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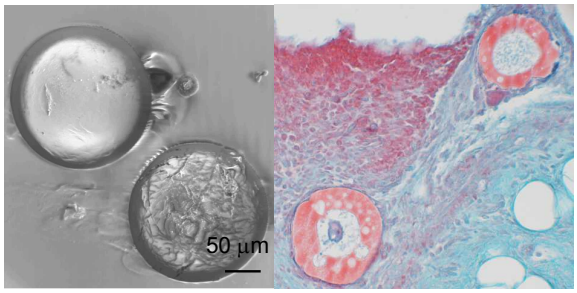

Core-shell PLGA/PLLA polymer microspheres sustained 2 weeks *in vivo* bupivacaine release, providing extended postoperative analgesia without infection or joint damage.

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ARTICLE

Sustained Release of Bupivacaine for Post-Surgical Pain Relief Using Core-Shell Microspheres

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Y. Shona Pek,[a] P. Pitukmanorom[a] and Jackie. Y. Ying[a*]

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[a] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669.
*Corresponding author. Tel: (+65) 6824 7100; fax: (+65) 6478 9020. E-mail address: jyying@ibn.a-star.edu.sg

Core-shell polymer microspheres with poly(D,L-lactic-co-glycolic acid) (PLGA) core and poly(L-lactic acid) (PLLA) shell were developed for sustained release of bupivacaine for postoperative pain relief after knee surgery. The PLLA-rich shell acted as a diffusion barrier, allowing linear release of bupivacaine in a goat model over an extended period of 2 weeks post-surgery. *In vivo* bupivacaine concentrations in the goat synovial fluid remained within therapeutic levels for the 2 weeks, whereas bupivacaine concentrations in the blood plasma remained safely below toxic levels. All animals survived until the end of the 28-day study, with no inflammation, infection or reduced mobility. Explantation at day 28 revealed some soft microsphere remnants in the para-patellar space of the knee joint. However, there was no damage to the articular surfaces, or interference with joint motion. Histological analysis of each knee compartment did not reveal any signs of osteoarthrosis or degeneration within the joints, and safranin-O staining showed intact cartilage surfaces with well-preserved proteoglycan content.

1. Introduction

Localized and sustained delivery of anesthetics for regional control of postoperative pain in knee surgery is currently achieved by continuous femoral or epidural infusion of drugs such as bupivacaine through a catheter[1–3]. In particular, due to the relatively short 3.5 hr half-life of bupivacaine, continuous infusion is necessary to achieve extended postoperative pain relief[4]. However, there is often a risk of infection or catheter malfunction[5,6]. In addition, post-operative hospitalization for the purpose of drug administration and catheter maintenance is also required, thus increasing patient cost. Therefore, localized and sustained delivery of bupivacaine using a one-time administration of biodegradable bupivacaine-loaded microspheres is expected to provide a safer and more effective treatment of pain. These bupivacaine-loaded microspheres could be implanted in the parapatellar space in the stifle joint at the end of the surgical procedure, thus eliminating the need for extended hospitalization and catheter infusion. However, it is difficult to achieve sustained release of anesthetics using hydrogels and single-walled polymer microspheres because these small water-soluble molecules can diffuse quickly through the polymer matrix, thus resulting in a large initial burst and rapid release of drugs. In fact, many delivery systems are unable to prolong the release of anesthetics by more than several days[7–14].

We had previously developed polymer-inorganic composite microspheres for tunable sustained and delayed release of proteins[15]. In this study, we showed that by modifying the conventional oil-in-water emulsion technique, core-shell polymeric microspheres could be formulated to provide for sustained *in vivo* release of bupivacaine over 2 weeks with minimal initial burst. These core-shell microspheres were formed by phase separation of two polymers, poly(L-lactic acid) (PLLA) and poly(D,L-lactic-co-glycolic acid (PLGA), which occurred during the solvent evaporation process[16–24]. As the solvent was removed from the oil-in-water emulsion, the polymer concentration in the oil droplets would increase, eventually leading to a phase separation. For the solution with a PLLA/PLGA mass ratio of 1:1, this phase separation resulted in a core-shell structure with the PLLA-rich phase enveloping the PLGA-rich phase[19–22] (Fig. 1a). By selecting the appropriate molecular weights of PLLA and PLGA such that the PLLA degrades much slower than the PLGA, a porous PLLA shell is obtained once the PLGA has degraded. This porous PLLA shell creates a diffusion barrier to reduce the initial burst release and maintain a constant release rate of bupivacaine from the core of the microspheres (Fig. 1b). By varying selected critical synthesis parameters such as synthesis buffer composition, buffer stirring speed and drug:polymer ratio, we could control the bupivacaine delivery profile so as to achieve the desired delivery rate while maintaining a high drug encapsulation efficiency.

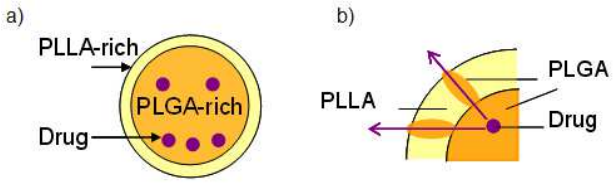

Figure 1. Schematic showing a) drug-loaded core-shell microsphere with a PLGA-rich core and PLLA-rich shell, with the drug in the PLGA phase, and b) PLGA degradation creates pores in PLLA-rich shell, thus enabling drug release from the core PLGA phase.

Due to the knee being an area that experiences frequent mechanical loading, it was also important to confirm that the microspheres implanted did not break when subjected to such mechanical forces, as this would cause the drug to be unintentionally released in a burst. This was demonstrated in a separate *ex vivo* study using microspheres loaded with a chlorophenol red dye, so that any breaking or leakage of the microsphere pellet after implantation could be observed more easily. This initial *ex vivo* test did not show any damage to the dye-loaded microspheres implanted in the knee joint despite being subject to typical mechanical forces during joint movement. For ease of handling and implantation, the required volume of bupivacaine-loaded microspheres for the 2-week bupivacaine release was made into a disc-shaped compact. These microsphere compacts were then implanted into the parapatellar space in the stifle joint of goats in order to determine their efficacy in releasing and maintaining therapeutic bupivacaine concentrations for 2 weeks in the synovial fluid. Therapeutic doses commonly used are 4 ml/hr of 0.25% (2.5 mg/ml) bupivacaine[4]. In addition, as the recommended dosage limit of bupivacaine is 400 mg/day[25] and excessively high bupivacaine concentrations (0.5%, 5 mg/ml) have been shown to be toxic to cartilage and chondrocytes[26,27], it was important that burst release did not occur. Therefore, bupivacaine levels in both the synovial fluid as well as the plasma were monitored throughout the 2-week duration of the microsphere delivery and for an additional 2 weeks to ensure the bupivacaine release from the microspheres had stopped. Bupivacaine concentrations in the knee joint were within desired therapeutic levels for the complete 2-week post-surgical duration after a one-time administration of bupivacaine-loaded microspheres. Bupivacaine concentrations in plasma were low and below toxic levels. After 4 weeks, the study was terminated and the goats were sacrificed. Macroscopic examination and histological analysis of the joint area did not show any signs of cartilage degeneration or inflammation, thus demonstrating the feasibility of using our core-shell microspheres for long-term post-surgical delivery of bupivacaine for pain relief.

2. Experimental

2.1 Materials

PLGA samples with molecular weights of 7 kDa (5050 DLG 1A), 24 kDa (5050 DLG 2.5A) and 33kDa (5050 DLG 3A) were purchased from Lakeshore Biomaterials. PLLA with a molecular weight of 100 kDa was purchased from Polysciences. For *in vitro* studies, bupivacaine hydrochloride was purchased from Sigma-Aldrich (Singapore). For *in vivo* studies, medical-grade bupivacaine hydrochloride was purchased from Aginko AG (Bern, Switzerland).

2.2 Synthesis of core-shell microspheres

The drug-loaded PLLA/PLGA core-shell microspheres were synthesized using a solid-in-oil-in-water emulsion solvent extraction technique. In a typical synthesis, two separate solutions of 125 mg of PLLA in 1 ml of dichloromethane (DCM) and 125 mg of PLGA in 1 ml of DCM were prepared. 90 mg of bupivacaine powder was then added to the PLGA solution to form a solid-in-oil suspension. In the case of the dye-loaded microspheres, instead of bupivacaine powder, 0.3 mg of chlorophenol red dye (Sigma) was added to the PLGA solution to form a solid-in-oil suspension. This suspension and the PLLA solution were then combined, mixed, and subsequently emulsified in 200 ml of 0.1% methyl cellulose solution in PBS using a mechanical stirrer (Heidolph RZR2051 Overhead Stirrer) with a stirring speed of 400 rpm to produce a solid-in-oil-in-water emulsion. The methyl cellulose solution used in this emulsification step was partially saturated with 1.8 ml of DCM to minimize unwanted extraction of DCM from the oil droplets by water, and prevent premature hardening of microspheres before the two polymers could phase separate. Controlled extraction of DCM and hardening of polymer droplets were later initiated after 30 min of stirring by adding another 200 ml of 0.1% methyl cellulose solution in PBS that contained no DCM with a peristaltic pump at a rate of 1 ml/min. The hardened microspheres obtained were filtered, washed with water, and freeze-dried overnight.

2.3 *In vitro* bupivacaine release studies

20 mg of microspheres were incubated in 1.5 ml of PBS buffer at 37°C. At the pre-determined time intervals, 0.75 ml of the incubated medium was withdrawn and replaced with 0.75 ml of fresh PBS buffer. The collected medium was then assayed for bupivacaine by using an ultraviolet-visible (UV-Vis) spectrophotometer (Biochrom Libra S22) to measure the absorbance at 240 nm. Each formulation of microspheres was tested in triplicates, and the cumulative bupivacaine released was reported as a percentage of the amount of drug loaded in the microspheres. The amount of drug loaded in the microspheres was determined by dissolving away the polymer matrix of the drug-loaded microspheres in DCM, leaving behind the insoluble drug.

2.4 *In vitro* microsphere degradation studies

Multiple batches of microspheres (20 mg per batch) were incubated in 1.5 ml of PBS buffer at 37°C. At the same time intervals as in Section 2.3, 0.75 ml of the incubated medium was withdrawn and replaced with 0.75 ml of fresh PBS buffer. At longer time intervals, 3 microsphere batches were selected at random and freeze-dried. The freeze-dried microspheres were then examined with environmental scanning electron microscopy (ESEM) using a FEI Quanta 200 environmental scanning electron microscope operating at an accelerating voltage of 10 kV. These images were compared with ESEM images of newly-synthesized microspheres, as well as empty microspheres without drug, in order to observe morphological changes in the microspheres as they released the drugs and degraded with time. Where possible, the microspheres were sliced in half with a surgical blade to observe the cross-section.

2.5 *Ex vivo* dye release studies

2.5.1 Preparation of dye-loaded microsphere compacts (MCs) for implantation

Core-shell microspheres were synthesized according to the protocol described in Section 2.2, but substituting the bupivacaine drug with 0.3 mg of chlorophenol red dye. This resulted in microspheres with a yellow color for easy visibility in the implanted site. The microspheres were compacted into cylindrical pellets for implantation into the parapatellar space of goats. This was done by pouring 10 g of microspheres into cylindrical molds of 2-cm diameter, and placing the filled molds in an oven at 45–50°C for 24 h. Holding the microspheres at the lower limit of the glass transition temperature of the PLLA outer shell enabled a slight softening of the PLLA, allowing the microspheres to stick to one another lightly. Upon removal from the molds, the microspheres remained in the desired shape of the molds, but could be separated again undamaged by applying a moderate force or friction. Any breakage of the individual microspheres due to mechanical force would result in the release of the dye, which would turn bright purple in the pH of the *in vivo* parapatellar environment. This was verified by intentionally cutting the dye-containing spheres open with a surgical blade and soaking the cut spheres in PBS. Intentionally cutting open the dye-containing microspheres resulted in the release of the dye, which turned bright violet in PBS.

2.5.2 *Ex vivo* implantation of dye-loaded microspheres

These dye-loaded microspheres were implanted into the parapatellar region of the stifle joint of a pre-sacrificed goat leg. The stifle joint was then surgically closed and moved 200 times to mimic the natural movement of the goat (see supplemental video). The joint was then re-opened and the implantation site was macroscopically examined. Each joint was then re-sutured and left still for 24 hr at room temperature. After 24 hr, the joints were re-opened, and the state of microspheres was examined again for any signs of breakage.

2.6 *In vivo* bupivacaine release studies

2.6.1 Preparation of bupivacaine-loaded MCs for implantation

The microspheres were compacted into cylindrical pellets for implantation into the parapatellar space of goats. This was done by pouring 10 g of microspheres into cylindrical molds of 2-cm diameter, and placing the filled molds in an oven at 45–50°C for 24 h. Holding the microspheres at the lower limit of the glass transition temperature of the PLLA outer shell enabled a slight softening of the PLLA, allowing the microspheres to stick to one another lightly. Upon removal from the molds, the microspheres remained in the desired shape of the molds, but could be separated again undamaged by applying a moderate force or friction. These MCs used for *in vivo* implantation were then sterilized with ethylene oxide.

2.6.2 Preparation of goats for surgery

The goats were fasted for 12 hr with free access to water prior to surgery. A permanent catheter was positioned into the jugular vein in order to facilitate blood drawing during the length of the experiment. This same catheter was used to administer pre-medication and anesthesia. Premedication was performed with 0.3 mg/kg midazolam (Dormicum, Roche Pharma, Reinach) and 0.2 mg/kg methadone (Methadon Streuli, Streuli Pharma AG, Uznach) intravenously. The anesthesia was induced with a combination of 3 mg/kg ketamine (Narkan 100; Dr. E Greub AG, Bern) and 1 mg/kg propofol (Propofol 1% Fresenius, Fresenius Kabi, Stans) intravenously. After endotracheal intubation, the anesthetic status was maintained with 1 MAC sevoflurane (2.3% ET) in O_2/air. Penicillin was used as a preoperative and postoperative antibiotic treatment. The goat's left hind limb from the tarsus bone to the middle of the femur was surgically prepared by clipping the hair using a veterinary hair clipper (Aesculap Favorita II 220 Volt, Provet AG, Switzerland), washing and disinfecting the skin with Betadine® (BASF, Switzerland) liquid soap and alcohol. The animal was positioned with a vacuum cushion on a surgical bench in a dorsal decubitus-supine position; the left hind leg was in a vertical position distally fixated; the tibia trochlea was in an upright position above the femoral condyle. The surgical field was covered with sterile drapes. An adhesive U-Drape was adhered around the incision; a second adhesive drape was used to cover the rest of the goat.

2.6.3 Creation of implantation site

A medial para-patellar skin incision of 7 to 9 cm long was performed with a scalpel blade just medial from the insertion point of the adductor magnus longitudinally to the border of the medial femoral condyle or the patellar ligament, until the insertion of the tendon of the fibularis tertius muscle. The approach was in respect of all anatomical layers (layer by layer): first the skin, then the aponevrosis, then the vastus medialis obliquis portion of the vastus medialis that overlies the superior aspect of the medial-femoral ligament in layer two of the medial stifle joint, then the patellar retinacular fibers of the joint capsule to finally arrive at the level of the joint. The medial aspect of the patella tendon was elongated proximally with a pair of scissors, avoiding collaterals of the popliteal arterioles localized more distally. The open joint and soft tissue surfaces were continually rinsed with a sodium chloride 0.9% solution. The joint and synovial fluids were examined for evidence of unacceptable pathology (osteoarthrosis, synovitis, foreign bodies).

2.6.4 Implantation of MCs

Bleeding was stopped by cauterization before the introduction of the MCs. Six goats were implanted with a standardized dose of bupivacaine-loaded microspheres (10 g MCs, made with an initial drug:polymer ratio of 90:250). Another six control goats were implanted with empty microspheres. The MCs were implanted into the parapatellar space of the stifle joint. The intrapatellar fat pad was retracted from the medial femoral condyle, and the knee was straightened to reduce the patella to avoid inadvertent contact of the soft tissues against the newly implanted MCs. The leg was cycled through a full range of motion. The retinaculum and subcutaneous tissue and joint capsule were closed with Vicryl 2-0, the sub-skin layer was closed with PDS 2-0. The skin was closed with Monocryl 2-0. Finally, the suture was vaporized with Op-Site® spray and a soft bandage was applied.

2.6.5 Post-operative care

The goat was disconnected from the anesthesia machine and extubated when swallowing reflexes were fully re-established. The goat was discharged and returned to its stall, where it was able to tolerate a standing position. Buprenorphine hydrochloride analgesic (Temgesic®, 10 µg/kg) was administered by intramuscular route until the animal was able to stand partially on the operated leg. Antibiotic (Penicillin, Ilocillin PS) was administered twice daily for 5 days. For the remainder of the survival period, the goats were kept in one farm environment, and had unrestricted motion until necropsy. The goats were also examined routinely for any gross abnormalities or signs of excessive discomfort.

2.6.6 Blood and synovial fluid collection

Blood samples were collected through the jugular catheter at fixed time intervals (0 hr, 4 hr, 8 hr, 24 hr, 48 hr, 72 hr, 7 days, 10 days, 14 days and 28 days). Samples were taken immediately onto ice and kept cool until centrifuged between 2°C and 8°C. After centrifugation, ~ 1 ml of plasma was collected in cryovials and stored at –80°C for later analysis. Before drawing synovial fluid, the animals were sedated. At fixed time intervals (24 hr, 48 hr, 72 hr, 7 days, 10 days, 14 days and 28 days), 0.25–0.5 ml of synovial fluid were drawn. The aspirate was centrifuged at 3000 rpm for 20 min, and the supernatant was collected in cryovials and stored at –80°C for later analysis. Bupivacaine levels in plasma and synovial fluid samples were determined by liquid chromatography-mass spectrometry (LC-MS) using an API 4000 LC/MS instrument (Applied Biosystem, Switzerland), according to a published standard protocol[28].

2.6.7 Animal necropsy

The goats were painlessly euthanized at 4 weeks post surgery by a lethal dose of pentobarbital. At the time of sacrifice, the joint cavity was opened; the joint surface was photographed for any remnants of MCs. The joint was subjectively evaluated for gross appearance of the articular cartilage (tibia and femur) and synovium. Macroscopic cartilage assessment was performed. The different compartments, medial femoral condyle (MFC), lateral femoral condyle (LFC), medial tibia plateau (MTP), lateral tibia plateau (LTP), were graded following India Ink application (grade 1= no uptake of India Ink, which indicates an intact surface, grade 2 = minimal focal uptake of India Ink, which indicates a minimal fibrillation, grade 3 = evident large focal dark patches of ink uptake, which indicates overt fibrillation, grade 4 = large general uptake of India Ink, which indicates erosion of cartilage). Knees were radiographed at necropsy, and the X-ray images were evaluated for signs of osteoarthrosis/osteolysis.

2.6.8 Histological processing

Samples were fixed in 10% neutral buffered formalin at room temperature for 96 hr. Cartilage samples were decalcified in EDTA, and embedded in paraffin. Four cuts per anatomical site (MFC, LFC, MTP, LTP, trochlea) were performed and stained with safranin-O, fast Green, and hematoxylin and eosin (HE). Part of the synovium was embedded in paraffin, cut and stained with hematoxylin and eosin. Synovial membranes were also collected randomly in the knee capsule. The membranes were embedded in several cassettes, fixed for 48 hr in formalin. Histological samples of synovial membrane were stained using HE, safranin-O and Masson trichrome. Two sections were analysed per sample. The sections were morphologically described by observing the cartilage and the bone compartment. The synovium was analyzed for signs of inflammation (according to Cake *et al.*[29]) and for the presence of foreign body material. All experiments were performed in compliance with the relevant laws and institutional guidelines under the International Guiding Principles for Animal Research[30].

3. Results

3.1. *In vitro* bupivacaine release studies

The effects of PLGA molecular weight on the release profiles of bupivacaine from PLLA/PLGA microspheres are shown in Fig. 2. Microspheres prepared with 7 kDa PLGA released most of the bupivacaine within 10 days, while those synthesized with 24 kDa PLGA were able to sustain a constant bupivacaine release rate for 17 days after which the release rate slowed down. On the other hand, microspheres synthesized with 33 kDa PLGA only started releasing bupivacaine after a delay of 15 days. Based on the results of the *in vitro* test, microspheres used for *in vivo* implantation were synthesized using 24 kDa PLGA in order to achieve the 2-week sustained release required.

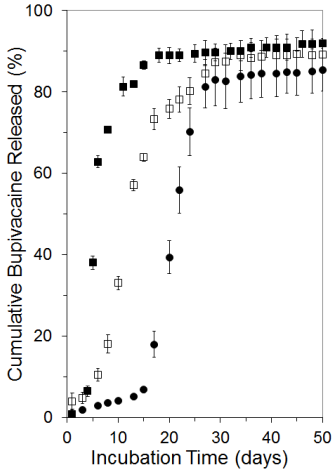

Figure 2. Cumulative release of bupivacaine from drug-loaded core-shell microspheres. The microspheres were prepared with 100 kDa PLLA and either (■) 7 kDa PLGA, (□) 24 kDa PLGA, or (●) 33 kDa PLGA. The microspheres were prepared using a drug:polymer ratio of 90:250, using an initial polymers concentration of 12.5% (w/v), with 1.8 ml of DCM in the non-solvent bath and 1 ml/min of non-solvent addition.

3.2 *In vitro* microsphere degradation studies

Figure 3 shows the changes in microsphere morphology after different incubation intervals. The newly synthesized microspheres have a smooth surface (Fig. 3a(i)) and a relatively homogeneous cross-section (Fig. 3a(ii)). After 10 days of incubation in PBS, pits were observed on the surface of the microspheres (Fig. 3b(i)), and the core appeared depleted in the cross-section (Fig. 3b(ii)), attributable to the degradation of PLGA regions in the PLLA-rich shell. After 80 days, the

microsphere was no longer of a defined spherical shape, and was reduced in size (Fig. 3c). There was extensive surface pitting (Fig. 3c(i)), and in some spheres, large chunks of the shell have degraded, revealing the hollow core (Fig. 3c(ii)). The spheres at this point also became much softer and stickier to handle, hence it was difficult to slice the individual spheres in half to obtain a cross-sectional view. After 150 days, the microspheres have mostly degraded, leaving only a sticky translucent residue and no defined features were visible under ESEM (Fig. 3d)

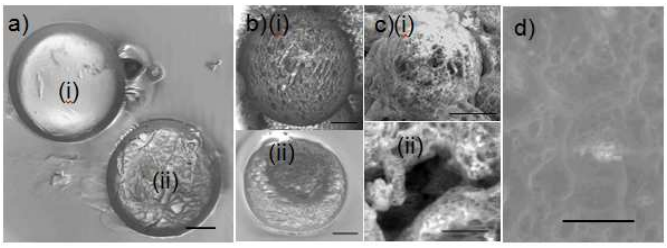

Figure 3. Core-shell microspheres (100 kDa PLLA/24 kDa PLGA) a) without bupivacaine, before incubation in PBS for drug release: (i) whole sphere and (ii) cross-sectional view of sphere; b) with bupivacaine, after incubation in PBS for 10 days: (i) whole sphere and (ii) cross-sectional view of sphere; c) with bupivacaine, after incubation in PBS for 80 days: (i) whole sphere and (ii) cross-section view of sphere; d) after incubation in PBS for 150 days. All scale bars represent 50 μm.

3.3 *Ex vivo* dye release studies

The chlorophenol red dye-loaded MCs remained intact after synthesis (Fig. 4a) and at the point of implantation (Fig. 4b). After the 24-hr joint movement, it was observed that the implanted MC had separated into the individual microspheres; however, the location and structure of the microspheres remained unchanged (Fig. 4c). There was also no visible change in the color of the microspheres, which would signal microsphere breakage due to the leakage of encapsulated chlorophenol red.

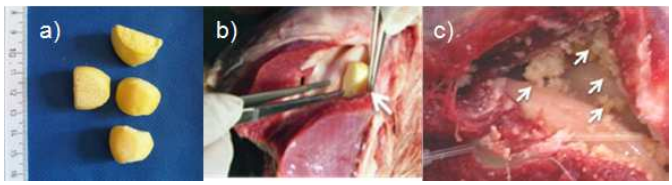

Figure 4. Dye-loaded microspheres a) in compact form after synthesis, prior to implantation; b) in compact form at the point of implantation into the parapatellar space of the goat stifle joint; c) in loose form as indicated by the arrows in the parapatellar space after 200 times of joint movement over 24 hr.

3.4. *In vivo* sustained release of bupivacaine

For goats implanted with bupivacaine-loaded microspheres, the concentration of bupivacaine in the synovial fluid of the goat knee joint over 28 days is shown in Fig. 5a. The concentration of bupivacaine in the blood plasma of the goats over 28 days is shown in Fig. 5b. In both cases, drug concentrations remained elevated for 14 days before decreasing towards the end of the experiment. Drug concentrations in the synovial fluid increased from 2 μg/ml at 24 h to 10 μg/ml at 48 h, and remained 10–15 μg/ml from day 2 until day 10 (Fig. 5a)), while the drug concentrations in the blood plasma remained below the plasma toxic limit of 2.5 μg/ml[27] (Fig. 5b).

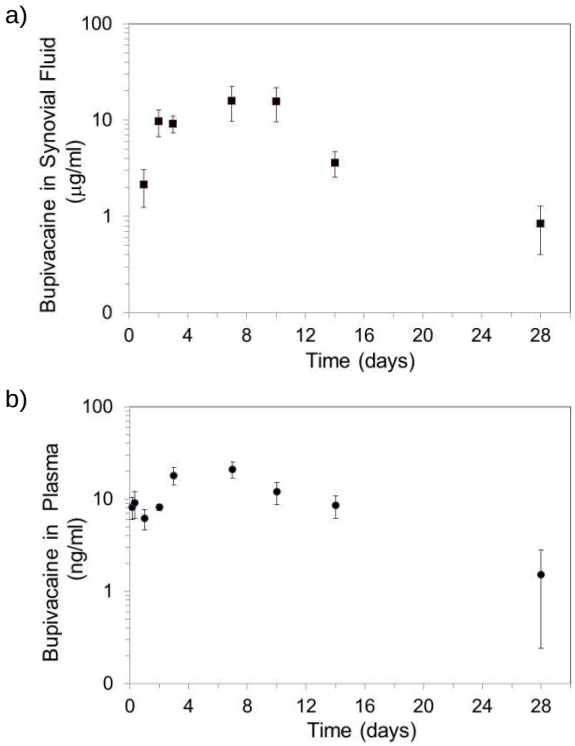

Figure 5. *In vivo* concentrations of bupivacaine in a) synovial fluid and b) blood plasma for goats implanted with bupivacaine-loaded microspheres prepared using 100 kDa PLLA and 24 kDa PLGA.

3.5 Gross examination, explantation and X-ray observations

In general, there was good healing with no signs of inflammation or infection. Post-operative buprenorphine analgesic treatment was slightly shorter for the animals with bupivacaine-loaded microspheres, as compared to animals with empty microspheres. All the animals survived until the end of the 28-day study without any side-effects or gait abnormalities attributable to either bupivacaine administration or microsphere implantation. There was no damage to articular surfaces; however, there were visible remnants of MCs still present, mostly in the para-patellar space (Fig. 6a). The MCs were large in some cases, but always soft to palpation and did not interfere with joint motion or adhere to the synovial membrane. Both anterior (Fig. 6b) and lateral (Fig. 6c) X-ray images taken on the day of explantation did not reveal signs of osteolysis or osteoarthritis in the implanted animals.

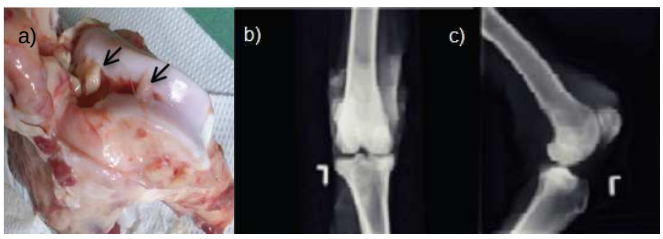

Figure 6. Goat knee joint at 28 days post-implantation of MCs. a) Remnants of MCs (marked by arrows) in the parapatellar space, and the corresponding b) anterior and c) lateral X-ray images.

3.6 Histological analysis

Based on histological analysis of each knee compartment, there was no sign of osteoarthrosis or degeneration within the joints. Cartilage surfaces were intact and the proteoglycan content was well preserved as shown by the intense safranin-O staining (Fig 7a). In all synovial membranes, microspheres were not completely degraded and were surrounded by a moderate inflammatory infiltrate made of histiocytes and macrophages. Masson's trichrome staining of a cross-section through the synovial lining showed the degraded microspheres to be hollow shells, with the center degraded and infiltrated by multinucleated giant cells (Fig. 7b).

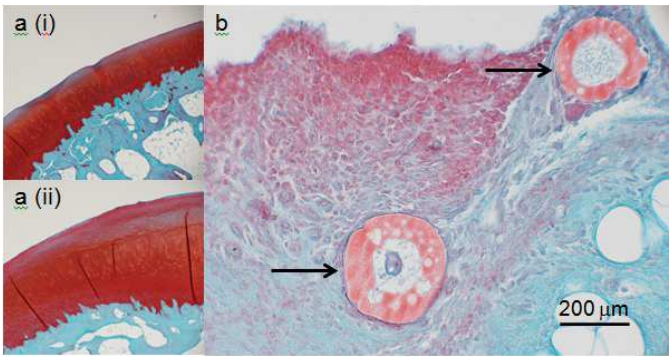

Figure 7. a) Safranin-O staining of i) medial femoral condyle and ii) lateral femoral condyle. b) Masson's trichrome staining of the synovial lining showing the remnants of two microspheres as indicated by the arrows at 28 days post-implantation.

4. Discussion

Regardless of eventual application, one of the primary concerns with sustained delivery vehicles is the possibility of accidental failure or breakage, causing the sudden and possibly dangerous burst release of large drug quantities. As there was no dye leakage during the initial *ex vivo* test, this indicated that very limited, if any, microsphere breakage occurred under the conditions employed. However, this test was conducted only over 24 hr; for the actual *in vivo* conditions several days after implantation in living animals, the degrading microspheres could be more vulnerable to breakage, particularly when subject to excessive mechanical loading or movement. In this particular study, however, there was no sudden increase observed in the bupivacaine levels in the synovial fluid and plasma for the critical initial 2 weeks after implantation (Fig. 5). In addition, the *ex vivo* dye release studies (Fig. 4) as well as the histological evidence of intact hollow microspheres at 28 days post-implantation after the drugs had been completely released (Fig. 7b) indicated that the microspheres were robust enough to maintain their core-shell structure for the entire 2-week drug release duration required. The *ex vivo* study also demonstrated the feasibility of creating MCs for easy implantation, as these MCs were shown to separate quickly into the individual microspheres within the first 24 hr post-implantation (Fig. 4c), thus eliminating the possibility that the drug release kinetics from the individual microspheres would be affected by implanting them in a compact form.

The *in vitro* drug release profile (Fig. 2) was compared with the drug concentration measured in the synovial fluid (Fig. 5a). While *in vitro* results indicated drug release for up to 21 days, with a linear release portion for the first 17 days, the *in vivo* synovial fluid concentrations were only relatively constant from day 2 to day 10, and started to drop after day 10, resulting in a much lower level by day 14. This could be due to the differences between the closed *in vitro* system and the more open system in the synovial space, where fluid transport would occur continuously in and out of the synovial space and fresh synovial fluid would be produced. In addition, even though the microspheres themselves did not burst, the constant mechanical loading experienced in the synovial joint might have resulted in faster drug release from the microspheres, as compared to the static environment in which the microspheres were incubated *in vitro*. Lastly, while the spheres were incubated in PBS for *in vitro* drug release measurements, the *in vivo* release occurred in the more complex environment of the synovial fluid, which would have variations in pH, viscosity and composition depending on individual disease and injury conditions[31], which could contribute to faster drug release *in vivo*. The initial low drug concentration in the synovial fluid at day 1 might be due to the temporary loss of synovial fluid when the joint was opened during the knee surgery, resulting in reduced hydration and fluid transport to the microspheres to induce PLGA core hydrolysis and drug release. There could also be a natural lag time for the drug concentration to reach steady-state levels after the surgery. As a result, additional analgesic support might be necessary in the initial 24–48 hr post-surgery. Also, as a steady-state concentration in the synovial fluid was maintained only from day 2 to day 10 (Fig 5a), it might be preferable to use microspheres that showed a longer *in vitro* release profile than the ones here, so as to sustain a steady-state concentration *in vivo* for up to day 14.

Figures 6a and 7b indicate the presence of microsphere remnants at the end of the study (28 days), despite the bupivacaine having been completely released. As the microspheres by the end of drug release would eventually become hollow and soft (Fig. 3c), they were not expected to pose an obstruction to joint movement or abrasion of the articulating surfaces. Indeed, the histological evidence in Fig. 7a shows that even after being in contact with the microspheres for 28 days, the integrity of the joint tissues was still well maintained. Based on *in vitro* degradation results in Fig. 3d, and since the PLLA shell material has previously been shown to be safely degradable *in vivo*[32–34], it was also expected that given sufficient time, complete clearance of the microsphere remnants should occur. However for other long-term drug delivery applications requiring multiple repeated infusions of microspheres to indefinitely maintain drug levels, there would be an issue of space constraints due to the continued presence of partially degraded non-drug-releasing microspheres. In such applications, the PLLA molecular weight might be reduced such that complete degradation of the shell would occur earlier, while still maintaining a diffusion barrier during the period of drug release. While we have considered using microspheres prepared with PLGA alone in order to avoid the additional degradation time required for the PLLA shell, our initial studies

showed that PLGA-only microspheres were not able to effectively encapsulate and prolong the release of bupivacaine at a constant rate, which was in general agreement with previous investigations[7–14].

5. Conclusions

We have demonstrated the feasibility of using our core-shell microspheres for sustained post-surgical delivery of bupivacaine for pain relief following a one-time implantation of bupivacaine-loaded microspheres at the end of knee surgery. This approach would eliminate the need for multiple daily injections or continuous catheter infusion, thus allowing the patient to regain mobility earlier with reduced hospitalization time and cost.

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