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Synthesis of hydrophilic intra-articular microspheres conjugated to ibuprofen and evaluation of anti-inflammatory activity on articular explants

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ABSTRACT

The main limitation of current microspheres for intra-articular delivery of non-steroidal anti-inflammatory drugs (NSAIDs) is a significant initial burst release, which prevents a long-term drug delivery. In order to get a sustained delivery of NSAIDs without burst, hydrogel degradable microspheres were prepared by co-polymerization of a methacrylic derivative of ibuprofen with oligo(ethylene-glycol) methacrylate and poly(PLGA-PEG) dimethacrylate as degradable crosslinker. Microspheres (40–100 μm) gave a low yield of ibuprofen release in saline buffer (≈2% after 3 months). Mass spectrometry analysis confirmed that intact ibuprofen was regenerated indicating that ester hydrolysis occurred at the carboxylic acid position of ibuprofen. Dialysis of release medium followed by alkaline hydrolysis show that in saline buffer ester hydrolysis occurred at other positions in the polymer matrix leading to the release of water-soluble polymers (>6–8000 Da) conjugated with ibuprofen showing that degradation and drug release are simultaneous. By considering the free and conjugated ibuprofen, 13% of the drug is released in 3 months. In vitro, ibuprofen-loaded MS inhibited the synthesis of prostaglandin E2 in articular cartilage and capsule explants challenged with lipopolysaccharides. Covalent attachment of ibuprofen to PEG-hydrogel MS suppresses the burst release and allows a slow drug delivery for months and the cyclooxygenase-inhibition property of regenerated ibuprofen is preserved.

1. Introduction

To date there is no known cure for osteoarthritis (OA) but treatments with non-steroidal anti-inflammatory drugs (NSAIDs) reduce pain and improve the mobility of patients (Jordan et al., 2003). Local action of drug within the joint cavity is required to achieve the relief of pain and inflammation. The systemic administration of NSAIDs for long time and repetition of the treatment periods are limited by side effects on digestive tract, kidney and cardiac functions (Dajani and Islam, 2008). Introduction of specific inhibitors of cyclooxygenase-2 (COX-2) does not remove the serious side effects associated to oral treatment with classical NSAIDs (Moodley, 2008).

Due to the lymphatic drainage of the joint cavity, the components of synovial fluid are regularly renewed as well as anti-arthritic drugs which filtrate from blood capillaries to joint synovial fluid (Netter et al., 1989). The half-life of anti-inflammatory drugs in joint cavity measured after oral administration is around 1–5 h (Larsen et al., 2008). Consequently, a regular treatment with NSAIDs is required to maintain in the joint cavity a constant therapeutic concentration of drug estimated at 8 μg/mL for ibuprofen (Mäkelä et al., 1981). In order to reduce secondary side effects during NSAIDs treatment of arthritic patients and to maintain a therapeutic concentration of drug in joint cavity, a novel therapeutic approach consists in the development of intra-articular drug delivery systems (DDS) with the ability to provide long-term sustained release. To obtain high drug concentrations at the site of action in joint cavity, microspheres (MS) made with natural or synthetic polymers were proposed (Gerwin et al., 2006; Larsen et al., 2008; Butoescu et al., 2009).

Drug loading was achieved by drug dispersion in polymer matrix during MS formation, and in theory, these biodegradable polymers

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release their drugs during their gradual degradation. But, experimentally, the main drawback of intra-articular MS loaded with NSAIDs is the important initial burst release of drug: diclofenac sodium (30% in the first hours) (Tunçay et al., 2000), flurbiprofen (50% in 6 h) (Lu et al., 2007), celecoxib (50% in first hours) (Thakkar et al., 2004), ibuprofen (14-21% in first hour) (Fernández-Carballido et al., 2004). The main limitation of these DDS is their impossibility to achieve a sustained and efficacious drug delivery for months, since the release was completed in less than one week (Tunçay et al., 2000; Thakkar et al., 2004; Fernández-Carballido et al., 2004). No intra-articular MS loaded with NSAIDs has shown therapeutic efficacy during preclinical studies (Tunçay et al., 2000; Thakkar et al., 2004), probably because of the initial burst, which precludes the long-term maintenance of a therapeutic concentration of drug within the joint cavity. This drawback probably explains why there is today no injectable device on the market for intra-articular delivery of NSAIDs.

A solution to suppress the burst effect and obtain a long-term release of NSAIDs from MS is to immobilize via a hydrolysable covalent linkage the drug to the polymer matrix. The drug release occurs according to the hydrolysis of the covalent bond (Rosario-Meléndez et al., 2013). It exists different ways to create polymers loaded with NSAIDs via degradable bonds: coupling of drug on preformed polymers or preparation of polymerizable prodrug monomers. For example, polymeric prodrugs were obtained by covalent attachment of diclofenac on preformed methacrylic polymers via an ester linkage (Babazadeh, 2007). Ibuprofen, ketoprofen and ibuprofen were coupled to polyvinyl polymers via amide bond (Babazadeh, 2008) or on chondroitin sulfate via ester linkage (Peng et al., 2006). Ibuprofen was covalently conjugated to a poly(glycerol-adipate-co-ω-pentadecalactone) polyester with an ester bond for the formulation of MS (Thompson et al., 2009). A methacrylic derivative of ibuprofen suitable for radical polymerization with methacrylic monomers was proposed by Babazadeh (2006). The drug release from the polymeric prodrugs occurred in saline buffer without burst effect and the release rates depend on the polymer composition.

Thus, to remediate to the burst release reported for the current intra-articular DDS, we have conceived a novel intra-articular MS containing a methacrylate derivative of ibuprofen. By using a prodrug, the formulation of intra-articular MS with synthetic polyesters or natural polymers is no longer necessary because the drug release occurs via ester hydrolysis rather than by polymer erosion. As platform for ibuprofen delivery we used a poly(ethylene-glycol) hydrogel formulated in MS which triggers a low inflammation in synovial membrane after intra-articular injection in sheep's shoulder joint (Bédouet et al., 2013). Nowadays, there is no study associating the concepts of a covalent linkage of an NSAID on hydrogel MS for intra-articular applications. Few hydrogels based intra-articular delivery systems are described (Inoue et al., 2006; Holland et al., 2007) while polymeric hydrogel-MS formed by crosslinking of hydrophilic polymer chains are a class of biomaterials that have demonstrated a great potential as drug-delivery systems (Amin et al., 2009). In theory, hydrophilic MS display several advantages over DDS composed of hydrophobic polyesters for intra-articular use. Swellable hydrophilic MS of high water content should reduce the amount of foreign material injected in joint cavity compared to more hydrophobic polyester MS and could improve the biocompatibility. Hydrophilic surfaces of biomaterials are known to inhibit monocyte adhesion and lymphocytes proliferation (MacEwan et al., 2005) and increase expression of anti-inflammatory cytokine IL-10 and (Brodbeck et al., 2002). The softness of hydrogel MS can facilitate intra-articular injection through small diameter needles used for IA injections.

The aim of this work is to synthetize and to evaluate degradable hydrophilic MS for a sustained delivery of ibuprofen after intra-articular injection. In this study, resorbable PEG-hydrogel MS (40–100 μm) were made by polymerization of poly(ethylene glycol) methyl ether methacrylate with a bi-functional hydrolyzable crosslinker, a tri-block of PLGA-PEG-PLGA and 2-methyl-acrylic acid 2-[2-(4-isobutyl-phenyl)-propionyloxy]-ethyl ester (HEMA-ibu) as ibuprofen prodrug. Then, the in vitro performances of MS were determined: rate of ibuprofen release in saline buffer, research and quantification of MS degradation products containing ibuprofen. Finally, the efficacy of MS covalently modified with ibuprofen on cyclooxygenase activity was investigated in vitro on articular cartilage and synovial membrane explants activated with lipopolysaccharides (LPS).

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMMA) of number-average molecular weight 300 g/mol, stannous octanoate (Sn(Oct)2), triethylamine (TEA), anhydride chloride, hydroxyethyl methacrylate (HEMA), dicyclohexylcarbodiimide, 4-dimethylaminopyridine, poly(vinylalcohol) (PVA) 88% hydrolyzed and poly(ethylene glycol) dimethacrylate (PEGDMA) of number-average molecular weight 750 g/mol were obtained from Aldrich (St Quentin Fallavier, France) and used as received. Tetraethylene glycol (TEG), 2,2′-azobisisobutyronitrile (AIBN) used as polymerization initiator was obtained from Acros Organic (Geel, Belgium). D,L-lactide and glycolide were obtained from Biovalley (Marne la Vallée, France). Analytical grade solvents were supplied by Carlo Erba (Val de Rueil, France).

2.2. Preparation of microspheres

2.2.1. Synthesis of 2-methyl-acrylic acid 2-[2-(4-isobutyl-phenyl)-propionyloxy]-ethyl ester (HEMA-iBu)

In a round bottom flask containing a magnetic stirring bar, ibuprofen (0.34 g; 1.65 mmol) and 4-dimethylaminopyridine (0.01 g; 0.09 mmol) were solubilized in dry CH_2Cl_2 (4 ml) under nitrogen atmosphere. Hydroxyethyl methacrylate (0.21 g; 1.65 mmol) and dicyclohexylcarbodiimide (0.34 g; 1.65 mmol) dissolved in 2 ml of dry CH_2Cl_2 were sequentially added at 0 °C. After reacting 24 h at 0 °C, the mixture was filtrate and the crude product was purified on silica gel column (cyclohexane/ethyl acetate: 2/1). Monoisotopic mass $[M+H^+]^+$ calculated for HEMA-iBu ($C_{19}H_{26}0_4$) was 319.19, experimental mass was 319.18.

^{1}H NMR in CD_3COCD_3: 0.88 (d, CH_3, isopropyl), 1.43 (d, CH_3—CH, ibuprofen), 1.85 (m, CH_3, methacrylate + CH-iPr, ibuprofen), 2.44 (d, CH_2-phenyl, ibuprofen), 3.75 (q, phenyl—CH—COO—, ibuprofen), 4.31 (m, CH_2, HEMA), 5.59-5.98 (m, CH_2=C), 7.16 (dd, C_6H_4).

2.2.2. Synthesis of poly(PLGA$_5$-TEG-PLGA$_5$) dimethacrylate

Tetraethylene glycol (5 mmol, 0.971 g), D,L-lactide (10 mmol, 1.441 g, glycolide (10 mmol, 1.161 g) and stannous 2-ethylhexanoate (34 mg) were introduced in a dry schlenk tube and subjected to several vacuum-argon cycles. The schlenk tube was then heated at 115 °C for 20 h under argon atmosphere with continuous magnetic stirring. After the reaction completed, the polymer was cooled and then dissolved in 40 mL of dichloromethane and precipitated two times in a 1 L equivolumic mixture of diethyl ether and petroleum ether at first, and, then, in petroleum ether to remove any traces of unreacted monomers. Purified polymer was dried under vacuum at room temperature and characterized by ^{1}H NMR. The final product is a slightly white gel (yield 95%). ^{1}H NMR ($CDCl_3$) δ (ppm): 5.18 (m, CH, LA), 4.82 (m, CH_2, GA), 4.31 (m, CO—O—CH_2 of TEG), 3.64–3.70 (m, CH_2 of TEG), 1.53 (m, CH_3, LA).

$PLGA_5$-TEG-$PLGA_5$ copolymer (3 g, 4.2 mmol) was introduced in a schlenk tube and dissolved in 30 mL of degassed ethyl acetate. The reaction mixture was cooled at 0 °C into a glass bath and after 5 min of gentle stirring triethylamine (6 equiv. per mol of polymer) was added dropwise under argon flow. Then, anhydride chloride (6 equiv.) was added dropwise under argon flow. The final solution was stirred for 1 h at 0 °C and heated at 80 °C for 8 h. The mixture is precipitated three times in petroleum ether to remove anhydride chloride and triethylamine excess. Purified polymer was dried under vacuum at room temperature and characterized by ^{1}H NMR. Yield = 95%.

^{1}H NMR ($CDCl_3$) δ (ppm): 6.22 (m, CH=), 5.64 (m, CH=), 5.18 (m, CH, PLA), 4.82 (m, CH_2, GA), 4.31 (m, 4H, TEG), 3.64-3.70 (m, 12H, TEG), 1.97 (m, CH_3 methacrylate), 1.53 (m, CH_3, PLA).

2.2.3. Synthesis of microspheres

An aqueous solution (115 mL) containing poly(vinylalcohol) at a concentration of 0. 5% (w/v) and 3% wt of NaCl was introduced into a 0.5 L reactor and was purged with nitrogen for 15 min. The dispersed phase containing HEMA-iBu (1.12 g; 3.5 mmol), PEGMMA (4.3 g; 14 mmol), ($PLGA_5$-TEG-$PLGA_5$) dimethacrylate (0.8 g; 1.1 mmol) and 30 mg AIBN solubilized in 4.6 g toluene was degassed by nitrogen bubbling for 15 min. The solution was added to the aqueous phase at 30 °C and stirred at 225 rpm by using a propeller type stirrer to obtain monomer droplets of suitable diameter. Temperature was increased to 80 °C and the mixture was stirred for 8 h. The MS were collected by filtration on a 20 μm sieve and washed extensively with acetone and water. The MS were then sieved with decreasing size of sieves (150, 100, 40 and 20 μm). Only the fraction between 40 and 100 μm was kept for evaluation. For "Mock MS", the process was the same as described above, except that no HEMA-iBu was added. All microspheres were freeze dried immediately after purification and stored at −20 °C until use.

2.2.4. Analytical methods

Products were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy using a Bruker DPX300 FT-NMR spectrometer using the solvent peak as reference. Mass spectra were acquired in the positive-ion mode on a tandem mass spectrometer time-of-flight (Q-STAR Pulsar I, Applied Biosystems) equipped with an electrospray ionization (ESI) source. Particles size analyses were performed with a laser granulometer (Coulter LS 230, Beckman Coulter, Fullerton, USA).

2.3. In vitro release experiments of ibuprofen in PBS

200 mg of MS were suspended in 100 mL of PBS (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl) pH 7.4 and maintained at 37 °C with occasional manual stirring. At days 7, 14, 30, 60 and 90, MS were suspended in medium and aliquots of 1 mL were removed. After centrifugation (30 s, 2000 × *g*), 500 μL of supernatants was injected for RP-HPLC analysis. Three independent incubations of ibuprofen modified MS were performed. "Mock microspheres" of same composition but without ibuprofen loading were treated in same conditions.

2.4. Swelling of microspheres in PBS

Diameter of "Mock microspheres" (n = 706) and ibuprofen loaded microspheres (n = 959) were measured after suspension in PBS and after one week, 2 months and 3 months at 37 °C. Pictures (×2.5) were taken using light microscope and diameters were determined using ImageJ software.

2.5. HPLC analysis of ibuprofen release

The high-performance liquid chromatography (HPLC) was performed onto a C_{18} column (SunFire, 5 μm, 4.8 mm × 150 mm, Interchim, France) with detection at 230 nm. The mobile phase used in chromatographic separations consisted of a binary mixture of solvents: acetonitrile (A) and water with acetic acid 0.1% (B) at a flow rate of 1 mL/min. The elution was isocratic for 3 min (70% B) then acetonitrile raised to 90% in 20 min (Panusa et al., 2007). After each run the column was washed with 90% acetonitrile for 5 min and conditioned for 5 min with the initial mobile phase.

2.6. Dialysis of release medium

The supernatant (5 mL) recovered after three months of incubation of MS in PBS was size-fractionated by dialyzing overnight against 30 mL of water at room temperature through a 500 Da cut-off membrane (Spectra/Por®, Float-A-Lyser®, Spectrum Laboratories). The retentate (>500 Da) was further dialysed overnight against 30 mL of water through a 6-8000 Da cut-off membrane (Spectra/Por®, Spectrum Laboratories). The dialysates and the retentates were divided in two fractions, one for ibuprofen assay after HPLC injection and the other was submitted to alkaline hydrolysis (see below) for research of ibuprofen upon HPLC fractionation.

2.7. Alkaline hydrolysis of release medium and dialysis sub-fractions

In order to check in PBS medium and in dialysis sub-fractions the presence of water-soluble fragments of MS containing ibuprofen, MS-free supernatant (500 μL) and fractions recovered after dialysis of release medium were incubated in 50 mM NaOH for 10 min at 60 °C. Neutralization of medium was performed with 1 volume of 200 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Then, neutralized samples were analyzed by RP-HPLC for ibuprofen assay.

2.8. Collection and co-culture of sheep joint explants

Cartilage and synovial membrane were obtained from the glenohumeral joint of adult Préalpes sheep. Full-thickness cartilage slices from humerus surface were obtained after scalpel shaving. The synovial tissue including the intima, the subinima layer and the fibrous capsule was removed from each joint. Explants were placed in sterile medium (DMEM high-glucose, 100 U/mL penicillin, 100 μg/mL streptomycin) and aseptically the cartilage and capsule fragments were cut in pieces (≈5 mm diameter) and placed into 24 well-plates (Costar). Wells were filled with 2 mL of culture medium (10% FBS, 2 mM L-glutamine, penicillin (50 U/mL), streptomycin (50 μg/mL), 10 mM HEPES, DMEM-high glucose) and the plates were incubated at 37 °C in 5% CO_2. Explants were equilibrated during 5 days before treatments.

2.9. Treatment of joint explants

Explants (n = 4) were challenged with 1 mL of culture medium as followed: unchallenged explants, LPS challenged at 10 μg/mL, LPS and simultaneous treatment with 1–10–50 and 100 μM of ibuprofen, LPS with 25–50 and 75 mg of MS conjugated with ibuprofen. Media collection was done after two days of culture and explants were dehydrated (90 °C for 60 h) before weighing. Ibuprofen concentration in explant supernatants was determined three times by RP-HPLC (see below).

2.10. Ibuprofen assay in cell culture medium

300 μL of cell culture medium was mixed with 1 volume of acetonitrile in order to precipitate proteins and the mixtures were centrifuged for 5 min (12,000 × *g*) at room temperature. The supernatants were analyzed by RP-HPLC.

2.11. Assay of prostaglandin E2 (PGE_2) and lactate dehydrogenase (LDH) in culture medium

PGE_2 in the explant supernatants was measured by ELISA using a commercial kit (R&D Systems, Parameters PGE_2). The PGE_2 secretion (ng) was normalized to the explants dry weight (mg). LDH leakage from joint explants to the culture medium was measured to quantify the cytotoxicity by using the "CytoTox 96® Non-radioactive Cytotoxicity Assay (Promega, France). Measures were done according to the manufactuter's instructions and absorbance values (450 nm) were normalized to the explant dry weight (mg) leading to an arbitrary unit of toxicity.

2.12. Data analysis and statistical tests

Statistical analyses were performed on StatView SAS 2000 (SAS institute, Cary, NC). Continuous variables were expressed as median ± median absolute deviation. For comparison of two groups, non-parametric Mann–Whitney (MW) test was used. The Kruskal–Wallis test was used to compare more than two groups. Significance was set at $p < 0.05$.

3. Results

3.1. Ibuprofen microspheres

The anti-inflammatory drug ibuprofen was chemically conjugated to the pendant hydroxyl groups of HEMA via an ester bond by reacting with the carboxylic group in ibuprofen (Fig. 1). MS containing 19 mol% of ibuprofen were then prepared by suspension polymerization using poly($PLGA_5$-TEG-$PLGA_5$) dimethacrylate as cross-linking agent and PEGMMA Mw300 as hydrophilic comonomer. The MS size range was between 40 μm and 100 μm with a mean diameter of 64 μm. MS had a spherical morphology with a smooth surface, they were homogeneously distributed and well proportional (Fig. 1).

3.2. Microspheres swelling

The diameter of "Mock microspheres" increased during incubation in PBS (2.1-fold in 2 months) and remained stable at 3 months (Fig. 2). On contrary, the swelling of ibuprofen loaded microspheres was significantly lower at each time of analysis compared to the "Mock microspheres". The swelling of ibuprofen loaded microspheres in PBS was delayed compared to "Mock microspheres", a 1.8-fold increase of diameter occurred after 3 months of incubation. The swelling of each group of degradable MS suggested some degradation of the crosslinking as already described (Censi et al., 2010; Nguyen et al., 2013).

3.3. In vitro drug release in saline buffer

3.3.1. RP-chromatography analysis of release medium

During the time course analysis performed from day 7 up to 3 months, the "Mock microspheres" released in medium polar compounds eluted at 5–6 min which absorbed UV light at 230 nm (Fig. 3). The chromatograms were stable during the 3 months study period without observation of additional peaks. For MS loaded with ibuprofen, the large peak eluted at 5–6 min was also observed as

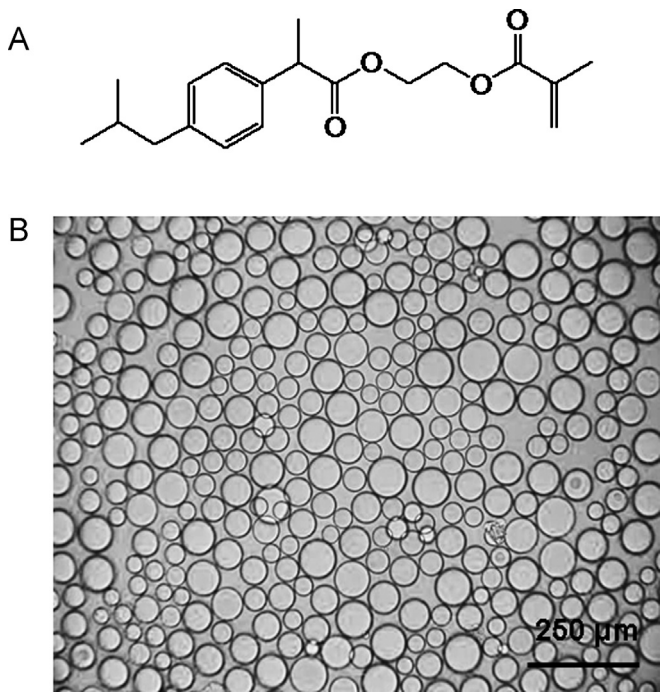

Fig. 1. Preparation of ibuprofen-conjugated microspheres. Structure of prodrug derivative of ibuprofen (2-methyl-acrylic acid 2-[2-(4-isobutyl-phenyl)-propionyloxy]-ethyl ester (HEMA-iBu) (A). The prodrug was synthesized upon coupling between alcohol function of HEMA and carboxylic group of ibuprofen in presence of dicyclohexylcarbodiimide in dichloromethane. Optical microscopy observation of topology of ibuprofen loaded MS after lyophilization and hydration in PBS (B).

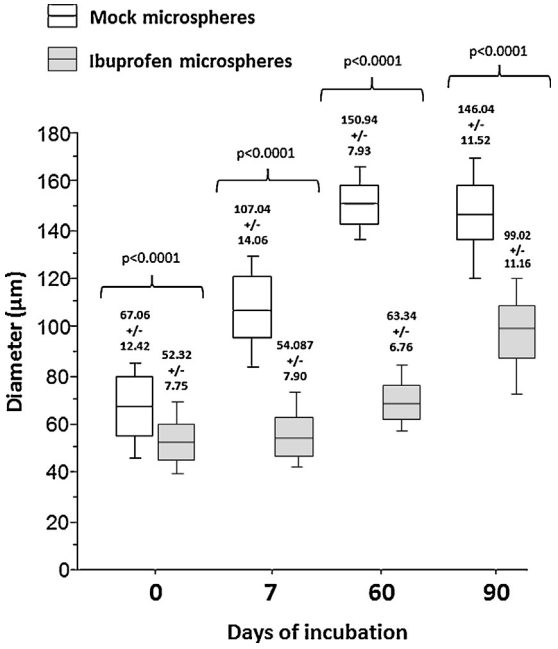

Fig. 2. Swelling of "Mock microspheres" and ibuprofen loaded microspheres in PBS. After microspheres suspension in PBS at day 0 and after 7, 60 and 90 days of incubation in PBS, aliquots of microsphere suspension were transferred on a glass slide for photography (×2.5). Diameters were determined using ImageJ. Data are median ± median absolute deviation. Comparisons between two groups of microspheres were done using Mann–Whitney (MW) test. Significance was set at $p < 0.05$.

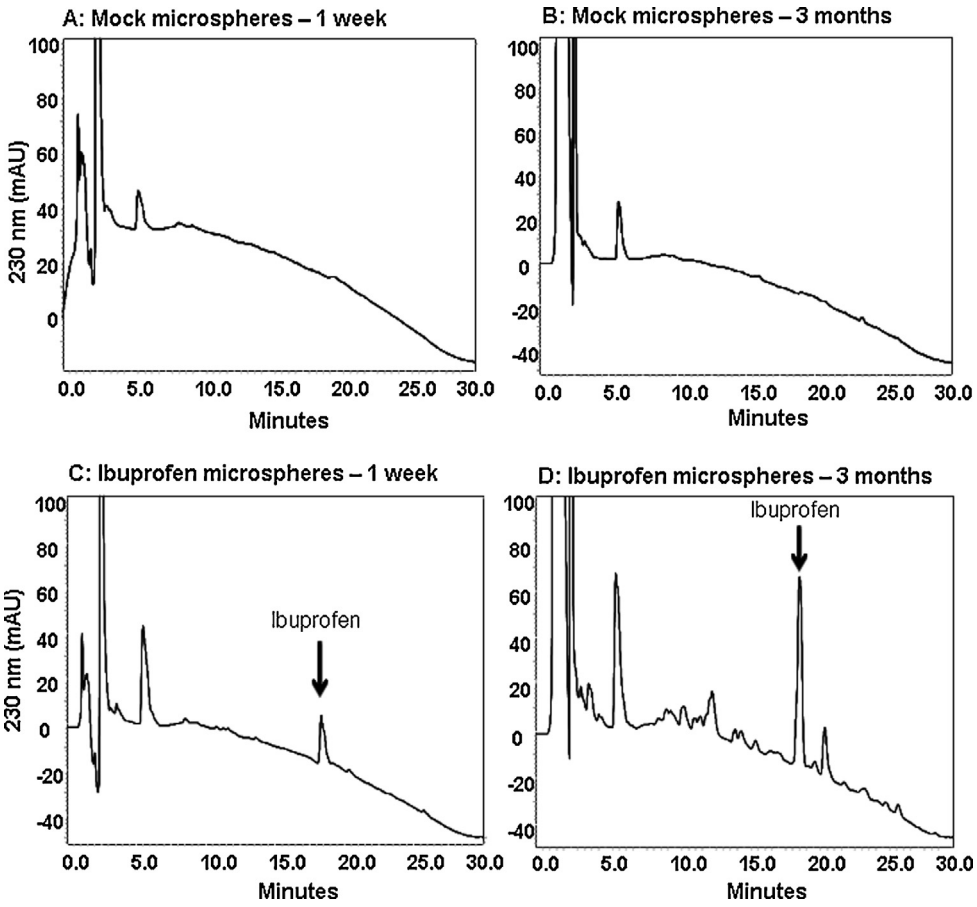

Fig. 3. Comparative HPLC pattern of "Mock microspheres" and ibuprofen-conjugated microspheres obtained during incubation in PBS. MS were incubated at 2 mg/mL in PBS (100 mL) at 37 °C, and after 1 week and 3 months of incubation medium was separated by RP-HPLC with UV detection performed at 230 nm. PBS medium of "Mock microspheres" at 1 week (A) and 3 months (B). PBS medium after 1 week (C) and 3 months (D) of incubation with ibuprofen conjugated-microspheres.

well as another peak at 19 min, which was attributed to ibuprofen. After 3 months, both peaks have raised with time but additional peaks have appeared in the chromatogram contrary to the "Mock microspheres". This suggests that more than one form of conjugates was present in medium and probably correspond to ibuprofen conjugates indicating that ester linkages in the MS polymer matrix may have been hydrolyzed at several positions. The fragments generated probably contain ibuprofen according to the 230 nm absorbance. Esterification of ibuprofen through its carboxylic acid moiety implies a molecular characterization of soluble ibuprofen present in medium in order to verify that true ibuprofen was regenerated during incubation of MS in PBS.

3.3.2. *Mass spectrometry control of free ibuprofen release*

Identity of ibuprofen in the peak eluted at 19 min was ascertained by ESI-MS analysis (Fig. 4). During ESI analysis, ibuprofen appeared as its mono-charged ion ($[M+H^+]^+ = 207.13$) and as its sodium adduct ($[M+Na^+]^+$) at m/z 229.12. The main ion at m/z 161.13 was identified as fragment of ibuprofen generated during ionization as confirmed by gas-phased fragmentation of ibuprofen ion (m/z 207.12). ESI analysis of ibuprofen peak recovered after HPLC separation of standard ibuprofen solution led to observation of the same low m/z ion at 161.13 as well as the mono-charged ibuprofen ion (m/z 207.13) with its sodium adduct (m/z 229.11). After RP-HPLC, the mass spectra were more noisy (ions at m/z 177 and 219.01) probably due to contaminants released by HPLC solvents from the plastic tubing. ESI-MS analysis of peak eluted at 19 min obtained from incubation medium of degradable MS showed the specific ions of ibuprofen at m/z 207.13 and 229.11, and the ibuprofen fragment ion (m/z 161.13). Both RP-HPLC and ESI-MS analyses indicated that hydrolysis of ester linkage between ibuprofen and matrix polymer had occurred releasing native ibuprofen molecule.

3.3.3. *Quantification of ibuprofen release*

Quantification of ibuprofen released from MS was made by the measure of the surface of ibuprofen peak observed after RP-HPLC fractionation of PBS medium (Fig. 5). The covalent bonding of ibuprofen precluded an initial burst release since after one week only 0.44% of ibuprofen was released. Then, a slow release was measured: 1% after 1 month, 1.5% after 2 months and 2.2% after 3 months. A mean release of around 0.03% of initial dose of ibuprofen per day of incubation in PBS (~3.6 μg ibuprofen for 100 mg of MS) could be calculated using the release values.

3.3.4. *Release of water-soluble MS fragments conjugated to ibuprofen*

The release of free ibuprofen was low in spite of 3 months incubation. According to the HPLC chromatograms (Fig. 3), we supposed that conjugated ibuprofen could be released from MS. The alkaline hydrolysis performed on the supernatant after 3 months of MS incubation in PBS led to an important increase (6-fold) of the ibuprofen peak area compared to non-treated medium (Fig. 6). Identity of ibuprofen release after the alkaline treatment was verified using ESI-MS analysis (data not shown). As control, the NaOH treatment of incubation medium of "Mock microspheres" did not induce release of any compounds measurable at 230 nm (Fig. 6). This experiment indicates that PBS medium contains different

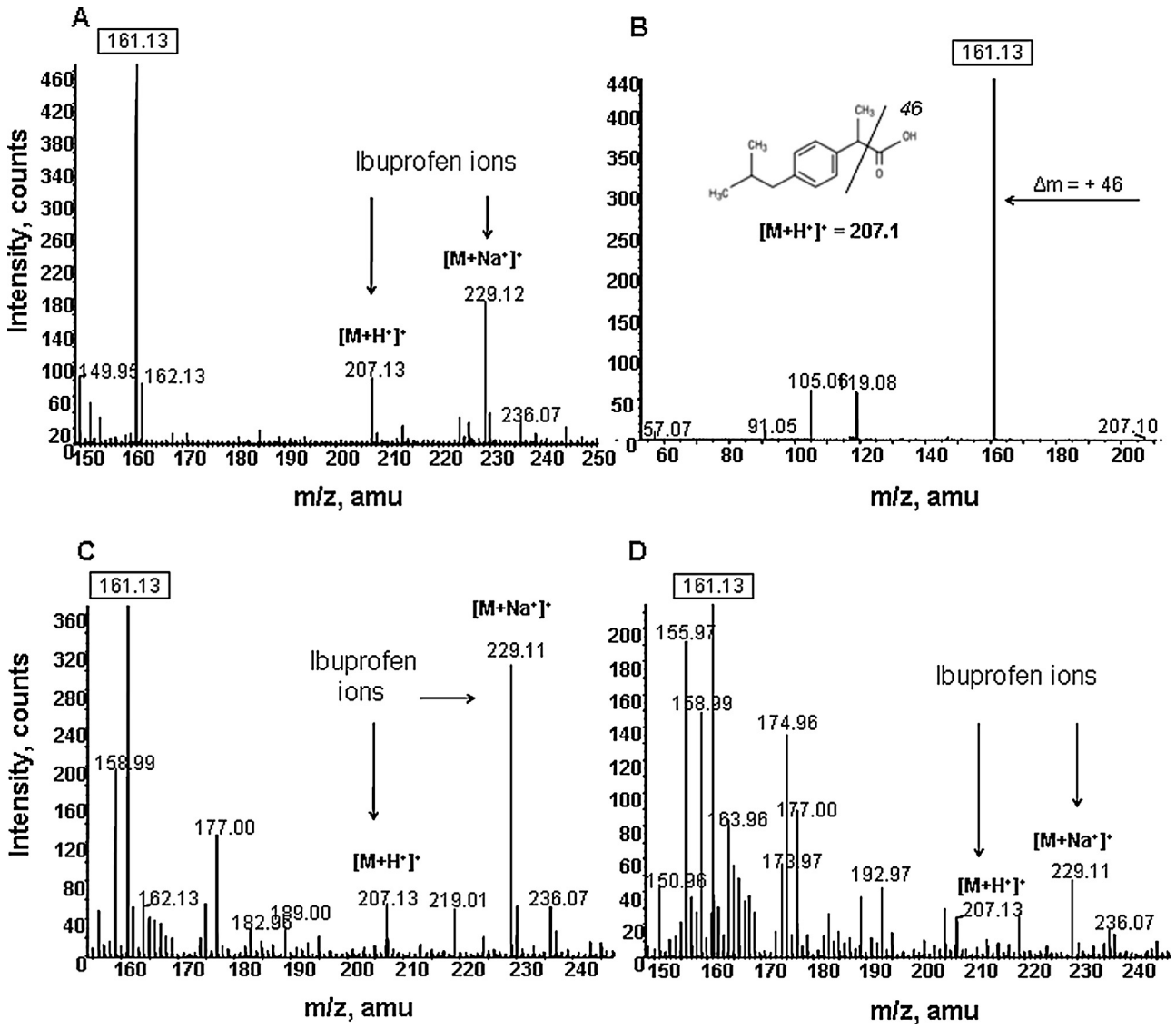

Fig. 4. Identification of ibuprofen released from microspheres in PBS was performed using ESI-MS experiment after injection of standard ibuprofen at 10 μM in PBS (A). MS/MS fragmentation of ibuprofen ion at *m/z* 207.1 led to formation of one main ion fragment at *m/z* 161.13 (boxed) (B). Ibuprofen peak recovered after RP-HPLC fractionation of a standard ibuprofen solution (2.5 μg/μL) (C). ESI-MS analysis of the peak at 19 min recovered after RP-HPLC fractionation of PBS medium recovered after 60 days of incubation with ibuprofen-conjugated MS (D).

forms of ibuprofen: free ibuprofen and ibuprofen linked to soluble MS fragments invisible during HPLC analysis without an alkaline hydrolysis step. Alkaline hydrolysis of PBS medium performed at days 30, 60 and 90 indicated that the amount of ibuprofen conjugated to soluble polymer fragments increased during time and the MS released more ibuprofen as soluble conjugate species than free ibuprofen (Fig. 7). By adding free and polymer linked ibuprofen around 13% of ibuprofen was released from microspheres during 3 months in PBS.

3.3.5. *Size-analysis of polymers released from ibuprofen MS*

For a better characterization of compounds released from MS, a size fractionation was performed by dialysing the PBS medium at the end of incubation period (90 days) through a 500 Da cut-off membrane yielding a dialysate and a retentate (Fig. 8). Free ibuprofen was only measured in the fraction <500 Da. Alkaline hydrolysis performed on a sample of each fraction led to a release of ibuprofen in fraction >500 Da while non-additional ibuprofen was released in fraction <500 Da. The fraction >500 Da was further dialysed against water through a 6–8000 Da cut-off membrane giving two sub-fractions devoid of free ibuprofen. Alkaline hydrolysis of samples of these two sub-fractions led to the release of ibuprofen only in fraction >6–8000 Da. This size-fractionation experiment of PBS release medium indicated that MS released ibuprofen conjugated to polymer fragments higher than 6–8000 Da.

3.4. *Efficacy of MS conjugated to ibuprofen on COX inhibition*

Sheep gleno-humeral joint explants activated with LPS were used to determine if regenerated ibuprofen released from MS could inhibit the prostaglandins synthesis activity of inducible COX-2 enzyme. In this aim, articular cartilage explants were mixed with synovial membrane and challenged with LPS in order to induce synthesis of PGE_2 (Fig. 9). Dilutions of ibuprofen (1–10–50 and 100 μM) as positive control for COX-2 inhibition were mixed with LPS and ibuprofen-conjugated MS (25–50 and 75 mg) were added to LPS-activated explants. After 2 days of culture, the LDH assay in explant supernatants indicated that microspheres did not induce cell lysis as observed for explants treated with free ibuprofen (Fig. 9A). In parallel, the immunoassay performed on explant media

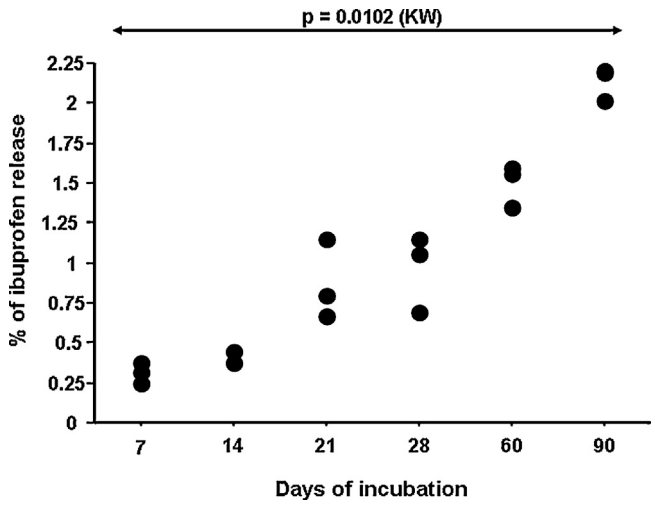

Fig. 5. Release of ibuprofen from biodegradable microspheres into PBS. Ibuprofen-conjugated MS (200 mg) were incubated in 100 mL of PBS (pH 7.4) during 3 months at 37 °C ($n = 3$). Soluble ibuprofen was determined after RP-HPLC separation of PBS supernatant by measuring the area of ibuprofen peak eluted at 19 min. The Kruskal–Wallis (KW) non-parametric test was used to compare the ibuprofen release at days 7, 14, 21, 28, 60 and 90. Significance was set at $p < 0.05$.

showed that expression of PGE_2 was stimulated with LPS compared to control explants group ($p < 0.0001$) while a significant inhibition of PGE_2 synthesis was obtained with free ibuprofen at 10 μM ($p = 0.0033$), 50 μM ($p = 0.0008$) and 100 μM ($p = 0.0008$) and for each concentration of MS ($p = 0.0008$) added to LPS-activated explants (Fig. 9B). RP-HPLC assay indicated that media contained 25 μM, 35 μM and 45 μM of ibuprofen for MS at 25 mg, 50 mg and 75 mg, respectively. Inhibition of PGE_2 synthesis revealed that ibuprofen regenerated from MS was functional. The inhibition of COX activity obtained with 50 mg and 75 mg of ibuprofen-loaded MS was equivalent to the inhibition achieved with 50 μM of free ibuprofen, a concentration close to the values measured in human synovial fluid after oral treatment (Mäkelä et al., 1981).

4. Discussion

Intra-articular DDS loaded with NSAIDs are proposed as a local therapeutic approach for treatment of pain and inflammation during arthritic diseases. Non-covalent loading of NSAIDs in MS obtained by drug dispersion into the polymer matrix led to an initial burst release which remove in few hours most of the drug content precluding a sustained drug delivery (Tunçay et al., 2000; Thakkar et al., 2004; Fernández-Carballido et al., 2004; Lu et al., 2007). In order to suppress the burst release and to achieve a long-term drug delivery, we have prepared hydrophilic degradable MS where

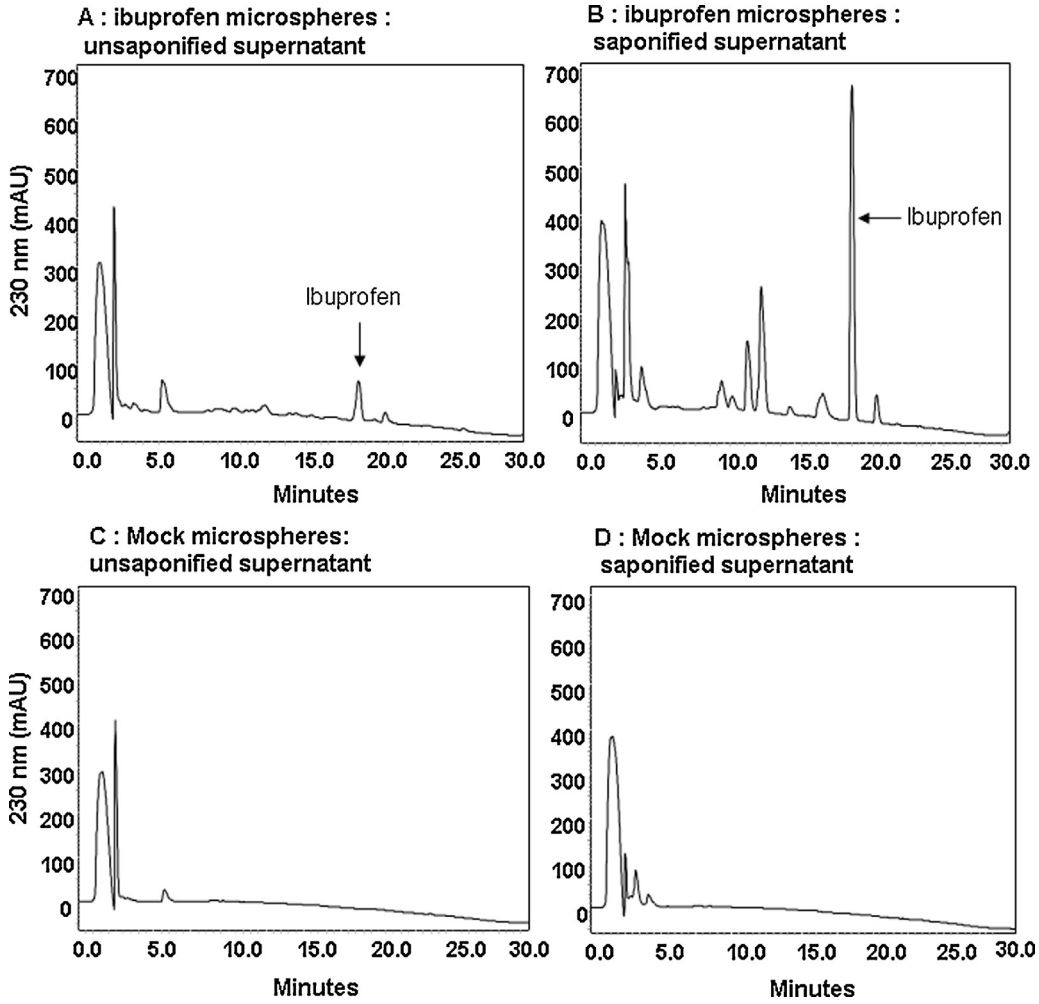

Fig. 6. Ibuprofen-conjugated microspheres released high-molecular weight material modified with ibuprofen. RP-HPLC fractionation of PBS medium after 3 months of incubation (37 °C) with ibuprofen-conjugated MS (A) or "Mock microspheres" (C). RP-HPLC chromatograms observed after alkaline hydrolysis (50 mM NaOH, 60 °C, 10 min) of the PBS medium collected from ibuprofen-conjugated MS (B) or "Mock microspheres" (D) after three months of incubation in PBS.

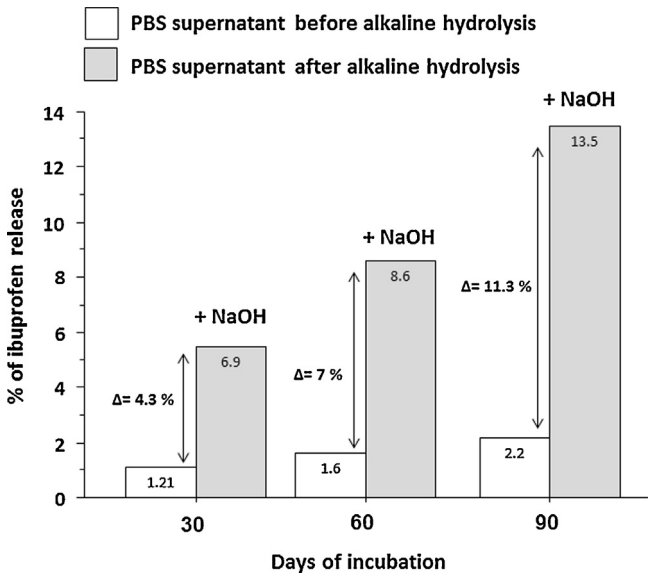

Fig. 7. Quantification of ibuprofen conjugated to soluble microsphere degradation products. After 30, 60 and 90 days of incubation, 1 mL of PBS medium was collected and divided in two sub-fractions. One fraction was directly fractionated by HPLC while the second was treated with 50 mM NaOH (10 min, 60 °C) before neutralization with HEPES and HPLC analysis. Δ indicates the difference between free ibuprofen present in PBS medium without saponification and the additional ibuprofen released after alkaline hydrolysis.

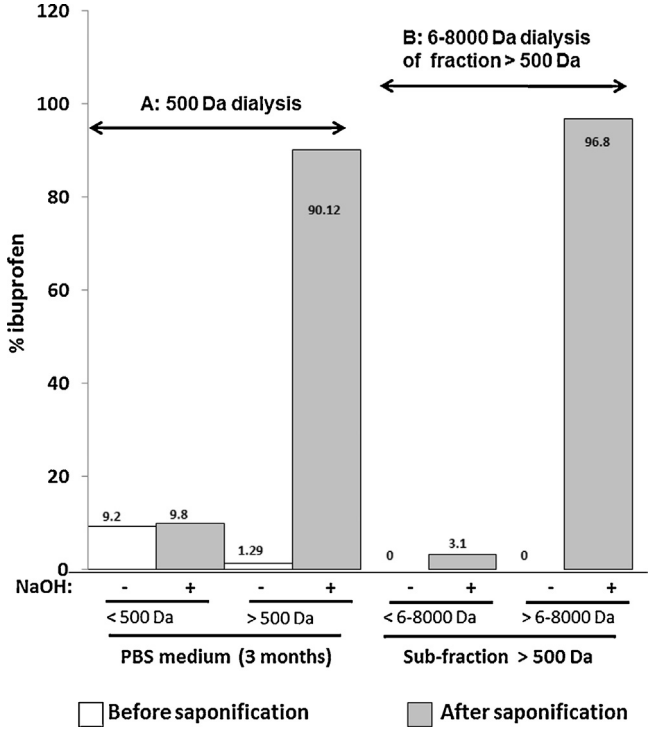

Fig. 8. Evidence for high molecular weight conjugates of ibuprofen bound to polymer material released from microspheres. At the end of incubation period of MS in PBS (90 days), medium (5 mL) was fractionated using dialysis through a membrane cut-off of 500 Da (A). The fraction >500 Da was further dialysed through a 6–8000 Da cut-off membrane (B). Both of dialysis fractions (permeates and retentates) were analyzed by HPLC before and after alkaline hydrolysis (50 mM NaOH, 60 °C) for ibuprofen assay. For each dialysis experiment, values are expressed as percentages of total ibuprofen (μg) determined in permeate and retentate fractions after saponification.

ibuprofen is conjugated via an ester linkage to a polymethacrylate backbone. In vitro properties of these PEG-hydrogel MS were determined in terms of ibuprofen release (amount, rate, free drug or drug conjugated to polymer fragments) and biological effect (anti-inflammatory activity).

4.1. Suppression of ibuprofen burst release for a sustained drug delivery

No initial burst release of ibuprofen during incubation of ibuprofen conjugated MS in PBS was observed. The release of free ibuprofen was sustained during 3 months (2.2% of the total amount of drug). Our results highlight that the methacrylic derivative of ibuprofen obtained by condensation with HEMA led to a sustained release of ibuprofen by hydrolysis as described by Babazadeh (2006). Our drug release data are consistent with other studies showing that the covalent conjugation of a drug to a polymer via an ester bond is a way to obtain a slow release. For example, in saline buffer, linear polyester prodrugs obtained by condensation of a ketoprofen glycerol ester derivative with poly(ethylene glycol) and divinyl sebacate, released ketoprofen without burst effect (Wang et al., 2010). After 14 days, between 10% and 30% of initial ketoprofen was released according to the composition of polyesters. Thompson et al. (2009) observed that incubation in phosphate buffer of MS composed of a polyester of poly(glycerol-adipate-co-ω-pentadecalactone) conjugated with ibuprofen via an ester linkage released slowly the drug for 17 days due to the stability of the covalent bonding between ibuprofen and the polymer. By contrast, absence of release of a drug conjugated to a polyester via an ester bond can also occurs as reported for poly(D,L-lactic acid) polymer modified with 5-iodo-2′-deoxyuridine incubated in phosphate buffer (Rimoli et al., 1999).

The slow ibuprofen release from the hydrophilic MS observed in our study could be explained by the stability of the ester linkage, with a half-life estimated at 3.3 years (Thompson et al., 2008). Secondly, the microenvironment within the MS could be unfavorable for the ester hydrolysis reaction. The presence of ibuprofen moiety (isobutyl-phenyl-propionyl pendant group) probably make the drug loaded microspheres more hydrophobic than the non-loaded ones as suggested by the delay for the MS swelling compared to control MS. To improve the drug release it seems necessary to prepare more hydrophilic MS in order to attract water close to the ester linkage of ibuprofen. Incorporation of PEG spacer within the prodrug is a strategy to enhance the rate of ester hydrolysis in polymer prodrugs (Peng et al., 2006) or the introduction of a more labile linkage such an anhydride function (Mizrahi and Domb, 2009) instead of an ester bond between ibuprofen and polymer.

4.2. Release of ibuprofen conjugated to degradation products of microspheres

In this study we have observed for the ibuprofen loaded MS a slow release of free (regenerated) ibuprofen, identified according to mass spectrometry, together with the simultaneous release of water-soluble degradation products of MS containing ibuprofen moiety. Thank to alkaline hydrolysis of release medium before HPLC assay of ibuprofen, we have quantified the amount of ibuprofen conjugated to polymers fragments. During incubation in PBS, around 5 times more ibuprofen was released from MS as conjugated molecules than in free form. We assume that in saline buffer, ester linkage hydrolysis reaction within the MS polymer matrix was complex leading to the release of a blend composed of solubilized conjugated forms of ibuprofen and the free drug. Ester linkage hydrolysis inside the MS may occur at various positions, between HEMA and ibuprofen and within the resorbable

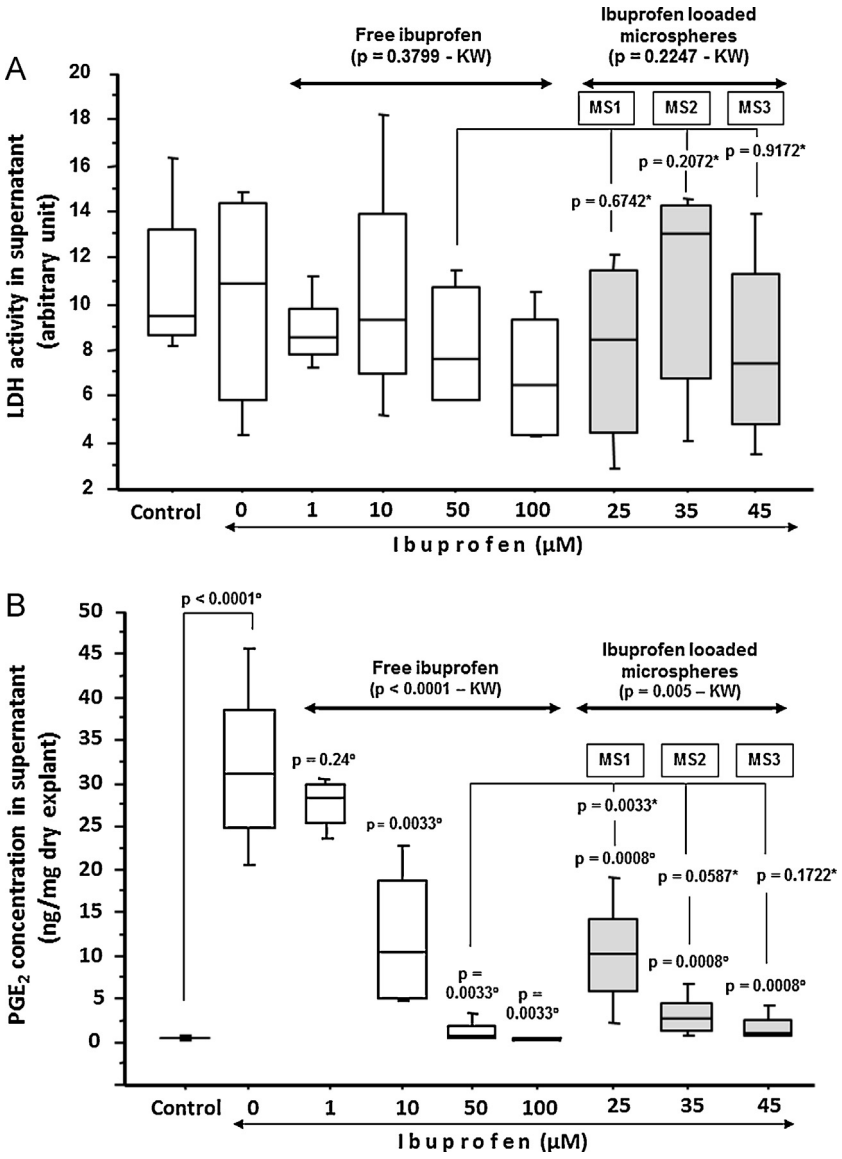

Fig. 9. In vitro analysis of anti-inflammatory activity of ibuprofen-conjugated microspheres. Cartilage and capsule explants collected from sheep shoulder joint were cultured in co-culture ($n = 4$) and challenged with LPS (10 μg/mL) and simultaneously treated with free ibuprofen as positive control of COX inhibition and 3 doses of microspheres: 25 mg (MS1), 50 mg (MS2) and 75 mg (MS3). LDH leakage from cells to culture medium (A) and PGE_2 concentration in supernatants (B) were assayed in duplicate at day 2 of culture. Values of LDH activity (OD450nm*1000) and PGE_2 (pg) were normalized to the explant dry weight (mg). Comparisons between two groups or more than two groups were done using Mann–Whitney (MW) or Kruskal–Wallis (KW) test, respectively. Significance was set at $p < 0.05$. ¤, comparison between each explant group with the LPS treated explant, *, comparison between each MS group and free ibuprofen (50 μM).

crosslinker in the same time. In vivo, we do not know whether the water-soluble ibuprofen conjugates will be released from MS as observed in PBS. Drug release and polymer degradation occurs simultaneously during incubation in saline buffer of the ibuprofen conjugated MS.

Such events were previously reported for a degradable prodrug polymer composed of ibuprofen conjugated with poly(glycerol-adipate-co-ω-pentadecalactone) where HPLC profile evidenced release of free ibuprofen and polyester fragments (Thompson et al., 2008). When, this prodrug was formulated in MS, a global assay of ibuprofen was done by UV analysis method, which cannot discriminate the different forms of ibuprofen (conjugated and free). The authors state that in vivo, it is likely that the conjugated ibuprofen would be rapidly degraded to the active drug (Thompson et al., 2009). We suppose that in plasma, the circulating esterases (Li et al., 2007) could split the ester linkage between ibuprofen and water soluble polymers as observed during in vitro studies (Rimoli et al., 1999; Peng et al., 2006). A strategy to reduce the release of MS degradation products conjugated with ibuprofen will be to slow down the rate of crosslinker hydrolysis by changing its composition, for example by increasing its lactide content.

4.3. Anti-inflammatory activity of ibuprofen loaded MS

Inhibition of COX-2 enzyme activity during arthritic diseases represents a therapeutic target since prostaglandins trigger several pathways of cartilage catabolism, increase vascular permeability and reduce threshold of nociceptors excitation (Martel-Pelletier et al., 2003). Contrary to previous intra-articular MS where unconjugated NSAIDs are entrapped in the polymer matrix (Tunçay et al., 2000; Thakkar et al., 2004; Fernández-Carballido et al., 2004; Lu et al., 2007) the covalent immobilization of ibuprofen to the degradable PEG-hydrogel MS led to the release of different species of drug (free and conjugated to polymer fragments), suggesting that

a fraction of initial drug will be available for COX inhibition. We assumed that ibuprofen species conjugated to hydrophilic polymer fragments are probably inactive on COX enzyme inhibition since ibuprofen must pass through the hydrophobic cytoplasmic membrane to reach the active center of COX enzymes located within the endoplasmic reticulum and nuclear membranes (Rao and Knaus, 2008). To prove the efficiency of ibuprofen released from MS, we have designed an in vitro experiment where ibuprofen-conjugated MS were added to LPS-activated articular cartilage and synovial membrane explants. We observed that 50 mg of ibuprofen-loaded MS significantly reduced the explants PGE_2 synthesis without cytotoxicity indicating inhibition of inducible COX-2 activity. Hydrophilic MS released around 6 μg of ibuprofen per mL of culture medium in two days leading to a final concentration of 30 μM, a value in accordance with the IC_{50} values of ibuprofen reported for COX-2 inhibition (Mitchell et al., 1994). In culture medium we could not determine if the PEG-hydrogel MS release only free ibuprofen or a blend of molecules containing degradation products conjugated with ibuprofen moiety. This in vitro test performed in a close system reveals that ibuprofen released from the PEG-hydrogel MS keeps its biological activity toward the intracellular COX-2 enzyme. In vivo, the amount of ibuprofen delivery should be enhanced to achieve a therapeutic concentration in the joint cavity. Fernández-Carballido et al. (2004) proposed that according to the intra-articular pharmacokinetic of ibuprofen, 2.4 μg of drug must be released from a DDS per mL of synovial fluid in one hour. At present, 100 mg of PEG-hydrogel release around 15 μg of free and conjugated ibuprofen in one day, suggesting that the release of ibuprofen should be at least four times greater in order to replace the drug removed from joint cavity by the synovial drainage.

5. Conclusion

The present study represents the first step in the development of hydrophilic MS dedicated to an intra-articular drug delivery. The three main findings are (1) incorporation of ibuprofen prodrug within a PEG-hydrogel MS suppressed as expected the initial burst release and allowed long term drug delivery, (2) ibuprofen released from pre-loaded MS keeps its anti-inflammatory activity, (3) drug release and crosslinker hydrolysis were simultaneous. As perspective, the hydrolysis reactions of resorbable cross-linker and ibuprofen prodrug must be adjusted in order to reduce the release of MS's degradation products conjugated with ibuprofen moiety. Then, it will be necessary to demonstrate that the covalent bonding of ibuprofen to PEG-hydrogel MS does not change the synovial targeting and the low inflammatory response of joint tissues to the degradable PEG-hydrogel MS (Bédouet et al., 2013).

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