAA were higher with bolus injections (45 μg/ml) than in the slow release system (12.5 μg/ml, $p < 0.05$), while bolus TAA was detectable in systemic circulation until 120-hour post injection and TAA released from microspheres 170-hour post injection, Fig. 4).

3.5. Joint histology

H&E stained sections were evaluated for possible remainders of microspheres but these were not encountered. Seven weeks after induction of OA, synovial inflammation (3 out of 9 on Krenn score) and cartilage degeneration (5 out of 14 on Mankin score) were mild (Fig. 5). There was no

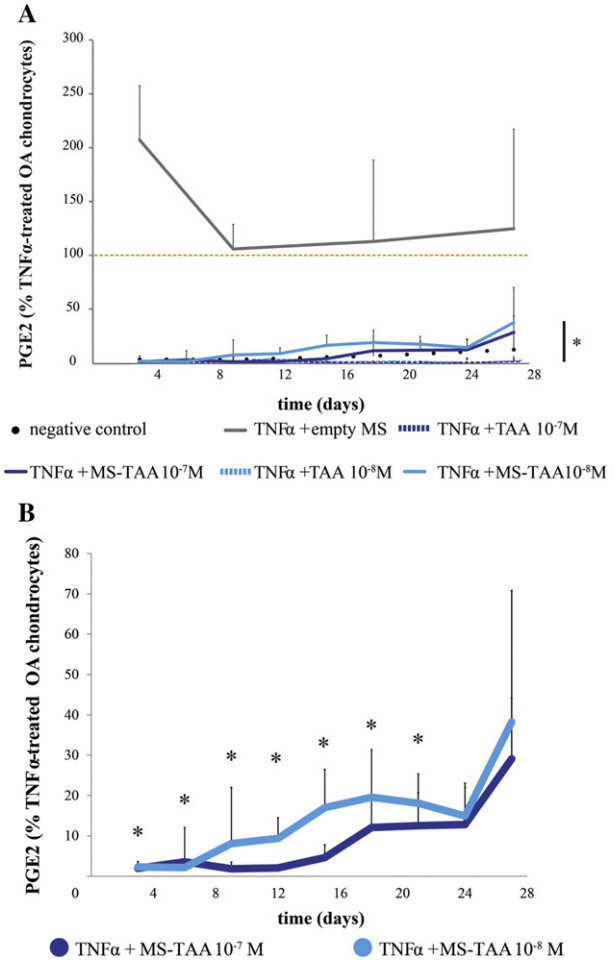

Fig. 2. PGE_2 production was inhibited during the total culture period upon TAA treatment, regardless of dose and formation, compared to chondrocytes treated solely with TNFα (100%, orange dotted line) except in the presence of empty microspheres (grey solid line). Negative control indicates chondrocytes without TNFα (black dotted line), TNFα + TAA 10^{-7} M (dark blue dotted line) and TNFα + TAA 10^{-8} M (light blue dotted line) represent free 0.1 or 0.01 μM TAA, respectively, added to the culture medium. Corresponding TAA dosages in microspheres are indicated with TNFα + MS-TAA 10^{-7} M (dark blue solid line) and TNFα + MS-TAA 10^{-8} M (light blue solid line). Results are shown as average mean ± SD for three donors with six replicates per donor. *$p < 0.05$.

evidence of an enhanced inflammatory reaction or additional degenerative changes in the joints injected with empty microspheres. However, TAA administered in microspheres significantly decreased synovial lining thickness and inflammatory infiltrate compared to microsphere saline-injected joints ($p < 0.05$). No clear effects were noted of either controlled release of TAA nor bolus TAA on cartilage integrity.

4. Discussion

Biomaterials encapsulating corticosteroids for OA treatment developed to sustain their release locally are a promising approach towards long term relief of OA-associated pain [37–39]. Here, a previously described polyester amide-based biomaterial platform was characterized for its safety, retention and applicability for local delivery in the joint over a relatively long period of time. The release of TAA from this platform lasted for over 60 days in PBS. Furthermore, in a chondrocyte-based *in vitro* system, inflammation was reduced to 5–20% over the full period of 28 days by microspheres loaded at a single dose of TAA equivalent to TAA added directly. Upon intra-articular (IA) injection *in vivo*, retention of PEA microspheres loaded with near infrared label was found for over two months after injection. At the histological level, the microspheres could not be traced back after seven weeks, nor did their presence in the joints induce enhanced inflammation or cartilage degeneration. TAA in microspheres nor as bolus resulted in improved cartilage integrity. However, synovial inflammation was effectively reduced in OA joints receiving sustained TAA release from microspheres but not by bolus TAA. Furthermore, controlled release of TAA from microspheres was confirmed by decreased peak serum levels compared to the bolus of the same amount of TAA, with a slightly prolonged presence in the systemic circulation.

Focussing on the anti-inflammatory action in the current study, TAA released from PEA-based microspheres remained bioactive over 4 weeks in an *in vitro* assay based on inflammatory OA chondrocytes. This assay is used to determine the potential of TAA to supress inflammation and was previously optimized for optimal inflammatory response of the cells, and hence did not aim to mimic "natural" chondrocyte behaviour [19]. In the same *in vitro* bioactivity assay investigating TAA release from PGLA microspheres, PGE_2 production was repressed up to 38% after 27 days, using a ten-fold lower dosage of TAA. When comparing the same amount of TAA released from both microspheres (435 ng, equivalent to 10^{-8} M TAA if released immediately), PGLA microspheres were only able to reduce PGE_2 production to 29% after 27 days as opposed to the 80% reduction found in this study after 21 days of culturing [19]. This is in line with available data on PLGA-based platforms, which typically show a release in culture medium or *in vivo* varying from days to weeks. For example, controlled temporospatial delivery of bovine serum albumin proteins by PLGA microspheres showed degradation in a relatively short period of 14 days subcutaneously in mice [22]. The prolonged stability and release of the current PEA-based platforms thus appears to confer an advantage over other faster releasing platforms. PEA compatibility was already shown in preclinical settings targeting the eye or intervertebral disc degeneration [25,26]. The current study shows the applicability of this platform in the highly immune responsive environment of the articular joint, which is in accordance with a recently published study evaluating the small molecule celecoxib in OA joints [27].

The importance of reducing the systemic load after intra-articular application of corticosteroids is shown in previous pharmacokinetics studies of IA injection of TAA, where TAA was detectable in plasma afterwards and incurred impaired wound healing, infection, muscle weakness and hormone imbalance [11,12,40]. In humans, hydrocortisone levels could be suppressed after IA injection of TAA suspension (40 mg) and remained repressed for three days, suggesting a systemic reaction with serum TAA levels even though these fell below the 1 ng/ml detection limit [40]. Therefore, minimizing systemic TAA load is an important feature of this platform since it indirectly decreases risks for adverse side effects caused by systemic TAA. After 120 h post IA injection, serum TAA levels were diminished to undetectable levels (<0.5 ng/ml). Surprisingly, compared to a previously described PLGA-based platform, systemic TAA levels in Lewis rats 120 h post IA injection of 0.28 mg TAA were still approximately 0.75 ng/ml, systemic TAA levels in the current study (250 μg TAA-MS) diminished faster [20]. Although loss of solutes such as cytokines in the human joint were shown to occur within 3 days [10], the prolonged systemic levels of TAA after intra-articular injection, sometimes up to 2 weeks after injections, can be explained by the low solubility of TAA in water, naturally forming crystals allowing for delayed delivery [40]. However, the TAA crystals in TAA suspension injections have shown to be cytotoxic in direct contact with cell membranes [41,42], which is another advantage of using slow release formulations.

Application of controlled release of TAA in the rat model of OA did not show a clear improvement of cartilage histology, however, neither did the TAA bolus. Even so, synovial inflammation was clearly decreased using TAA-loaded microspheres. Possibly the limited room for improvement in terms of cartilage degeneration has precluded a more pronounced effect on cartilage integrity of the administration of either

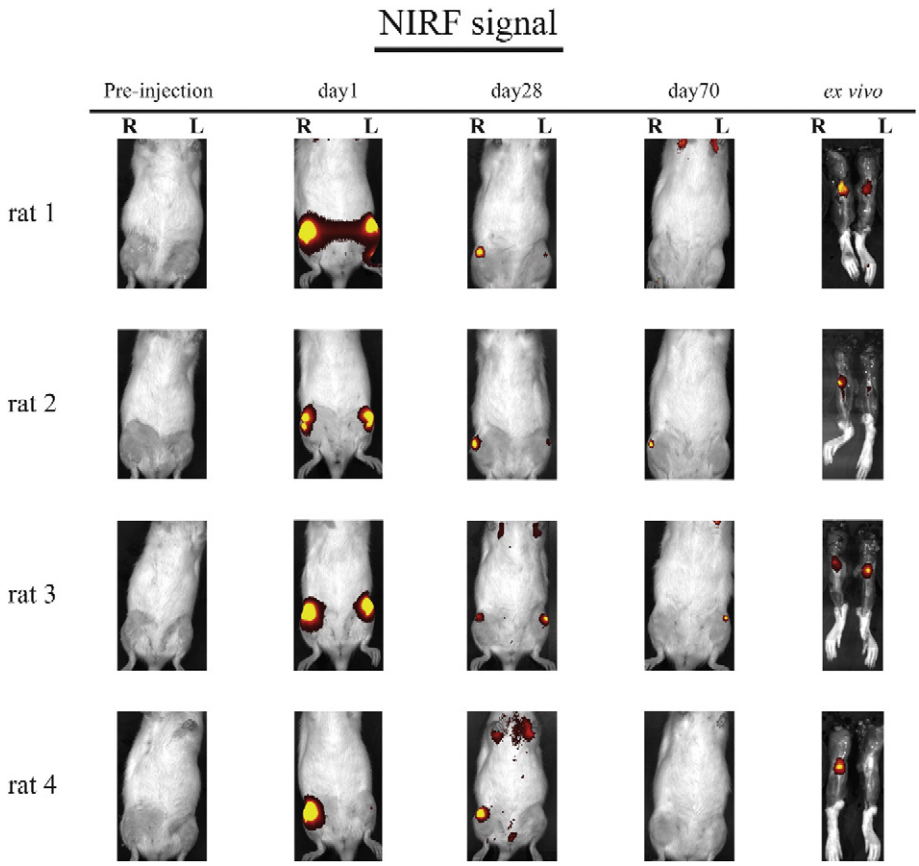

Fig. 3. PEA microsphere retention *in vivo*. In four rats, OA was induced in the left knee and contralateral knee served as healthy control. All joints were intra-articularly injected with PEA microspheres, loaded with IR 780 iodide. Here, rats are shown that were subjected to fluorescence optical imaging pre-injection, at day 1, 28, 70 and at day 70 *ex vivo*. The signal obtained around the front paws and lower abdomen was autofluorescence.

bolus or microsphere-encapsulated TAA. On the other hand, TAA might influence cartilage metabolism processes by its effect on synovial tissue. A previous study already have demonstrated an inhibition in GAG production in single cartilage explants while this effect was counteracted by TAA in cocultures of synovium and cartilage explants [43]. Also, other more severe OA models may possibly enable intra-articularly applied TAA to show a pronounced effect. The effect of corticosteroids on joint tissue integrity *in vivo* has been studied to a limited extent. Decreased cartilage ECM synthesis or degradation after treatment *in vitro* [44–46] as well as *in vivo* were found [47,48]. Notably, these studies were based on corticosteroid formulations of which the vehicle excipients were shown to be toxic [49,50], so it cannot be excluded that these negative effects were not caused by the corticosteroids. In contrast, many OA animal studies demonstrated a decrease of OA progression or a protective role of corticosteroid injections, based on histological and biochemical findings [20,51–55]. Whether this is representative of the human situation is unknown. In the current study, dosing may have been suboptimal. Although the used dosages were based on human *vs* rat synovial fluid joint volumes as conversion factor, which is superior to dosing based on body weight scaling [52], species specific characteristics in TAA signalling may require higher dosages for effects on cartilage degeneration. Clinical studies might in fact represent the best tool, with no severe harmful effects on arthritic joints found at the radiographic level following repeated intra-articular TAA injections [23,56–58]. A systematic review described beneficial effects of IA corticosteroid suspension injections, Kenalog® mostly used, lasting up to 4 weeks although it was argued unlikely that clinical effects remain after this period [59]. Whether systemic side-effects of controlled release of TAA by IA injections arise has not been investigated extensively yet. Only one recent phase-II randomized double-blind clinical study assessed the efficacy of IA injection of TAA PGLA-microparticles and described no adverse effects clinically and at the radiographic level during the seven weeks study period [23]. Typically the clinical outcome parameter of clinical studies on OA is pain, and in the latter study this was suppressed more strongly with the microspheres than

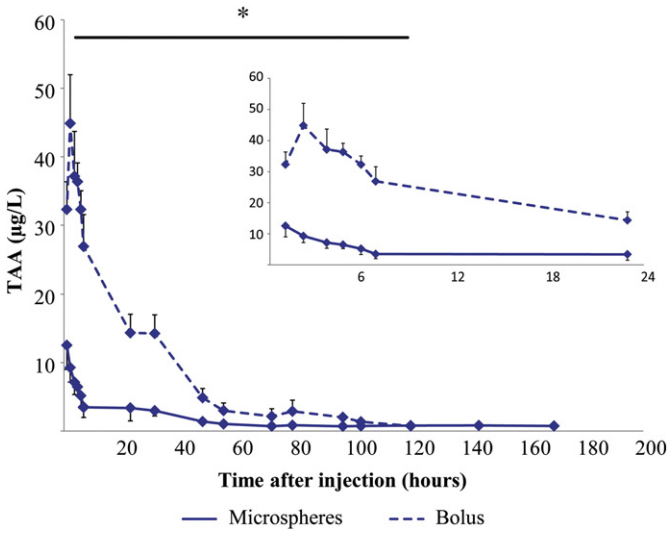

Fig. 4. Serum TAA levels of rats were determined at several time-points. Release profiles of bolus and TAA-loaded microspheres intra-articular injections in OA joint represented in dashed and solid lines, respectively. Bullets show mean ± SEM for six rats per group. Insert provides a more detailed overview of serum TAA levels of the first 24 h post-injection. *$p < 0.05$.

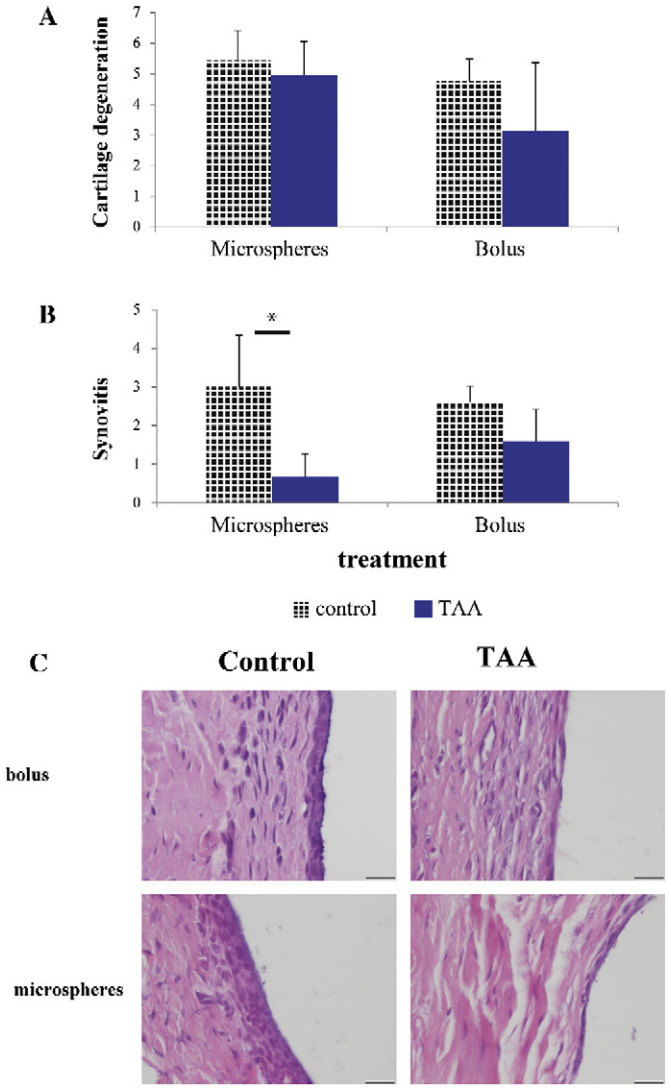

Fig. 5. Cartilage degeneration (A) and synovitis (B) scores of rats treated with microsphere or bolus TAA injections. In twelve rats, OA was induced in both joints. Cartilage and synovial tissue histology was determined using Mankin and Krenn scores, respectively, of six rats intra-articularly injected with TAA bolus or microsphere formation (dotted bars). Contralateral joints were injected with saline and served as control (solid bars). Mankin score is ranging from healthy grade 0 to total joint destruction grade 14. Krenn score indicates no synovitis with grade 0–1 and high grade synovitis with grade 5–9. Bars represent mean ± SD for six rats per group. $^{*}p < 0.05$. Synovial tissue stained for Haematoxylin & Eosin (C) of all four treatments. Scale bar = 20 μm.

using the same quantity of TAA in suspension at the same dose. Possibly pain would in *in vivo* OA models in general also be a more relevant parameter, provided no adverse effects at the histological and systemic level are noted.

Other delivery platforms, in addition to PGLA and the current biomaterial, were recently developed for sustained drug release in preclinical studies [17,60,61]. Nanoparticles, scaffolds or hydrogels of diverse biomaterials such as chitosan, hyaluronic acid or PLGA have been tested in rodent OA joints and while in general these did not evoke an additional inflammatory effect, their therapeutic effect on tissue regeneration or pain was often inconclusive and needs further investigation [62,63]. PEA-based platforms are rather newly used as controlled release systems and their retention and possible therapeutic effects *in vivo* have not been extensively described before. Here, polymer retention was shown to be prolonged compared to other platforms with signal from the near infrared marker present up until 70 days after injection indicating not all microspheres were degraded at this time point. While we cannot with certainty exclude that the loss of NIR signal is due to the loss of microspheres from the joint, the presence of the endothelial barrier in the synovium makes this highly unlikely. The prolonged retention of NIR-loaded microspheres in the joint is well in line with the <100% TAA released in PBS found in the same time period. Although this cannot directly be extrapolated towards the *in vivo* release of a hydrophobic small molecule drug such as TAA, this suggests much longer release periods are possible using this biomaterial platform.

PEA based platforms are attractive for novel controlled release strategies. They have already shown to provide effective controlled release *in vitro* by releasing anti-inflammatory drugs and thereby attenuating foreign body inflammatory responses *in vitro* or only provoke slight foreign body reactions reflecting in inflammatory cell infiltrate *in vivo* [25,26,31,64]. In the knee, no foreign body reaction was observed histologically *in vivo*, nor was an additional degenerative effect noted, in line with a previous finding showing a lack of synovial thickening and necrosis after injection [23]. However, we cannot exclude that these responses have occurred at earlier time points. Microsphere size has been shown important for their entrapment in joint tissues after IA injection [21,23,57]. The microspheres used in the present study could be expected to be sequestered as subsynovial granulation plaques due to the present macrophages [27]. However, PEA microspheres were not recognized in the histological sections, possibly due to the embedding and histological staining procedures [21]. Complete degradation of the microspheres is an unlikely explanation for the lack of an inflammatory response, as NIR label incorporation showed retention of label-containing microspheres at 10 weeks. Previously, hydrolysis of knee joints, twelve weeks after IA injection showed still 20% of PEA polymer, while PEA degradation was significantly higher in OA rat joints compared to contralateral healthy joints injected with empty microspheres [27]. In the current study, a milder OA model was used, but PEA microspheres loaded with TAA still decreased synovial inflammation successfully. This suggests that these PEA microspheres loaded with the corticosteroid often used in the clinics can potentially be an improved pain treatment for OA patients by reducing inflammatory responses.

5. Conclusion

A safe route of administration of incorporated triamcinolone acetonide in PEA microspheres was confirmed by intra-articular injection in OA joints. Moreover, the slow degradation of the microspheres makes it a suitable tool for relieving inflammation and pain locally for a longer period of time. We have shown here that this new generation of microspheres provide a safe platform, by 1) no considerable foreign body reaction, 2) decreased peak serum TAA levels and 3) decreased synovial inflammation, which renders it an applicable controlled release platform for clinical use. Further studies should focus on the appropriate dosing of corticosteroids locally in mild to severe OA joints and their effect on suppressing pain.

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