Pharm Res (2015) 32:680–690
DOI 10.1007/s11095-014-1495-z

RESEARCH PAPER

Applicability of a Newly Developed Bioassay for Determining Bioactivity of Anti-Inflammatory Compounds in Release Studies – *Celecoxib and Triamcinolone Acetonide Released from Novel PLGA-Based Microspheres*

Hsiao-yin Yang • Maarten van Dijk • Ruud Licht • Michiel Beekhuizen • Mattie van Rijen • Martina Källrot Janstål • F. Cumhur Öner • Wouter J. A. Dhert • Detlef Schumann • Laura B. Creemers

Received: 10 September 2013 / Accepted: 15 August 2014 / Published online: 28 August 2014

ABSTRACT

Purpose : To develop a bio-assay for measuring long-term bioactivity of released anti-inflammatory compounds and to test the bioactivity of celecoxib (CXB) and triamcinolone acetonide (TA) released from a new PLGA-based microsphere platform.

Methods : Human osteoarthritic chondrocytes were plated according to standardized procedures after batch-wise harvest and cultured for 3 days to prevent cell confluency and changes in cell behaviour. Prostaglandin E2 (PGE_2) production stimulated by TNFα was used as a parameter of inflammation. A novel microsphere platform based on PTE-functionalised PLGA was used to incorporate CXB and TA. Loaded microspheres were added to transwells overlying the cells, with transfer of the wells to new cell cultures every 3 days. Inhibition of PGE_2 production was determined over a period of 21 days.

Results : PLGA(75:25)-PTE microspheres were prepared and loaded with CXB and TA at 86 and 97% loading efficiency, respectively. In the bioactivity assay, PGE_2 levels induced by TNFα were reduced to an average of 30% using microspheres loaded with 0.1 nmol CXB per transwell; with microspheres loaded with 0.1 nmol TA, PGE_2 production was initially reduced to 3% and gradually recovered to 30% reduction. At 1 nmol loading, PGE_2 was inhibited to 0–7% for CXB-loaded microspheres, and 0–28% for TA-loaded microspheres.

Conclusions : We present a novel sustained release bioactivity assay which provides an essential link between *in vitro* buffer-based release kinetics and *in vivo* application. Novel PLGA-based microspheres loaded with TA and CXB showed efficient anti-inflammatory effects over time.

KEY WORDS celecoxib · chondrocytes · PGE_2 · PLGA-PTE microspheres · triamcinolone acetonide

ABBREVIATIONS

CXB	Celecoxib
Mn	Number-average molecular weight
Mw	Weight-average molecular weight
OA	Osteoarthritis
PBS	Phosphate buffered saline
PDI	Polydispersity index
PGE_2	Prostaglandin E2
PLGA-PTE	Poly(lactic-co-glycolic acid) with poly(thioester) linkages
TA	Triamcinolone acetonide
TNFα	Tumor necrosis factor alpha

INTRODUCTION

With the ageing of the population in industrialized countries, the incidence of wear-related disease is ever increasing. In particular, degenerative diseases of the musculoskeletal system impose a huge burden on the society (1). Of these, osteoarthritis (OA) and low back pain associated with intervertebral disc degeneration are major degenerative musculoskeletal diseases (2, 3). In the United States, for example, 12.1% of the Americans aged 25–74 years are affected with OA, whereas a 26.4% prevalence of low back pain was found in the population aged 18–74 years (2, 4).

H.-y. Yang • R. Licht • M. Beekhuizen • M. van Rijen • F. C. Öner • W. J. A. Dhert • L. B. Creemers (✉)
Department of Orthopaedics, University Medical Center Utrecht
Heidelberglaan 100, PO Box 85500, 3508 GA Utrecht,
The Netherlands
e-mail: l.b.creemers@umcutrecht.nl

M. van Dijk • M. K. Janstål • D. Schumann
DSM Biomedical B.V. Geleen, The Netherlands

W. J. A. Dhert
Faculty of Veterinary Medicine, University of Utrecht
Utrecht, The Netherlands

Although the etiology of OA and disc degeneration is still largely unknown, inflammatory mediators are suggested to correlate to the initiation or maintenance of tissue degeneration and the consequent symptoms (5, 6). As no real cures are available as yet, common strategies for treating the symptoms of joint diseases consist of the use of either non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids, mainly for pain relief (7, 8).

In spite of their effectiveness, systemic administration of these anti-inflammatory compounds has several disadvantages. As clearance of systemically administered drugs is fast, continuous administration at high doses is required. In addition, several side effects are reported. For instance, celecoxib (CXB) may cause gastrointestinal and cardiovascular side effects (9), and the use of corticosteroids in patients with rheumatoid arthritis has been suggested to induce bone loss and hyperglycemia (10, 11).

These side effects are mostly related to the systemic burden caused by the route of administration of these drugs, usually orally, and may be avoided by local administration (12). Local administration could achieve therapeutic concentrations *in situ* while maintaining low systemic levels and also reduce the risk of drug-drug interactions (13). Intra-articular and intra-discal corticosteroid injections, used for treatment of chronic pain, have indeed shown positive but invariably temporary results (14, 15) which were most likely due to fast clearance *in situ*. Therefore, a delivery method allowing for sustained local release of these drugs will reduce systemic drug levels and prolong their presence, hence minimizing side effects and ultimately prolonging clinical effects.

A common delivery method is based on the use of particles releasing encapsulated active compounds. In induced arthritis models, intra-articular administration of liposomes loaded with triamcinolone acetonide (TA) showed a prolonged local delivery compared to free drug (16). Chitosan particles encapsulating CXB showed a limited capacity to diminish the number of arthritic lesions in rats with induced arthritis(17). Sustained release of cortisol from liposomes in patients with rheumatoid arthritis has also been reported in a case study showing improvements in the Ritchie articular index (18).

In vitro release kinetics of therapeutic agents typically is determined to further tailor and adjust the biomaterial properties of sustained release vehicles before their final application *in vivo*. However, release kinetics is usually determined in aqueous media such as PBS or saline. Even if physiological pH and osmolarity can be controlled, the results do not provide information on the bioactivity of the released therapeutic agents. The total absence of proteins in these assays typically would not provide information on the effects of binding occurring *in vivo* by body fluid proteins, especially occurring with hydrophobic small molecules. Binding to plasma proteins does not only affect pharmacokinetics and pharmacodynamics, but also the biological activity of a drug (19). Therefore, bioactivity-based *in vitro* assays are required to show the efficiency and applicability of such release systems. The aim of the current study, was to develop such a bioactivity assay based on TNFα –stimulated OA chondrocytes and use this assay to evaluate the applicability of a new PLGA-PTE-based microsphere platform. Addition of thioester bonds allows for functionalization of the polymer chains (20, 21), hence enabling tissue- or cell-specific targeting, which would further fine tune the biological activity of compounds incorporated. This platform was subsequently tested for its capacity to release CXB or TA for a period of 21 days using the new bioassay.

MATERIALS AND METHODS

Synthesis and Characterization of PLGA-PTE Polymer

The copolymers of D-, L-lactide and glycolide coupled with poly(thioester) linkages (PLGA-PTE) were synthesized according to the method described previously (22). In brief, the synthesis is composed of three steps. PLGA diol was first synthesized with D-, L-lactide, glycolide (Purac Biomaterials, Gorinchem, The Netherlands) and diethylene glycol (Sigma-Aldrich, St. Louis MO, USA) before being converted to PLGA diene using pentenoyl chloride (used as received). At last, PLGA-PTE was synthesized by photo curing PLGA diene with dithio adipic acid (DSM, Heerlen, The Netherlands) under UV irradiation. The degree of conversion in each step was determined by ^{1}H NMR.

The number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity index (PDI) of the polymer was determined using Gel Permeation Chromatography (GPC). GPC was performed on a Waters 515 HPLC pump (Waters Corporation, Milford, MA, USA), a Waters 410 differential refractometer and a Severn Analytical SA6503 Programmable Absorbance Detector (Severn Analytical, UK) equipped with a Waters Styragel columns (HR 2, 3, 4 and 5, Waters) using tetrahydrofuran (THF) (VWR, Radnor, PA, USA) as mobile phase at a flow rate of 1 ml/min. The polymers were dissolved in THF and calibrated against EasiCal polystyrene standards (Agilent Technologies, Santa Clara, CA, USA).

CXB- and TA-Loaded PLGA-PTE Microspheres

The microspheres were prepared by a double emulsion technique (23). For compound encapsulation, approximately 20 mg of CXB (Woburn, MA, USA) or TA (Sigma-Aldrich) was dissolved together with 200 mg of the PLGA-PTE polymer in 8 mL of dichloromethane (Merck, Darmstadt, Germany). To this solution 300 μL de-ionized water was added and this mixture was vortexed (3,000 rpm, 30 seconds) to obtain

an O/W emulsion. The emulsion was immediately transferred to a 50 mL spinner flask (Bellco glass) loaded with an aqueous solution consisting of 40 ml polyvinyl alcohol (1%*w/v*) (Sigma-Aldrich) and stirred (300 rpm) for 16 hours to evaporate the dichloromethane. Subsequently, the particles were spun down. The pellet was washed three times with deionized water and lyophilized to yield the microspheres as a white solid. Microspheres were sterilized with 25 K Gy gamma radiation while cooled by dry ice. In addition to hydrophobic therapeutic compounds shown in the study, the novel platform of PLGA-PTE microspheres could also allow encapsulation of hydrophilic drugs owing to the addition of water during the preparation of loading the compounds to microspheres.

Size Distribution of Microspheres

The particle size of loaded-microspheres was analyzed using laser diffraction (Mastersizer MS2000, Malvern Instruments Ltd, Worcestershire, United Kingdom), by which volume mean diameters and particle size distributions (denoted as span) were measured. Water was used as dispersant. Morphology of the microspheres was documented by scanning electron microscopy (SEM) (Phenom-World, Eindhoven, The Netherlands).

Loading Efficiency of CXB and TA into Microspheres

To determine the loading efficiency of the CXB- and TA-loaded microspheres, the active compound was extracted and quantified by reversed phase HPLC. For compound extraction, 20 mg of compound-loaded microspheres was dissolved in 1 mL DMSO. Subsequently, 9 mL of methanol (Merck) was added to precipitate the polymer and the suspension was shaken for 30 min. The residue was filtered and the amount of active compound was analyzed by reversed phase HPLC using a Waters 2695 Controller equipped with a UV/VIS detector (Waters Corporation). Drug quantification analyses were performed on a Zorbax eclipse XDB C18 column (4.6× 150 mm, particle diameter 5 μm, pore size 80 Å) (Agilent Technologies). For CXB, the mobile phase used was methanol/H_2O 75:25 (*v/v*) at a flow of 1.25 mL/min (24). The run time for the assay was 10 min and the absorption was measured at 254 nm. The mobile phase for TA measurements was methanol/H_2O/H_3PO_3 75:25:0.5 (*v/v/v*) at a flow rate of 1 mL/min (25). The run lasted 7 min and the absorbance was measured at 240 nm.

Release Kinetics in Aqueous Media

CXB- and TA-loaded microspheres were suspended to an amount of 10 mg/ml in 3 ml Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+}, Mg^{2+} (pH 6.9) (Biochrom AG, Berlin, Germany) to simulate the pH in a mildly degenerated joint (26, 27). Additionally 0.02% sodium azide (Sigma-Aldrich) was added to the saline. To increase the solubility of the active compounds in aqueous media, 0.2% of Tween-80 (Sigma-Aldrich) and 0.2% of SDS (Sigma-Aldrich) were added respectively to the release media for CXB- and TA-loaded microspheres (28, 29). The suspensions were incubated at 37°C with gentle shaking. At each time point, 1 mL buffer was collected for analysis and replaced by fresh buffer. The amount of compound in the collected buffer was determined by reversed phase HPLC. The samples for CXB measurement were stored at 4°C and samples for TA measurement were stored at room temperature (to prevent precipitation) until analysis. Triplicates were included in each condition.

Cell Isolation and Culture

Articular cartilage was harvested from knee joints derived from patients undergoing arthroplasty. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in the University Medical Center Utrecht (30). Chondrocytes were isolated by a 3 hours 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 consisting of DMEM (Gibco® Life Technologies, Carlsbad, CA, USA) containing 4.5 mg/ml glucose, 0.8 mg/ml glycyl-L-glutamine, supplemented with 10% fetal bovine serum (FBS) (HyClone® Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco® Life Technologies) and supplemented with 10 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA). The cells were cultured at 37 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.

Release and Bioactivity in TNFα-Stimulated Chondrocyte Culture

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; on the next day, the medium was renewed before starting the experiment. PLGA-PTE microspheres with

PLGA 75:25 were used for the experiment. Both non-loaded or loaded PLGA-PTE microspheres with CXB or TA were dispersed in culture medium and placed in Transwell® baskets (0.4 μm pore size, polycarbonate membrane) (Corning, Amsterdam, The Netherlands). In transwell baskets, the resuspended microspheres were washed once in culture medium before being transferred to each well with cells seeded the day before. Cells and microspheres were co-incubated for 4 hours at 37°C, at 5% CO_2 and 95% humidity. Subsequently, tumor necrosis factor alpha (TNFα) (eBioscience, San Diego, CA, USA) was added at a final concentration of 10 ng/ml to the culture medium.

To study the effect of microspheres on modulating cellular response when stimulated with TNFα, 5 μg of non-loaded microspheres was used. For determining bioactivity of released compounds, microspheres containing 0.1 or 1 nmol of therapeutic compounds were used. This corresponds to 0.5 μg or 5 μg of CXB-containing microspheres, and 0.55 μg or 5.5 μg of TA-containing microspheres. PLGA-PTE microspheres added at a total of 0.1 nmol or 1 nmol loading of CXB are denoted as CXB(0.1)−microspheres or CXB(1)−microspheres. This principle of denotation applies to TA-loaded microspheres as well. According to the release kinetics in PBS, the expected concentration of the anti-inflammatory compounds released from loaded microspheres was calculated to be 0.01 nmol and 0.1 nmol, equivalent to 0.01 and 0.1 μM in 1 mL culture medium, within the first 24 hours. 5 μg of non-loaded microspheres was used as a control.

For bioactivity determination, cells and microspheres were co-incubated for 72 hrs before the microspheres were transferred to another new 24-well culture plate containing cells seeded according to the procedure described above. This procedure was repeated 6 times amounting to a release period of 21 days. Cells treated with 0.01 and 0.1 nmol CXB or TA (equivalent to 0.01 and 0.1 μM in 1 mL culture medium), as a single dose, to the medium were included as comparisons at each time interval. Each condition was analyzed in 4 replicates ($n=4$) and experiments were performed for 3 different donors. Results shown are from one representative donor.

At every 72 hours 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.

Viability Analysis

Cytotoxicity of PLGA-PTE microspheres was examined by measuring lactate dehydrogenase (LDH) secreted in to culture medium from cells using the Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science) according to the manufacturer's instruction. The colorimetric signals were measured at 490 nm subtracted by a background reference at 655 nm, using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

PGE_2 Release Measurement

Cell culture medium was collected at each time point and stored at −80°C; samples were brought to room temperature immediately prior to PGE_2 measurement. The amount of PGE_2 in the samples was measured using the enzyme immunoassay Prostaglandin E_2 Parameter Assay Kit (R&D Systems) following the manufacturer's instructions. The colorimetric intensity was determined using the Benchmark Microplate Reader (Bio-Rad) at 450 nm. The readings were subtracted 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. For determining bioactivity of released anti-inflammatory compounds on cellular PGE_2 levels, normalized PGE_2 amount was further compared to controls in which cells received TNFα only. The average values of controls were set to be 100%.

DNA Content Measurement

The DNA content from each cell lysate was measured using the Quant-iT^{TM} PicoGreen® dsDNA Kit (Life Technologies) following the manufacturer's instruction. The fluorescent signal was measured using a FlexStation® 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices, Downingtown, PA, USA) at Ex/Em 485/538 nm.

Statistics

Statistical analysis was performed using SPSS 20 software (SPSS Inc., Chicago, IL, USA). Results are presented as mean ± standard deviation. Statistical significance was considered when p values were less than 0.05. Differences in PGE_2 production between released CXB and free CXB solution and differences between released TA and free TA solution were determined by univariate analysis of variance using a randomized block design. A post-hoc test with Bonferroni correction was applied when 4 conditions were compared to each other.

RESULTS

Synthesis of PLGA-PTE

PLGA copolymers containing thioester bonds were characterized by ^{1}H NMR. The general chemical structure of the polymers is shown in Fig. 1. Characteristics of the final products are summarized as *PLGA(50:50)-PTE* ^{1}H-NMR

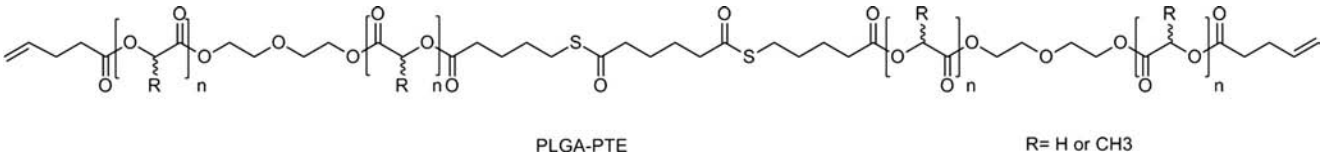

Fig. 1 Chemical structure of PLGA-PTE analyzed by ^{1}H-NMR.

(300 MHz, $CDCl_3$, TMS): δ (ppm) = 5.31-5.15 (m, 37H, CH(lac)); 4.95-4.61 (m, 74H, CH_2(gly)); 4.31 (m, 4H, —(C=O)OC$\underline{H}_2$CH_2O—); 3.69 (m, 4H, —(C=O)OCH_2C$\underline{H}_2$-O—); 2.86 (t, 4H, —(C=O)SC$\underline{H}_2$CH_2CH_2CH_2—); 2.51 (m, 8H, —(C=O)C$\underline{H}_2$CH_2CH_2C$\underline{H}_2$(C=O)—); 2.55-2.38 (m, 8H, —(C=O)SCH_2CH_2CH_2C$\underline{H}_2$—); 1.90-1.43 (m, 127H, CH_3(lac), —(C=O)CH_2C$\underline{H}_2$C$\underline{H}_2$CH_2(C=O)— and —(C=O)SCH_2C$\underline{H}_2$C$\underline{H}_2$CH_2—); GPC: Mn = 13.7 kDa,, Mw = 29.3 kDa, PDI = 2.14.

PLGA(75:25)-PTE ^{1}H-NMR (300 MHz, $CDCl_3$, TMS): δ (ppm) = 5.34-5.02 (m, 71H, CH(lac)); 4.91-4.59 (m, 44H, CH_2(gly)); 4.30 (m, 4H, —(C=O)OC$\underline{H}_2$CH_2O—); 3.69 (m, 4H, —(C=O)OCH_2C$\underline{H}_2$O—); 2.88 (t, 4H, —(C=O)SC$\underline{H}_2$-CH_2CH_2CH_2—); 2.51 (m, 8H, —(C=O)C$\underline{H}_2$-CH_2CH_2C$\underline{H}_2$(C=O)—); 2.65-2.29 (m, 8H, —(C=O)SCH_2CH_2CH_2C$\underline{H}_2$—); 1.91-1.35 (m, 233H, CH_3(lac), —(C=O)CH_2C$\underline{H}_2$C$\underline{H}_2$CH_2(C=O)— and —(C=O)SCH_2C$\underline{H}_2$C$\underline{H}_2$CH_2—); GPC: Mn = 13.5 kDa,, Mw = 26.9 kDa, PDI = 2.0.

Characteristics of CXB- and TA-Loaded Microspheres

The spherical particles prepared by the w/o/w method resulted in a mean diameter ranging from 40 to 55 μm when loading with CXB, and a mean diameter less than 40 μm when TA was loaded. For determining the loading efficiency of loaded microspheres, DMSO was first used to dissolve the polymer; the subsequent addition of methanol was used to completely dissolve CXB or TA, and yet to precipitate the polymer. Prior to HPLC analysis, the residue was filtered in order to remove possible residuals of polymer which might interfere with the measurement. The CXB loading was 7% for PLGA(50:50)-PTE and 8% for PLGA(75:25)-PTE, which correspond to 76% and 86% loading efficiency, respectively. In the case of TA, loading was 11% for PLGA(50:50)-PTE and 9% for PLGA(75:25)-PTE, corresponding to 96% and 97% loading efficiency, respectively. Characteristics of loaded microspheres are summarized in Table I. A SEM image of non-loaded PLGA-PTE microspheres is shown in Fig. 2.

Release Kinetics in PBS

The release kinetics of encapsulated CXB and TA were monitored for up to 16 and 10 weeks, respectively (Fig. 3). As the final aim was to investigate the bioactivity of microspheres loaded with therapeutically relevant doses, high loading was chosen. The release rate of CXB from either PLGA(50:50)-PTE or PLGA(75:25)-PTE was faster in the first 14 days during which 50% of the encapsulated CXB was released. No significant difference between the release profiles of the two PLGA types was noticed within the first 28 days. However, the release rate of CXB decreased gradually afterwards and differed between the two PLGA types. Overall, a complete release of CXB was measured by day 55 and day 110 from PLGA(50:50)-PTE and PLGA(75:25)-PTE, respectively. As the release of CXB was more sustained from PLGA(75:25)-PTE, the release profile of TA was only tested for this polymer ratio. For TA release the overall release rate is higher than for CXB, with a 50% release of TA from PLGA(75:25)-PTE measured within the first week. By day 28, over 90% of encapsulated TA was released (Fig. 3).

Viability

No cytotoxicity was found in any of the conditions. At day 3 and day 21, viability of cells co-incubated with microspheres encapsulating anti-inflammatory compounds measured over 99% (data not shown).

Inhibition of PGE_2 Production in TNFα-Stimulated Chondrocytes by Sustained Release of CXB and TA

The set up of the *in vitro* bioassay for evaluating the anti-inflammatory activity of the released CXB and TA is presented schematically in Fig. 4. P2 osteoarthritic chondrocytes produced low basal levels of PGE_2; stimulation of cells by adding TNFα increased PGE_2 levels 2–5 fold (Fig. 5a). Addition of non-loaded microspheres did not affect PGE_2 production (Fig. 5a), even at later time points (data not shown).

Addition of CXB(0.1)−microspheres to TNFα-stimulated cell cultures reduced PGE_2 production to an average of 30%

Table I Characteristics of Celecoxib (CXB)- and Triamcinolone Acetonide (TA)-Loaded Microspheres with PLGA(50:50)-PTE or PLGA(75:25)-PTE

PLGA-PTE Ratio L:G	Compound loaded	Yield (%)	Loading efficiency (%)	Loading (w%)	Average diameter (μm)	Span
50:50	CXB	75	76	7	40.3	0.82
75:25	CXB	67	86	8	54.6	1.15
50:50	TA	73	96	11	36.8	0.79
75:25	TA	70	97	9	38.1	0.76

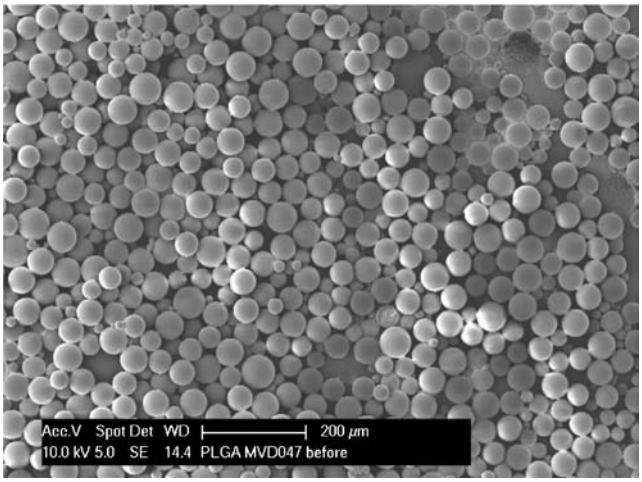

Fig. 2 Scanning electron microscopic (SEM) image of spherical PLGA-PTE microspheres.

of controls, with variable inhibition over the entire release period (Fig. 5b). Also 0.01 nmol free CXB (0.01 μM) seemed to show similar variable effects on PGE_2 level. With CXB(1)− microspheres, a consistent and steady inhibition to 0-7% of controls was found. At each time point, the efficacy of the CXB released from CXB(1)− microspheres was comparable to the 0.1 nmol free CXB (0.1 μM) added directly to the culture medium at each 3-day interval ($p > 0.05$; Fig. 5b).

When using TA(0.1)− microspheres, the PGE_2 level was reduced to 3% until day 6, yet the PGE_2 increased again from 26 to 74% in the following two time points and the levels remained over 70% in the rest of the release period, whereas 0.01 nmol free TA (0.01 μM) in each time interval resulted in inhibiting PGE_2 production to an average of 9% during the whole release period (Fig. 5c). TA released from the microspheres containing the higher dosage, TA(1)− microspheres, resulted in a comparable inhibiting effect as using 0.1 nmol free TA (0.1 μM) until day 15 (0-12%, Fig. 5c). After day 18, the PGE_2 level was slightly higher when cells were co-incubated with TA(1)− microspheres (27- 28%) compared to 0.1 nmol free TA (0.1 μM) (6-10%) ($p < 0.05$; Fig. 5c).

Images of CXB-loaded microspheres microspheres were shown to illustrate changes in appearance macroscopically after a release period of 21 days, co-incubated with cells. Before co-incubation with cells, microspheres were round and spherical with a smooth surface, but after 21 days the microspheres seemed to lose their initial shape (Fig. 6).

DISCUSSION

In the current study, we developed a bioactivity assay based on TNFα-stimulated chondrocytes as an *in vitro* inflammatory model to measure the bioactivity of anti-inflammatory agents released over time from biomaterial-based delivery systems. This bioassay was used to test the release of the anti-inflammatory therapeutic compounds celecoxib (CXB) and triamcinolone acetonide (TA) from a novel polymeric microsphere platform. Poly (lactic-co-glycolic acid) (PLGA), a commonly used copolymer as a carrier in drug delivery (31), was modified by linking poly(thioester) into the polymer (PLGA-PTE).

In this novel platform CXB and TA were incorporated. First, release kinetics in PBS was determined over 16 weeks. Subsequently the newly developed bioactivity assay was used, to show that anti-inflammatory compounds released from PLGA-PTE microspheres inhibited PGE_2 production over a release period for up to 21 days. A more pronounced and constant inhibition was found using CXB(1)– microspheres and TA(1)– microspheres, especially those formulated with TA. During the entire release period, the anti-inflammatory effects of the microspheres with CXB(1)− microspheres and TA(1)− microspheres showed comparable results to 0.1 nmol free anti-inflammatory agents (equivalent to 0.1 μM) added directly to the culture medium. No cytotoxicity was observed either at early or late time points in culture.

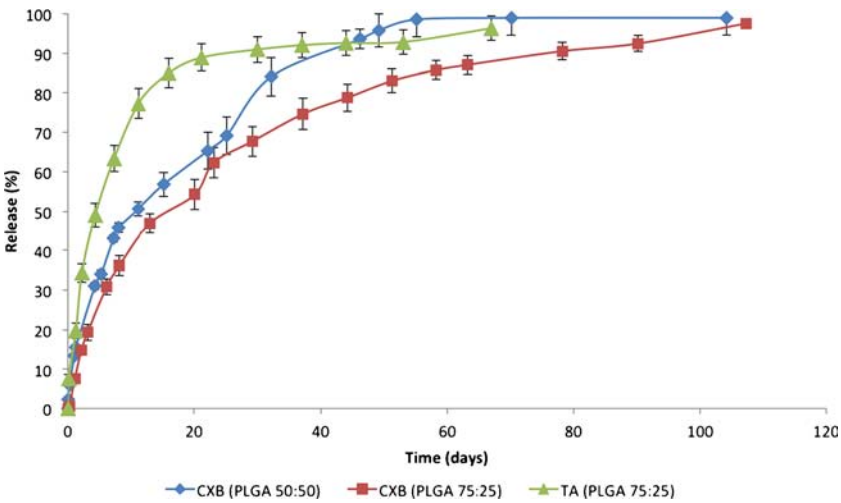

Fig. 3 Release profile of CXB- and TA-loaded microspheres, with either PLGA(50:50)-PTE or PLGA(75:25)-PTE, in PBS buffer pH 6.9 at 37°C.

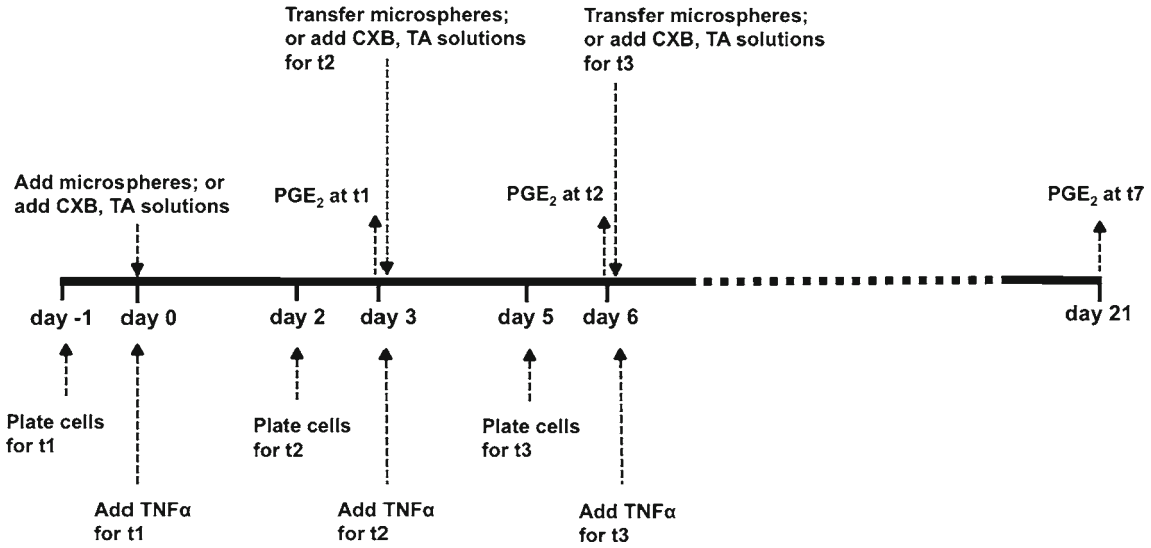

Fig. 4 Schematic overview of the bioassay used for evaluating bioactivity of released CXB and TA in inhibiting PGE_2 production in TNFα-stimulated chondrocytes over 21 days.

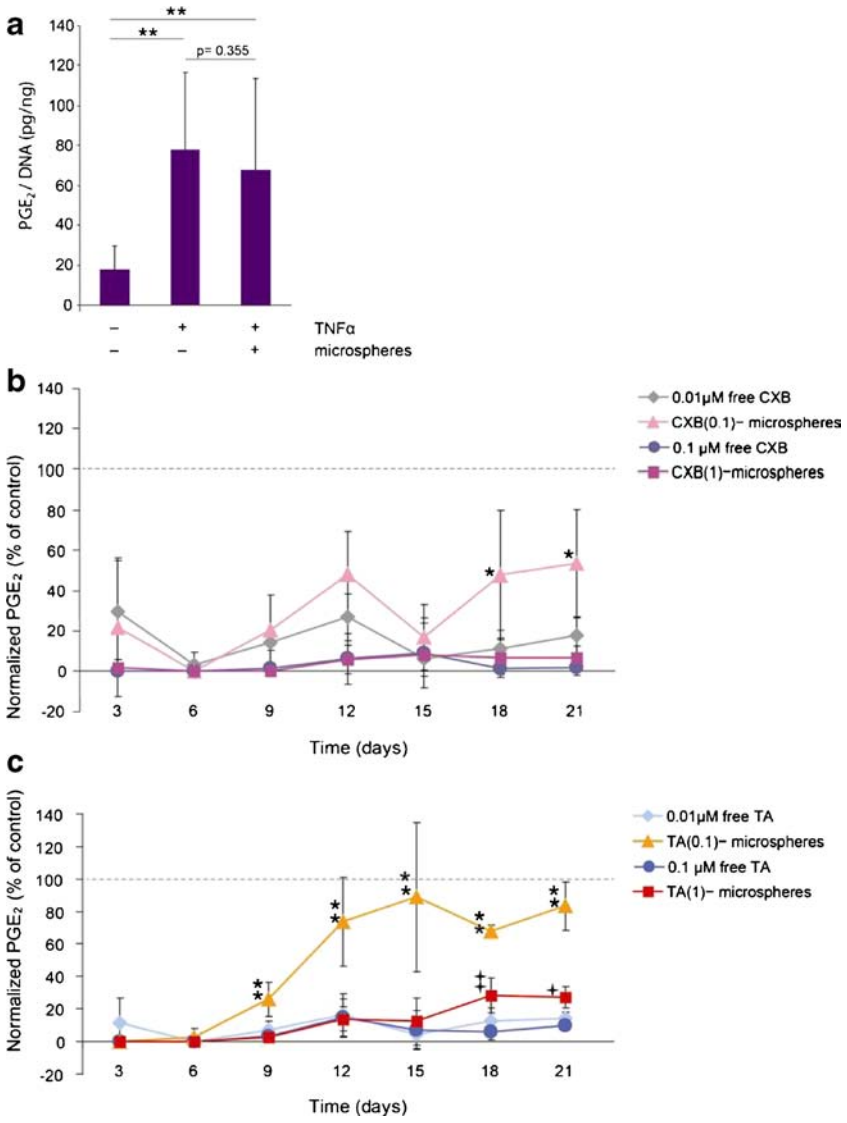

Fig. 5 PGE_2 levels in OA chondrocyte cultures stimulated with TNFα, and the inhibitory effects of CXB- or TA-loaded PLGA-PTE microspheres compared to cells treated with directly addition of 0.01 or 0.1 nmol therapeutic compounds (i.e. CXB or TA) as a single dose to the medium (equivalent to 0.01 or 0.1 μM). PGE_2 levels were first normalized to DNA content and then compared to controls. (**a**) Unloaded microspheres did not affect PGE_2 levels, but a clear stimulation by TNFα was noted. (**b**) PGE_2 levels of TNFα-induced cells was reduced when using CXB-loaded microspheres, albeit with a fluctuating pattern when CXB(0.1) − microspheres were used. (**c**) TA − loaded microspheres in cell culture also resulted in a decreased PGE_2 production, although it was restored to over 50% after day 12 when TA(0.1) − microspheres were used. However, consistent inhibition of PGE_2 can be found in the cell culture when using CXB(1)- or TA(1)-microspheres (**b**, **c**). The average values of controls were set to be 100%. * $p < 0.05$ compared to 0.01 μM free CXB. ** $p < 0.001$ compared to 0.01 μM free TA; + $p < 0.05$ compared to 0.1 μM free TA, ++ $p < 0.001$ compared to 0.1 μM free TA.

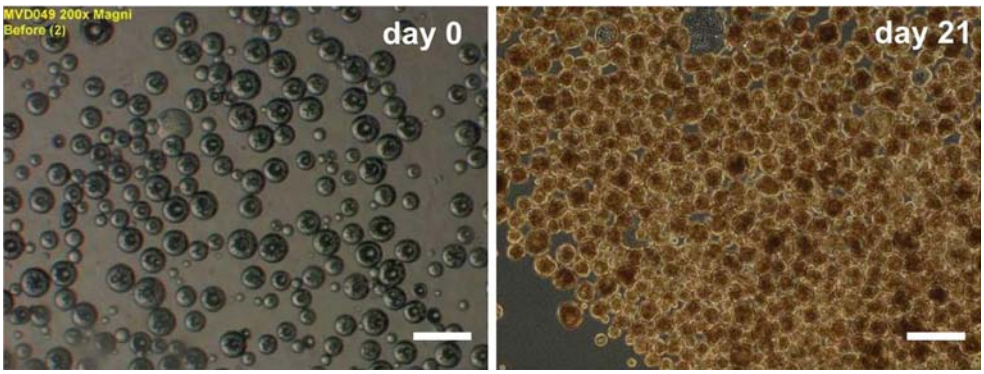

Fig. 6 PLGA-PTE microspheres before and after a release period of 21 days, co-incubated with cells. The scale bar represents 100 μm.

Since the final aim was to investigate the bioactivity of microspheres loaded with therapeutically relevant doses, high loading was chosen for the release studies. As CXB and TA display low solubility in aqueous solution (32, 33), Tween-80 and SDS, respectively, were used before for studying the release kinetics of CXB and TA (28, 29). Most likely, addition of these surfactants or emulsifiers would also affect the release kinetics by changing, for example, porosity of the microspheres, polymer chain mobility and density, as well as consequent hydrolysis rate and pH in the release medium which all contribute to drug release from microspheres (34, 35). However, such-like release studies are extremely difficult to extrapolate to the final *in vivo* situation.

By using a standardized cell culture protocol, the application of TNFα-stimulated osteoarthritic chondrocytes was found to be promising for determining the bioactivity of anti-inflammatory compounds released from biomaterial-based microspheres, also including evaluation of long-term actions. Hence, this assay provides an essential link between the commonly used *in vitro* buffer systems for release kinetics and the final *in vivo* application. The concentration of small molecules released into the culture medium was not determined in the current study. In particular hydrophobic small molecules are known to bind to proteins, including serum proteins such as albumin, the most abundant protein in plasma (19, 36), thus altering their bioavailability. Therefore concentration would not have correlated with activity, which was the major reason for developing the current bioassay. A similar system has been used previously for determining the activity of BMP-2 released from several biomaterials to stimulate osteogenesis (37). The reported system in combination with ELISA for protein detection, was shown to effectively distinguish between scaffolds releasing inactive and those releasing active protein, which also correlated with *in vivo* bone formation. The current bioactivity assay has the added value of using primary, non-cell line, human cells actually derived from tissue affected during degenerative disease of cartilaginous tissues, i.e. osteoarthritic chondrocytes, which have also been shown to display many similarities to degenerated intervertebral disc cells (3, 6). In addition to general inflammatory inhibitors, other factors known to interfere with the inflammatory response (e.g. NF-κB inhibitors) could also be tested in this *in vitro* assay.

The PLGA-PTE microspheres provide a suitable platform for the sustained release of small molecule drugs, with the PTE modification to allow for functionalization, e.g. through coupling of bioactive peptides or proteins. Release rates can be tailored by changing the ratio of lactic acid to glycolic acid (38, 39). In the current study, release profiles of CXB studied in buffer indeed showed that microspheres with PLGA(75:25)-PTE displayed a slower release rate compared to PLGA(50:50)-PTE. The residual of the solvent dichloromethane used for preparing the loaded microspheres was not determined in the study, as dichloromethane has been commonly used and its efficient elimination in the course of the preparation procedure is well characterized, including for application *in vivo* (40–42). Also in the current study, the absence of cytotoxicity supports proper removal of dichloromethane residue. However, quantifying residuals of organic solvents used for preparing formulations is of importance for future clinical applications.

Quite surprisingly, the response in terms of PGE_2 production to microspheres containing 0.1 nmol of CXB or to 0.01 nmol free CXB added to the culture medium (equivalent to 0.01 μM), was variable. This phenomenon was not found for TA-mediated inhibition of inflammation. One explanation may lie in the complex pathways involved in cyclooxygenase (COX)-mediated PGE_2 synthesis. In the PGE_2 synthesis pathway, COX activity generates PGH_2, which can be further converted to PGE_2 by one of the PGE synthases, for example, microsomal prostaglandin E synthase-1 (mPGES-1). mPGES-1 is responsive to pro-inflammatory cytokines in various cell types including synovial fibroblasts (43). When up-regulation of mPGES-1 would coincide with a submaximal inhibition of COX-2 activity, sufficient amounts of PGH_2 for mPTGS-1 may have been generated and thus resulted in more PGE_2 than expected. Another explanation may lie in a compensatory up-regulation of COX-1, normally constitutively expressed (44). These phenomena further stress

the importance of using bioassays as described in the current study for investigating release kinetics and bioactivity.

Although the severe side effects associated with systemic administration of CXB or corticosteroids (45) (10) were shown to be circumvented by intra-articular injection as a treatment for OA patients with severe pain (46, 47), the treatment effects were typically short (12, 48). Even with long-term administration of injections for up to 2 years, improvement of pain was measured only during the first year, despite an improvement in the range of motion (49). In an OA-induced rat model although intra-articular injection of COX-2 inhibitors resulted in effective inhibition of OA progression, the regimen involved high doses of COX-2 inhibitor (100 mg/ml) and frequent injections in the course of 5 weeks (50). In intervertebral disc degeneration, intradiscal corticosteroid injections also have been suggested to be temporarily effective in a subset of patients displaying MRI signal intensity changes in adjacent vertebral bodies (51). However, repeated intra-articular injections increase the risk of infection (52, 53). Biomaterial-based controlled release of CXB or TA holds advantages over systemic or local bolus administration by increasing efficacy and prolonging therapeutic effects, as well as reducing adverse effects. In an induced arthritis model, intra-articularly injected liposomes were used to release TA, which resulted in its prolonged retention in the joint cavity compared to free drug, although only 38% was still remaining after 8 hrs (16). In a limited pilot study, sustained release of cortisol from liposomes showed improvement in thermographic index for 14 days in rheumatoid arthritis patients; however, no further improvements was found (18).

Despite the promising advances of biomaterial-based platforms for releasing therapeutic compounds, application of these platforms for the release of anti-inflammatory agents will require thorough evaluation of the effects on tissue integrity and functionality of both the materials and their therapeutic components in animal models. Although systemic levels of the anti-inflammatory agents will be low, local levels will be sustained for a prolonged period, which may affect the local tissues differently from a single high dose. Furthermore, to achieve full therapeutic efficacy, higher loading of the therapeutic compounds may be required, which might be challenging. Increased loading of carriers can be achieved by, for example, adjusting PLA/PGA ratios of PLGA to enhance carriers' stability, increasing the size of the carriers (54), or by using alternative shapes such as rods (55). However, these approaches will partially compromise injectability of the controlled release platform, and often larger-sized carriers are less resistant to the shear stress induced by injection. Alternatively compound-loading may be increased, although this would also compromise carrier stability. Dose adjustment will therefore require a balanced approach based on these considerations before full clinical application is possible.

CONCLUSION

In the current study, we report the development and application of PLGA-PTE microspheres for delivery of CXB and TA and a bioassay for determining their bioactivity, with the ultimate goal of treating patients with inflammatory joint diseases. While additional studies *in vivo* are required in order to fully understand the biological response to the release system *per se* and to the prolonged released therapeutic agents, the results presented here demonstrate a high efficiency in reducing inflammation over 3 weeks in cells by a single administration of therapeutic agents loaded microspheres.

ACKNOWLEDGMENTS AND DISCLOSURES

This research forms part of the Project P2.01 IDiDAS of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs. The financial contribution of the Dutch Arthritis Association is gratefully acknowledged.

REFERENCE

1. The burden of musculoskeletal diseases at the start of the new millennium. World Health Organization. 2003; Technical Report Series no 919.
2. Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, *et al*. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. Arthritis Rheum. 2008;58(1):26–35.
3. Urban JP, Roberts S. Degeneration of the intervertebral disc. Arthritis Res Ther. 2003;5(3):120–30.
4. Deyo RA, Mirza SK, Martin BI. Back pain prevalence and visit rates: estimates from U.S. national surveys, 2002. Spine (Phila Pa 1976). 2006;31(23):2724–7.
5. Clark AG, Jordan JM, Vilim V, Renner JB, Dragomir AD, Luta G, *et al*. Serum cartilage oligomeric matrix protein reflects osteoarthritis presence and severity: the johnston county osteoarthritis project. Arthritis Rheum. 1999;42(11):2356–64.
6. Podichetty VK. The aging spine: the role of inflammatory mediators in intervertebral disc degeneration. Cell Mol Biol. 2007;53(5):4–18.
7. Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American college of rheumatology subcommittee on osteoarthritis guidelines. Arthritis Rheum. 2000;43(9):1905–15.
8. Roberts S, Butler RC. Inflammatory mediators as potential therapeutic targets in the spine. Curr Drug Targets Inflamm Allergy. 2005;4(2):257–66.
9. Solomon SD, Pfeffer MA, McMurray JJ, Fowler R, Finn P, Levin B, *et al*. Effect of celecoxib on cardiovascular events and blood pressure in two trials for the prevention of colorectal adenomas. Circulation. 2006;114(10):1028–35.
10. Saag KG, Koehnke R, Caldwell JR, Brasington R, Burmeister LF, Zimmerman B, *et al*. Low dose long-term corticosteroid therapy in rheumatoid arthritis: an analysis of serious adverse events. Am J Med. 1994;96(2):115–23.

11. Donihi AC, Raval D, Saul M, Korytkowski MT, DeVita MA. Prevalence and predictors of corticosteroid-related hyperglycemia in hospitalized patients. Endocr Pract. 2006;12(4):358–62.
12. Gaffney K, Ledingham J, Perry JD. Intra-articular triamcinolone hexacetonide in knee osteoarthritis: factors influencing the clinical response. Ann Rheum Dis. 1995;54(5):379–81.
13. Heyneman CA, Lawless-Liday C, Wall GC. Oral versus topical NSAIDs in rheumatic diseases: a comparison. Drugs. 2000;60(3): 555–74.
14. Pyne D, Ioannou Y, Mootoo R, Bhanji A. Intra-articular steroids in knee osteoarthritis: a comparative study of triamcinolone hexacetonide and methylprednisolone acetate. Clin Rheumatol. 2004;23(2):116–20.
15. Cao P, Jiang L, Zhuang C, Yang Y, Zhang Z, Chen W, *et al*. Intradiscal injection therapy for degenerative chronic discogenic low back pain with end plate modic changes. Spine J. 2011;11(2): 100–6.
16. Lopez-Garcia F, Vazquez-Auton JM, Gil F, Latoore R, Moreno F, Villalain J, *et al*. Intra-articular therapy of experimental arthritis with a derivative of triamcinolone acetonide incorporated in liposomes. J Pharm Pharmacol. 1993;45(6):576–8.
17. Thakkar H, Sharma RK, Mishra AK, Chuttani K, Murthy RS. Celecoxib incorporated chitosan microspheres: in vitro and in vivo evaluation. J Drug Target. 2004;12(9–10):549–57.
18. de Silva M, Hazleman BL, Thomas DP, Wraight P. Liposomes in arthritis: a new approach. Lancet. 1979;1(8130):1320–2.
19. Rowland M. Plasma protein binding and therapeutic drug monitoring. Ther Drug Monit. 1980;2(1):29–37.
20. Hoyle CE, Lee TY, Roper T. Thiol–enes: chemistry of the past with promise for the future. J Polym Sci Part A: Pol Chem. 2004;42(21): 5301–38.
21. van Dijk M, Rijkers DTS, Liskamp RMJ, van Nostrum CF, Hennink WE. Synthesis and applications of biomedical and pharmaceutical polymers via click chemistry methodologies. Bioconjug Chem. 2009;20(11):2001–16.
22. Dias A, Boerakker M, Nijenhuis A. Polymers comprising thioester bonds. 2007:WO/2007/028612.
23. Sinha VR, Trehan A. Biodegradable microspheres for protein delivery. J Control Release. 2003;90(3):261–80.
24. Baboota S, Faiyaz S, Ahuja A, Ali J, Shafiq S, Ahmad S. Development and validation of a stability-indicating HPLC method for analysis of Celecoxib (CXB) in bulk drug and microemulsion formulations. Acta Chromatogr. 2007;18:116–29.
25. Ahn JS, Choi HK, Chun MK, Ryu JM, Jung JH, Kim YU, *et al*. Release of triamcinolone acetonide from mucoadhesive polymer composed of chitosan and poly(acrylic acid) in vitro. Biomaterials. 2002;23(6):1411–6.
26. Goldie I, Nachemson A. Synovial pH in rheumatoid knee-joints. I. eff synovectomy Acta Orthop Scand. 1969;40(5):634–41.
27. Nachemson A. Intradiscal measurements of pH in patients with lumbar rhizopathies. Acta Orthop Scand. 1969;40(1): 23–42.
28. Chandran S, Ravi P, Saha RN. Development and in vitro evaluation of oral controlled release formulations of celecoxib using optimization techniques. Yakugaku zasshi: J e Pharm Soc Jpn. 2006;126(7):505–14.
29. Center for Drug Evaluation and Research. Approval package for: application number ANDA 090164 " Triamcinolone Acetonide Injectable Suspension USP 40 mg/mL". FDA. 2009.
30. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. For Bmj. 2002;325(7365):648–51.
31. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Preat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release. 2012;161(2):505–22.
32. DrugBank database. Celecoxib (DB00482). http://www.drugbank.ca/.
33. Royal Society of Chemistry. Tiamcinolone acetonide. http://www.rsc.org/learn-chemistry.
34. Fredenberg S, Wahlgren M, Reslow M, Axelsson A. The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems–a review. Int J Pharm. 2011;415(1–2):34–52.
35. Faisant N, Akiki J, Siepmann F, Benoit JP, Siepmann J. Effects of the type of release medium on drug release from PLGA-based microparticles: experiment and theory. Int J Pharm. 2006;314(2):189–97.
36. Seedher N, Bhatia S. Mechanism of interaction of the non-steroidal antiinflammatory drugs meloxicam and nimesulide with serum albumin. J Pharm Biomed Anal. 2005;39(1–2):257–62.
37. Kempen DH, Lu L, Hefferan TE, Creemers LB, Maran A, Classic KL, *et al*. Retention of in vitro and in vivo BMP-2 bioactivities in sustained delivery vehicles for bone tissue engineering. Biomaterials. 2008;29(22):3245–52.
38. Lu L, Garcia CA, Mikos AG. In vitro degradation of thin poly(DL-lactic-co-glycolic acid) films. J Biomed Mater Res. 1999;46(2):236–44.
39. Witschi C, Doelker E. Influence of the microencapsulation method and peptide loading on poly(lactic acid) and poly(lactic-co-glycolic acid) degradation during in vitro testing. J Control Release. 1998;51 (2–3):327–41.
40. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials. 2000;21(23):2475–90.
41. Cui F, Cun D, Tao A, Yang M, Shi K, Zhao M, *et al*. Preparation and characterization of melittin-loaded poly (DL-lactic acid) or poly (DL-lactic-co-glycolic acid) microspheres made by the double emulsion method. J Control Release. 2005;107(2):310–9.
42. Hazekawa M, Sakai Y, Yoshida M, Haraguchi T, Uchida T. Single injection of ONO-1301-loaded PLGA microspheres directly after ischaemia reduces ischaemic damage in rats subjected to middle cerebral artery occlusion. J Pharm Pharmacol. 2012;64(3):353–9.
43. Cheng S, Afif H, Martel-Pelletier J, Pelletier JP, Li X, Farrajota K, *et al*. Activation of peroxisome proliferator-activated receptor gamma inhibits interleukin-1beta-induced membrane-associated prostaglandin E2 synthase-1 expression in human synovial fibroblasts by interfering with Egr-1. J Biol Chem. 2004;279(21): 22057–65.
44. Kirtikara K, Morham SG, Raghow R, Laulederkind SJ, Kanekura T, Goorha S, *et al*. Compensatory prostaglandin E2 biosynthesis in cyclooxygenase 1 or 2 null cells. J Exp Med. 1998;187(4):517–23.
45. Moore RA, Derry S, Makinson GT, McQuay HJ. Tolerability and adverse events in clinical trials of celecoxib in osteoarthritis and rheumatoid arthritis: systematic review and meta-analysis of information from company clinical trial reports. Arthritis Res Ther. 2005;7(3):R644–665.
46. Hollander J, Brown EMJ, Jessar RA, Brown CY. Hydrocortisone and cortisone injected into arthritic joints: Comparative effects of and use of hydrocortisone as a local antiarthritic agent. J Am Med Assoc. 1951;147(17):1629.
47. Hollander JL, Jessar RA, Brown EMJ. Intra-synovial corticosteroid therapy: a decade of use. Bull Rheum Dis. 1961;11:239–40.
48. Dieppe PA, Sathapatayavongs B, Jones HE, Bacon PA, Ring EF. Intra-articular steroids in osteoarthritis. Rheum Rehabil. 1980;19(4): 212–7.
49. Raynauld JP, Buckland-Wright C, Ward R, Choquette D, Haraoui B, Martel-Pelletier J, *et al*. Safety and efficacy of long-term intraarticular steroid injections in osteoarthritis of the knee: a randomized, double-blind, placebo-controlled trial. Arthritis Rheum. 2003;48(2):370–7.
50. Jean YH, Wen ZH, Chang YC, Hsieh SP, Tang CC, Wang YH, *et al*. Intra-articular injection of the cyclooxygenase-2 inhibitor parecoxib attenuates osteoarthritis progression in anterior cruciate ligament-transected knee in rats: role of excitatory amino acids. Osteoarthr Cartil. 2007;15(6):638–45.

51. Lee JW, Choi SW, Park SH, Lee GY, Kang HS. MR-based outcome predictors of lumbar transforaminal epidural steroid injection for lumbar radiculopathy caused by herniated intervertebral disc. Eur Radiol. 2013;23(1):205–11.
52. Papavasiliou AV, Isaac DL, Marimuthu R, Skyrme A, Armitage A. Infection in knee replacements after previous injection of intra-articular steroid. J Bone Joint Surg (Br). 2006;88(3):321–3.
53. Kaspar S. de VdBJ. Infection in hip arthroplasty after previous injection of steroid. J Bone Joint Surg (Br). 2005;87(4):454–7.
54. Wischke C, Schwendeman SP. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. Int J Pharm. 2008;364(2):298–327.
55. Laeschke K. Biocompatibility of microparticles into soft tissue fillers. Semin cutaneous med surg. 2004;23(4):214–7.