Biorelevant release testing of biodegradable microspheres intended for intra-articular administration

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Abstract (100-200 words)

Characterization of controlled release formulations used for intra-articular (IA) drug administration is challenging. Bio-relevant synovial fluids (BSF), containing physiologically relevant amounts of hyaluronic acid, phospholipids and proteins, were recently proposed to simulate healthy and osteoarthritic conditions. This work aims to evaluate the performance of different controlled release formulations of methylprednisolone (MP) for IA administration, under healthy and disease states simulated conditions. Microspheres differed in grade of poly(lactide-co-glycolide) and in the theoretical drug content (*i.e.* 23 or 30% w/w). Their performance was compared with the commercially available suspension of MP acetate (MPA). Under osteoarthritic state simulated condition, proteins increased the MPA release and reduced the MPA hydrolysis rate, over 48h. Regarding microspheres, the release patterns over 40 days were significantly influenced by the composition of BSF. The pattern of the release mechanism and the amount released was affected by the presence of proteins. Protein concentration affected the release and the concentration used is critical, particularly given the relevance of the concentrations to target patient populations, *i.e.* patients with osteoarthritis.

Keywords (5-10 words)

Biorelevant synovial fluids, corticosteroids, intra-articular, proteins; methylprednisolone, microspheres, PLGA, release testing

1. Introduction

Corticosteroids locally administered by intra-articular injections represent one of the major treatment for arthritis, osteoarthritis or musculoskeletal disorders to reduce pain and inflammation, facilitate motion and function [1]. Due to lymph drainage of synovial fluids, the drug residence into the joint is very short even when prodrugs are used [2,3] and, thus, systemic side effects have been frequently reported [2]. To overcome this limitation, the controlled release of drugs loaded in microspheres made of poly(lactide-*co*-glycolide) [PLGA] have been proposed [4,5] and the efficacy and bioavailability of methylprednisolone loaded in PLGA matrix has been demonstrated in an animal model [6]. The optimization of these drug delivery systems is challenging, as compendial *in vitro* drug release tests are not described in the main Pharmacopoeias. The sample-and-separate or modified USP 4 apparatus methods using a buffer as release medium and sink conditions have been proposed in the literature for the screening of different PLGA-based microspheres formulations and the evaluation of batch-to-batch variability [7]. An *in vitro* release experimental set-up reflecting the *in vivo* conditions, which would assist in formulation development and prediction of the *in vivo* performance, is missing. For example, sink conditions which are generally applied in quality control testing are not bio-relevant in some anatomic sites, such as in the sub-cutaneous or the intra-muscular environment [8,9]. Moreover, simple buffers do not reflect the composition of physiological fluids in healthy or disease states. In the case of joints synovial fluids of healthy subjects and osteoarthritic patients, it has been demonstrated that these fluids significantly differ qualitatively and quantitatively in their composition [10], and they present different physicochemical properties, such as viscosity, osmolarity, surface tension and pH [11]. The simulation of the synovial fluid in both healthy and disease states can play a crucial role in the development of *in vitro* release/dissolution testing for intra-articular formulations. Up to date, there are no synovial fluid-simulating media approved by Regulatory Agencies, and limited information on the impact of their composition on the drug release and dissolution are reported. For instance, the addition of hyaluronic acid in a buffer system is the main focus of the published simulated synovial fluids used as release media [12–15]. Recently, bio-relevant synovial

fluids containing physiologically relevant amounts of hyaluronic acid, phospholipids and proteins were proposed to evaluate the release profile of an approved triamcinolone suspension and predict performance of intra-articular formulations [16].

The main goal of the present study was to evaluate the in vitro release behaviour of PLGA microspheres loaded by methylprednisolone, in media simulating synovial fluids under healthy and disease state conditions. Microspheres were prepared by using two grades of PLGA, differing in the lactide/glycolide ratio, and encapsulating different amounts of drug. Preliminarily, the proposed bio-relevant synovial fluids were used to test the release of methylprednisolone acetate from the aqueous suspension of the drug available on market (Depo-Medrone®), approved for the treatment of joint disease such as osteoarthritis. For both the types of formulations (*i.e.* drug loaded PLGA microspheres and drug aqueous suspension) the influence of the main components of bio-relevant synovial fluids was evaluated.

2. Materials and Methods

1.1. Materials

Two different grades of poly(D,L-lactide-*co*-glycolide) (PLGA) were kindly donated by Corbion Purac Biomaterials (Netherlands): Purasorb® PDLG 5002 (PLGA 5050) and Purasorb® PDLG 7502 (PLGA 7525; their characteristics are presented in **Table S1**). A grade of hydroxypropyl methylcellulose at low viscosity (Methocel® K100 LV, HPMC) was kindly provided by Colorcon (Italy). Methylprednisolone (MP) was obtained by Farmalabor (Italy), and Depo-Medrone® (methylprednisolone acetate [MPA] aqueous suspension, 40 mg/ml) was purchased from Pfizer Ltd (UK). Hyaluronidase from bovine testes type VIII (lyophilized powder, range of activity between 300 and 1000 Units/mg) and γ-globulin from bovine blood were purchased by Sigma-Aldrich (UK). Sodium hyaluronate 95% (HA) and bovine serum albumin (BSA) were obtained from Fischer Scientific (UK). Egg phosphatidylcholine (PC) was purchased from Lipoid (Germany) and

polysorbate 80 (Tween® 80) from Croda (Italy). Glass microfiber membrane and syringe filter (GF/D, pore size 2.7 μm) and regenerated cellulose syringe filter (RC, pore size 0.45 μm) were obtained from Whatman GE Healthcare Life Sciences (UK). Nitrocellulose membrane filters (NC, pore size 1.2 μm) were purchased by Millipore (Italy). Syringe filters of 0.2 and 0.45 μm pore size were purchased by VWR International (USA). All the other chemicals were bought by Fischer Scientific (UK) and all the solvents used were of analytical grade.

1.2. Methods

1.2.1. Bio-relevant synovial fluids preparation

Bio-relevant synovial fluids (BSF) were prepared according to the compositions and the protocols reported by Nikolettos I. [16]. BSF reflecting healthy (H-BSF) and osteoarthritic (OA-BSF) conditions contained physiologically relevant amounts of HA and PC with their ratio being 1:1.7 and 1:0.6 for OA and H conditions, respectively, γ-globulin and BSA. The pH of H-BSF was 7.4 and the pH of OA-BSF was 8. The OA-BSF was also prepared without BSA and γ-globulin [OAwp-BSF] in order to investigate the influence of the presence of proteins on the drug solubility and its release from the formulations under test.

1.2.2. Bio-relevant synovial fluid-sample treatment

The hyaluronidase type VIII solution was freshly prepared by dissolving the enzyme at the concentration of 1 mg/mL in sodium phosphate buffer at pH 7.0 with 77 mM NaCl and 0.01% w/v BSA. Biorelevant synovial fluid samples were treated with hyaluronidase solution, in order to facilitate sample filtration and HPLC analysis [17]. Hyaluronidase solution prepared according to manufacturer (initial concentration of 300-1000 U/mg) was added to the samples being treated, to obtain a final concentration of 150 units/mL of the enzyme.

1.2.3. Solubility study

The MP solubility was studied by the shaking-flask method in healthy and disease states BSF and in PBS (pH 7.4) containing 0.02% w/v of SDS (PBS/SDS). Briefly, after the addition of an excess amount of the drug to each medium studied, the suspension was vortexed and incubated in a horizontal shaking water bath (Fisher Scientific, UK) at 37.0±0.5°C for 48h. Samples were withdrawn at 24 and 48h, filtered through a 0.45 μm RC (samples from PBS/SDS) or 2.7 μm GF (samples from BSF) filters to remove the undissolved particles and the amount of dissolved drug was quantified by HPLC. Before injection, samples from BSF were treated with a hyaluronidase solution (section 1.2.2), filtered through 0.45 μm RC filter and diluted accordingly. Solubility studies were performed in triplicate.

1.2.4. Preparation of drug loaded microspheres

MP loaded microspheres were produced by the solid-in-oil-in-water (S/O/W) method, as described by Cilurzo et al., with minor modifications [5]. Briefly, MP was dispersed in 1 mL of 20 wt. % PLGA solution in dichloromethane by sonication with an ultrasound probe (UP200St, 7 mm diameter, Hielscher, G) at an amplitude of 20% for 5 s, and cooling the sample in an ice-bath. The amount of MP was fixed to obtain a theoretical drug loading of 23 or 30% (**Table 1**). The S/O suspension was added dropwise into 25 mL of 2.5 % w/v solution of HPMC at 4.0±0.5 °C, under mechanical stirring with a propeller (600 rpm, 5min). The S/O/W system was poured into 250 mL of ultrapure water cooled at 4.0±0.5 °C and the temperature was gradually increased till 30±1 °C. Hardened particles were recovered by filtration under vacuum using a 1.2 μm NC membrane filter, washed with ultrapure water, suspended in water and freeze-dried (Martin Christ Alpha 1-4 LSC Plus, G). Dried samples were stored under vacuum at 5±3 °C until use. All formulations were prepared in duplicate.

1.2.5. Determination of polymer molecular weight

Molecular weight distribution of raw polymers and loaded microspheres before and after the 40-day release studies were measured by gel permeation chromatography (GPC). Samples of about 5-6 mg were dissolved in dichloromethane and filtered through a 0.45 μm PTFE syringe filter prior the injection, to remove the undissolved particles. The instrument was equipped with a G1379A degasser, a G1310A isocratic pump, a G1313A auto-sampler, a G1316A thermostated column compartment and double detector: refractive index detector G1362A and UV/visible detector (G1314A) set at λ=230 nm. Three columns (Phenogel™ 300x4.6 mm, Phenomenex, I) with gel pore size of 10^4 Å, 10^3 Å and 500 Å were connected in series. The mobile phase was dichloromethane at a flow rate of 0.35 mL/min at a temperature of 25.0±0.1 °C. An injection volume of 70 μL was used. The weight-average molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were calculated using monodisperse polystyrene standards with M_w ranging from 486 to 188,000 Da and a GPC-Addon HP ChemStation software (Hewlett-Packard Co., USA) to compute molecular weight distribution. Dispersity index (DI) was calculated by the ratio between M_w and M_n.

1.2.6. Microspheres size distribution and morphology

The mean particle size and the size distribution of microspheres suspended in 0.05% polysorbate 80 solution were evaluated by the single particle optical sensing (SPOS) technique, using an Accusizer 770 (PSS Inc. USA). The results were expressed as undersize cumulative percentages and the dispersion of the size distribution as Span [**Eq. (1)**].

$$Span = \frac{d_{90}-d_{10}}{d_{50}} \qquad \text{Eq. (1)}$$

where d_{10}, d_{50} and d_{90} represent the diameters at 10, 50 and 90% of the size volume distribution, respectively.

All the samples were analysed in triplicate and the results reported as mean ± SD.

Microspheres morphology before and after the 40-day release was investigated by scanning electron microscopy (SEM, JEOL 6480 LV, JEOL, USA), at an electron beam voltage of 10 kV. Dried samples were rigidly mounted on an aluminium stub using a carbon adhesive and placed under vacuum overnight in order to remove residual moisture. Before images collection and to improve their resolution, samples were coated with a thin layer of gold, using a sputter coater S150B (Edwards, UK) for 5 min.

1.2.7. MP content in the microsphere formulations

The amount of MP encapsulated in the microspheres was quantified by the HPLC method described in section 1.2.10. MP was extracted from 10 mg of dried microspheres placed in 50 mL of a water/acetonitrile mixture (1:1) for 24h at room temperature. Each sample was filtered through a 0.2 μm nylon syringe filter before the HPLC analysis. All the measurements were performed in triplicate. The experimental MP loading % and the encapsulation efficiency (EE) % were calculated based on **Eq. (2)** and **Eq. (3)**, respectively.

$$Experimental\ MP\ loading\ \% = \frac{amount\ of\ MP\ entrapped\ in\ microspheres}{mass\ of\ microspheres} x\ 100 \qquad \text{Eq. (2)}$$

$$EE\ \% = \frac{amount\ of\ MP\ entrapped\ in\ microspheres}{theoretical\ amount\ of\ MP} x\ 100 \qquad \text{Eq. (3)}$$

1.2.8. ATR-FTIR spectroscopy

IR spectra were recorded using a Spectrum™ One spectrophotometer (PerkinElmer, USA) equipped with a diamond crystal mounted in a ATR cell (PerkinElmer, USA). Samples of MP and drug loaded microspheres were scanned with a resolution of 4 cm^{-1} over a wavenumber region between 4000 and 650 cm^{-1}. 64 scans for each sample were collected. Baseline and ATR correction were performed on each spectrum.

176 1.2.9. *In vitro* release studies

177 All the release studies were carried out by the sample-and-separate method in a 50 mL-glass bottle
178 closed by screwed cap at 37.0±0.5 °C, in a horizontal shaking water bath set at the mild agitation of
179 250 strokes/min. An exact volume of MPA aqueous suspension from the marketed formulation
180 (Depo-Medrone®), corresponding to 2 mg of MP, was placed in 20 mL of PBS/SDS or BSF (H-BSF,
181 OA-BSF and OAwp-BSF). After 0.5, 1, 2, 3, 7, 24 and 48h a 4 mL-sample was withdrawn through a
182 2.7 μm GF filter and the sampled volume was replaced with fresh medium. The quantification of
183 MPA and its hydrolysis product (MP) was performed by HPLC (section 1.2.10). The formation rate
184 constant of MP (k_{MP}) was calculated from the first order fitting of MP amount over time [**Eq. (4)**]
185 using OriginPro® 2015 software (OriginLab Corporation, USA).
186
187 $$Y = Y_{\max}(1 - e^{k_{MP}t}) \qquad \text{Eq. (4)}$$
188
189 where Y is the % amount of MP formed at time t and Y_{max} is the maximum % of MP formed over
190 time. The goodness of the fit was evaluated by the adjusted R^2 and by the residual sum of squares.
191 The release profiles of MPA were also corrected for hydrolysis by transformation of the MP
192 quantified amount to MPA amount.
193 Similarly, drug loaded microspheres were exactly weighed to obtain 2 mg of MP, properly dispersed
194 in 1 mL of the buffer used for the preparation of each BSF and then transferred in the release medium,
195 reaching a final volume of 20 mL. After 1, 3, 7, 24h, 3, 7 days and then once a week until 40 days, a
196 4 mL-sample was withdrawn as described above. Each sample from the studies in BSF was treated
197 with a hyaluronidase solution prior to HPLC analysis (section 1.2.2). A release study of formulation
198 MS 50-23 in PBS with 0.02% w/v SDS was also performed. Sink conditions were achieved for all
199 the release studies. All release studies were performed in triplicate.
200 The release rate constant was calculated according to Higuchi's model (**Eq. 5**).

$$\frac{M_t}{M_\infty} = kt^{0.5} \quad \text{Eq. (5)}$$

where M_t represents the amount of MP released at time t, M_∞ is the amount of MP loaded in the matrix and k is the constant rate of drug diffusion.

After 40 days and prior to the recovery of the microspheres, the pH of each medium was measured and reported as mean ± SD. Microspheres were then recovered by centrifugation (Hettich 1605-13 Universal 32 Centrifuge, G) at 8,000 rpm for 10 min at room temperature, washed three times with ultra-pure water, filtered (1.2 μm RA membrane) and dried under vacuum before GPC and SEM analyses.

1.2.10. HPLC analysis

MP and MPA were quantified by HPLC, using an Agilent HP1200 series (Agilent, UK) equipped with a G1312A binary pump, a G1329A auto-sampler, a G1316A thermostated column compartment and a G1315D UV detector. A Phenomenex® Inertsil ODS-2 (C18, 250 x 4.60 mm, 5 μm) column was used for the reversed phase chromatography [18]. The mobile phase was a mixture of water and methanol in the volume ratio of 70:30, at the flow rate of 1 mL/min. The injection volume was 50 μL and the temperature 35 °C. The detection of MP and MPA was performed at 247 nm. For the BSF samples, a gradient method was applied after the isocratic elution of MP and MPA, increasing the water content up to 90% over 12 min. MP and MPA calibration curves in the range of 1-10 μg/mL were freshly prepared in each medium prior each experiment ($R^2 > 0.99$). Standard solutions of MP and MPA in BSF were prepared by adding the relative amount of MP or MPA working solution at 20 μg/mL (in PBS) to the BSF and treating them as reported previously (section 1.2.2).

1.2.11. Statistical analysis

Release data from marketed formulation were analysed by one-way ANOVA followed by Tukey test for pair-wise means comparison (α=0.05), using OriginPro® 2015 software (OriginLab Corporation, USA).

Comparison of two experimental means from solubility data and release data from microsphere formulations were performed using unpaired student t-test to determine two-tailed p values at 95% confidence level.

3. Results and discussion

3.1. Solubility study

The equilibrium solubility of MP in healthy and disease states BSF (with and without proteins) was reached after 24h and the results are summarized in **Table 2**. Among the different BSF studied, the highest value of MP solubility was found in the osteoarthritic medium with proteins ($p<0.05$ in all the cases); while the lowest in the same medium without proteins (OAwp-BSF *vs* H-BSF $p=0.035$ and OAwp-BSF *vs* OA-BSF $p<0.01$). The MP solubility in OAwp-BSF was similar to the one in PBS/SDS ($p=0.190$), in accordance also with the value reported in literature [19]. The lower solubility values in the media without proteins compared to the ones where they were present can be attributed to the fact that particularly BSA can bind molecules through hydrophobic and electrostatic interactions [20] and act like a solubilizing agent, as in the case of ketoconazole, danazole, felodipine [21], itraconazole [22] and cholesterol [23]. Considering that MP has an albumin binding of approximately 78% [24], BSA can act similarly in the tested BSF influencing its solubility, as also revealed by the higher value resulted in H-BSF compared to PBS/SDS (H-BSF *vs* PBS/SDS $p=0.011$, **Table 2**). Being a surfactant [25], PC impacts MP solubility, even though in a lower extent, as demonstrated by the comparison of the values in OA-BSF and H-BSF (**Table 2**).

3.2. Microsphere formulations: preparation and characterization

The S/O/W method allowed to prepare particles suitable for the intra-articular administration [4], with a size ranging between 10 and 43 µm, a narrow size distribution and satisfactory drug encapsulation (**Table 1**).

SE micrographs of MP loaded microspheres (**Fig. 1**) showed that all the particles were spherical in shape. When the lowest amount of MP was loaded (formulation MS 50-23 and MS 75-23), small pores were evident on the surface of the particles, while when the highest amount of drug was encapsulated (formulation MS 50-30), particles had many holes since that the polymeric matrix seemed to be not completely formed.

Experimental MP loading and encapsulation efficiency were slightly higher when PLGA 5050 was used (formulation MS 50-23) instead of PLGA 7525 (formulation MS 75-23, **Table 1**), probably due to the lower rigidity of PLGA with a similar content between lactic and glycolic acids (**Table S1**). The increase of MP amount led to a higher loading of the drug in the microspheres with PLGA 5050 (MS 50-30 *vs* MS 50-23), with an encapsulation efficiency comparable to the one obtained for formulation MS 75-23. The GPC data confirmed that the use of an ultrasound probe to prepare microspheres did not have a detrimental effect on polymers, since the M_w calculated for all the microsphere formulations were superimposable at time 0 (**Table 4**). Additionally, the ultrasounds or the evaporation of the solvent during microspheres' formation did not change the solid state of MP that was encapsulated as Form I. This is evidenced by the three strong absorption bands between 1800 and 1580 cm^{-1} of the ATR-FITR spectrum that were attributed to the stretching of carboxylic acid and ketone groups (1650 and 1720 cm^{-1}, respectively) and to the aromatic bending (1592 cm^{-1}) [**Fig. 2**] [26].

3.3. *In vitro* release studies

3.3.1. Marketed formulation of methylprednisolone acetate

The release profiles of MPA aqueous suspension in all BSF and PBS/SDS showed that both the release and the hydrolysis of MPA were depended on the medium composition (**Fig. 3a**). After 24h the highest MPA release from the suspension was observed in the OA-BSF (29.2±2.2%, $p<0.05$ compared to the other media tested), whereas a lower and similar release was seen in the H-BSF and in PBS/SDS (22.6±1.6% and 21.6±1.9% respectively, $p=0.557$). This trend confirms the influence of proteins on drug release and this was even more evident from the significantly lower amount of MPA released in OAwp-BSF (8.2±1.2 %) compared to OA-BSF. In all media tested, MPA underwent hydrolysis according to first order kinetics (**Fig. 3b**). The rate of MP formation was medium-depended (**Table 3**), with the MPA hydrolysis rate constant in H-BSF being significantly different than the hydrolysis rate constants in all the other media ($p<0.05$). These differences reveal the importance of the simulation of healthy and pathological status during the *in vitro* studies. After the correction of the release profiles of MPA to account for the hydrolysed drug (**Table 3**), at 48h the highest MPA release was observed in OA-BSF ($p<0.05$). MPA release from the suspension in the other media was lower, following the rank order: H-BSF, OAwp-BSF and PBS/SDS. The biorelevant simulation of the synovial fluid under healthy and osteoarthritic conditions is critical and the release in these conditions was significantly different than in a simple buffer with a surfactant (% corrected MPA release at 48h: PBS/SDS *vs* OA-BSF $p=0.008$ and PBS/SDS *vs* H-BSF $p=0.023$). The presence of proteins in the release medium led to an increased % amount of MPA released, suggesting that their presence should be carefully considered (OA-BSF *vs* OAwp-BSF $p=0.038$, **Table 3**).

3.3.2. Methylprednisolone loaded PLGA microspheres

In all tested media, a high burst release of MP from the formulation with the highest drug loading (MS 50-30) was observed in the first hour (**Fig. 4a**). This effect can be explained based on the IR

spectrum of this formulation in which the high intensities of MP bands at 1592 and 1650 cm^{-1} was attributed to the high amount of surface-associated drug particles (**Fig. 2**). Moreover, the high discontinuity of PLGA matrix at microspheres' surface (**Fig. 1**) allowed the medium to enter quickly, fill the empty channels and dissolve the drug that then diffused out [27]. The burst effect of MS 50-30 was less pronounced in OAwp-BSF (MP released in 1h in OA-BSF=47.5±0.7% and in OAwp-BSF=29.4±1.1%, $p<0.05$), suggesting that the presence of proteins in the release medium influenced not only the drug solubility, but also the wettability of the matrix, as revealed by the SEM (**Fig. 5a-d**). After the burst effect, MP release reached a plateau after 3 days only in H-BSF and OA-BSF (49.6±4.3% and 46.3±2.5% in H-BSF and OA-BSF, respectively). On the contrary, a constant release occurred in OAwp-BSF ($k_{50\text{-}30\_OAwp}$: 0.032±0.002 $days^{-0.5}$; R^2=0.96±0.03). In this medium, a 59.4±2.6% MP was released after 40 days, that is significantly higher than the % MP released in the other media (OAwp-BSF *vs* H-BSF p=0.004, OAwp-BSF *vs* OA-BSF p=0.048), revealing the effect of proteins on the amount of drug released from MS 50-30.

Concerning the formulation MS 75-23, only the burst MP release of around 20% after the first hour was seen in the H-BSF (**Fig. 4b**). The SE micrograph of the microspheres recovered after 40 days in this medium showed that the particles had a completely smooth surface, called the "skin" type structure (**Fig. 5e**), probably due to a remodelling/healing process occurred in the plasticized particles over time [28]. This phenomenon determined the occlusion of the pores and the inability of the drug to be continuously released out of the particles. MS 75-23 behaved differently in OA-BSF as a typical tri-phasic release was noticed, with a burst effect similar to the other two media (22.4±0.2 % at t=1h, $p>0.05$), a lag-phase of about 7 days and a second release phase which fitted the Higuchi model ($k_{75\text{-}23\_OA}$=0.051±0.024 $days^{-0.5}$; R^2=0.90±0.04) [**Fig 4b**]. After day 21, a plateau on MP release was reached (37.5±2.3 %). In absence of proteins (OAwp-BSF), a completely different shape of the MP release profile was obtained: after the burst effect, a continuous release of MP without lag phase was obtained and the release data was well characterized by the Higuchi model ($k_{75\text{-}23\_OAwp}$=0.025±0.002 $days^{-0.5}$; R^2=0.84±0.11, $p>0.05$ compared to $k_{75\text{-}23\_OA}$), indicating that the MP release was mostly

governed by the diffusion, as also reported for the MS 50-30. However, at day 40, the % MP released was similar to the one in OA-BSF (about 37%). This can be explained by both morphological analysis (**Fig. 5f and h**) and GPC data of the recovered microspheres (**Table 4**) which demonstrated that the degradation of PLGA 7525 occurred similarly in OA-BSF and OAwp-BSF, with a reduction of the molecular weight of about 7 KDa.

Also in the case of MS 50-23, the MP release was dependent on the composition of the release medium (**Fig. 4c**), and a prolonged and controlled MP release was reached. The burst effect of about 6% at 1h was similar in H-BSF, OA-BSF and PBS/SDS ($p>0.05$). The % MP released from these microspheres in the first hour and after day 1 were lower than the corresponding ones from the MS 50-30 and MS 75-23. This difference can be attributed to the different distribution of the drug within the polymer matrix during the microsphere preparation when PLGA 5050 and the lowest theoretical drug loading was used (MS 50-23) [29]. In H-BSF and PBS/SDS, MP release started after a lag phase of about 28 days, revealing that for releasing the drug it was necessary that polymer chains degradation reached a certain critical PLGA M_w. According to the SEM of the recovered microspheres after 40 days (**Fig. 5i** and **l**), a bulk-erosion controlled release was observed, that is typical of microspheres made of PLGA with relatively low molecular weight (as PLGA 5050 used in this study) and encapsulating poorly soluble small molecules [30]. Despite the similarity in the release profiles, the microspheres did not behave in the same way in PBS/SDS and H-BSF, with microspheres recovered from H-BSF having a wrinkled surface compared to the ones from PBS/SDS (**Fig. 5i** and **l**). GPC data confirmed that MS 50-23 underwent a greater degradation in H-BSF than in the PBS/SDS, with a molecular weight loss of about 74% and 37%, respectively (**Table 4**). In the case of the MP release in BSF mimicking the disease state (OA-BSF), even though the burst effect was similar to the one observed in the simulated healthy conditions (H-BSF), MP release started after day 7, reaching a 27.8±1.6 % MP released at day 40, indicating that the simulation of disease state had an impact on the release. The absence of proteins resulted in a decrease of the burst effect, as a burst of 4.4% was measured in OAwp-BSF ($p=0.004$). Furthermore, in the same BSF medium, there was no

lag phase and the MP release was characterised by the Higuchi model ($k_{50\text{-}23\_OAwp}$=0.050±0.004 $days^{-0.5}$; R^2=0.95±0.05, **Fig. 4c**), with about 37% of MP released after 40 days. Conversely, the MP released from MS 50-23 in OA-BSF seemed to follow the tri-phasic release: burst effect, lag phase of about 1 week and second pulse zero-order release from day 7 to day 21, fitting the Higuchi model with $k_{50\text{-}23\_OA}$=0.119±0.014 $days^{-0.5}$ (R^2=0.84±0.11). Afterwards, a plateau was reached (27.8±1.6 % after 40 days) probably due to the occurrence of healing processes, as evident in the SE micrograph (**Fig. 5j**). No significant differences in terms of M_w were detected in the microspheres recovered from OA-BSF and OAwp-BSF after 40 days (**Table 4**).

The overall release data from the performed studies clearly indicated that the presence of proteins influenced significantly the drug release, both in terms of the amount released and the release mechanism of MP from all types of microspheres. The presence of proteins in the bio-relevant synovial fluid has to be carefully considered. Their interactions with other components of the synovial fluid are not completely understood, but they affect properties of the synovial fluid, such as the surface tension, and consequently the performance of a drug delivery system inside the joint [31,32]. For these reasons, the disease state BSF was prepared with and without the proteins. Proteins interact with hydrophobic polymers, such as PLGA, and they can be adsorbed in a selective and a competitive manner onto the surface of nanoparticulate PLGA systems, forming the so called “protein corona” [33]. Among them, BSA, which has a good sequence identity with human serum albumin, is adsorbed onto PLGA nanoparticles better than other proteins, such as γ-globulin [34,35]. Based on these considerations, it can be assumed that similar interactions could also occur in the release studies carried out in BSF, determining the different mechanisms in MP release from the different microsphere formulations. A tri-phasic MP release in OA-BSF was observed from formulations MS 75-23 and MS 50-23, reaching a plateau at day 21 probably due to a polymer remodelling that closed the surface pores of microspheres, as previously discussed (**Fig. 5f** and **j**). These formulations after 40 days of incubation in OAwp-BSF presented a sponge-like structure (**Fig. 5g**, **h** and **k**) which favours the release of MP according to a drug diffusion mechanism. For all the formulations, the

protein content of the tested media also influenced the Higuchi constants, with the highest values obtained when proteins were added to the medium.

Both types of PLGA used in the microspheres (MS 50-23 and MS 50-23 prepared with PLGA 5050 and MS 75-23 prepared with PLGA 7525) underwent a more pronounced degradation after incubation in H-BSF compared to the other media. The hydrolysis of PLGA ester bonds starts immediately upon contact with the release medium and the acidic degradation by-products accumulate within microsphere until a critical M_w is reached. As a result, the drop of micro-environmental pH catalyses the hydrolysis reaction, causing in some cases a heterogeneous degradation inside PLGA matrices [36,37]. The high viscosity of H-BSF could slow down the diffusion of PLGA oligomers allowing the establishment of the auto-catalysis phenomenon which accelerated PLGA degradation. Afterwards, the diffusion of PLGA degradation by-products outside microspheres determined the acidification of the H-BSF medium, which presents a low buffering capacity. On the other hand, in both the osteoarthritic media (OA-BSF and OAwp-BSF) the lower viscosity and the basic pH allowed a better oligomers' diffusion out of microspheres and their further neutralization. This hypothesis is supported by the pH of the BSF measured after the 40-day release (**Table 4**). The massive degradation occurred in H-BSF determined the greater drop in the pH value compared to the osteoarthritic media.

3. Conclusions

In the design and quality control of long-term release drug delivery systems, the availability of *in vitro* testing to characterize their biopharmaceutical performance is fundamental. This aspect is of great importance in the case of PLGA microspheres intended to locally administer a drug in a specific anatomic site. In the case of joints, the composition of the synovial fluid depends on the state of the subject (healthy *vs* pathological state) and such differences can impact the efficacy of an intra-articular medicinal product. In the present work, we proposed an advanced way to characterize MP loaded PLGA microspheres, simulating healthy and osteoarthritic status of the synovial fluid, that set the stage for the bio-relevant approach in an *in vitro* set up. The experimental results suggested that

the release from both the marketed and microsphere formulations was affected by the medium composition, with a significant impact by the presence of proteins on the release mechanism and the hydrolysis rate. Furthermore, the proposed bio-relevant conditions permitted to discriminate among all formulations and individuate a possible candidate able to control MP prolonged release over a 30 day-period.

401 **Figure captions**

402

403 **Fig. 1 –** SE micrographs of MP loaded microsphere formulations. (a: formulation MS 50-23; b:
404 formulation MS 75-23; c: formulation MS 50-30).

405

406 **Fig. 2 –** ATR-FTIR spectra of MP (black line), formulation MS 50-23 (blue line), formulation MS
407 50-30 (red line) and formulation MS 75-23 (green line).

408

409 **Fig. 3 –** Cumulative percentage of MPA released (a) and MP formed (b) in the tested media from
410 Depo-Medrone® formulation (osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins
411 [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS]).

412

413 **Fig. 4 –** Cumulative percentage of MP released from formulation (a) MS 50-30, (b) MS 75-23 and
414 (c) MS 50-23 in the tested media with the sample-and-separate method (osteoarthritic BSF [OA-
415 BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with
416 0.02% w/v SDS [PBS/SDS]); insert graphs: close up of first 7h.

417

418 **Fig. 5 –** SE micrographs of MP loaded microsphere formulation recovered after day 40 of release;
419 formulation MS 50-30 in (a) H-BSF, (b) OA-BSF, (c) and (d) OAwp-BSF ad different magnitudes;
420 formulation MS 75-23 in (e) H-BSF, (f) OA-BSF, (g) and (h) OAwp-BSF at different magnitudes;
421 formulation MS 50-23 in (i) H-BSF, (j) OA-BSF, (k) OAwp-BSF and (l) PBS/SDS.

422

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536
537

538 **Table 1 –** Characterization of microspheres in terms of particle size distribution (undersize
539 cumulative percentage of the volume distribution), and polydispersity of the size distribution (Span).
540 All the results are expressed as mean ± SD (n=3).
541

Form. ID	L/G ratio	*Drug loading*			*Size distribution*			
		Theoretical (%, w/w)	**Actual (% w/w)**	**EE%**	**d_{10}[a] (µm)**	**d_{50}[b] (µm)**	**d_{90}[c] (µm)**	**Span**
MS 50-23	50:50	23	18.8±0.3	81.7±1.2	11.1±1.2	14.4±1.7	31.6±3.7	1.2±0.1
MS 75-23	75:25	23	17.0±0.4	73.5±1.9	10.7±1.0	23.1±2.8	42.5±3.7	1.4±0.1
MS 50-30	50:50	30	22.1±0.5	73.8±1.8	9.6±0.2	18.6±0.5	36.4±2.4	1.4±0.1

542 [a] 10% of microparticle population were smaller than the number reported;
543 [b] 50% of microparticle population were smaller than the number reported;
544 [c] 90% of microparticle population were smaller than the number reported.
545

546 **Table 2** –MP solubility in different media after 24h, expressed as mean ± SD (n=3).
547

Medium	**Ratio % HA:PC**	**Ratio % BSA:γ-globulin**	**MP solubility (μg/mL)**
PBS/SDS	-	-	118.7 ± 5.3*
OAwp- BSF	95:5	-	112.8 ± 0.9*
OA-BSF	95:5	87:13	161.3 ± 0.2
H-BSF	98:2	87:13	136.9 ± 3.2

548 *MP solubility in PBS/SDS and OAwp-SDS were not statistically different ($p>0.05$)
549 Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with
550 0.02% w/v SDS [PBS/SDS].
551

552

Table 3 – Maximum % MPA released from Depo-Medrone® corrected for hydrolysis and goodness (adjusted R^2 (Adj R^2) and residual sum of square (RSQ)) of the first order fitting of the MP formation (k_{MP}). Results are reported as mean ± SD (n=3).

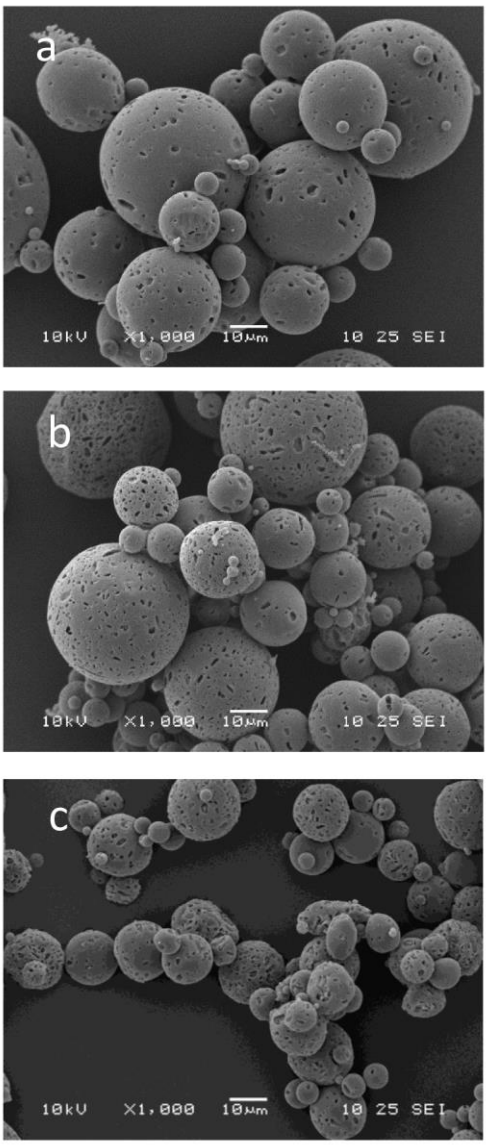
Release medium	MPA (%)	k_{MP} (h^{-1})	Adj R^2	RSQ
PBS/SDS	29.4±2.7	0.053±0.003	0.99±0.00	0.001±0.000
OAwp-BSF	34.7±2.2	0.166±0.015	0.87±0.04	0.163±0.060
OA-BSF	43.1±3.7	0.084±0.051	0.93±0.02	0.051±0.019
H-BSF	36.6±2.0	0.293±0.080	0.93±0.01	0.018±0.006

Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS].

Table 4 – GPC data of PLGA microspheres after preparation (t=0 day) and after the 40-day release in different media. Molecular weight distribution is reported as weight average molecular weight (M_w) and polydispersity index (DI). The pH value of the release medium was measured after the 40 day-release and reported as mean ± SD (n=3).

Release medium	Time (days)	MS 50-23			MS 75-23			MS 50-30		
		M_w (KDa)	DI	pH	M_w (KDa)	DI	pH	M_w (KDa)	DI	pH
--	0	20.1	1.6	--	22.2	1.6	--	20.2	1.5	--
PBS/SDS	40	12.6	1.4	7.23±0.03	n.d.	--	n.d	n.d	--	n.d
OAwp-BSF	40	11.8	1.7	7.93±0.04	15.1	1.7	7.98±0.04	14.1	1.7	7.98±0.02
OA-BSF	40	10.5	1.5	7.39±0.05	13.7	1.7	7.38±0.02	12.6	1.6	7.43±0.12
H-BSF	40	5.2	1.3	5.85±0.09	9.7	1.6	5.76±0.24	4.7	1.3	6.03±0.03

Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwp-BSF]; healthy state BSF [H-BSF]; PBS with 0.02% w/v SDS [PBS/SDS].



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574 Figure 1

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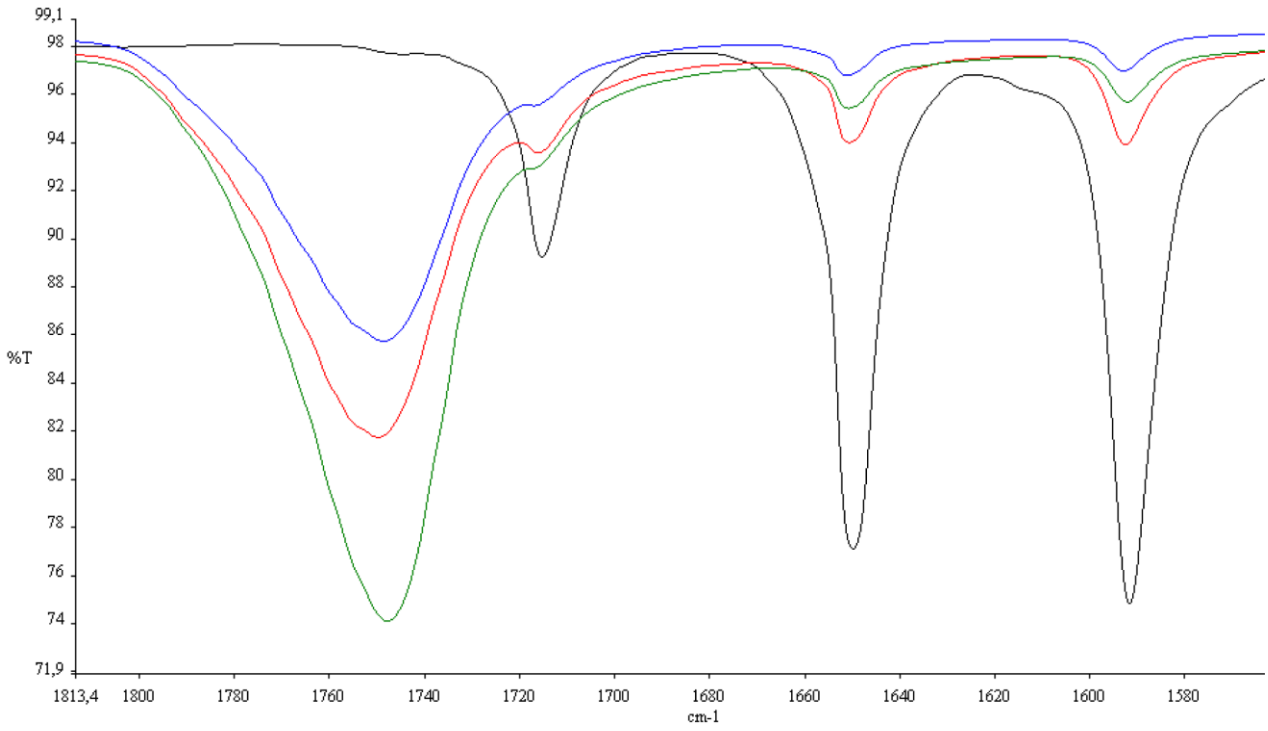
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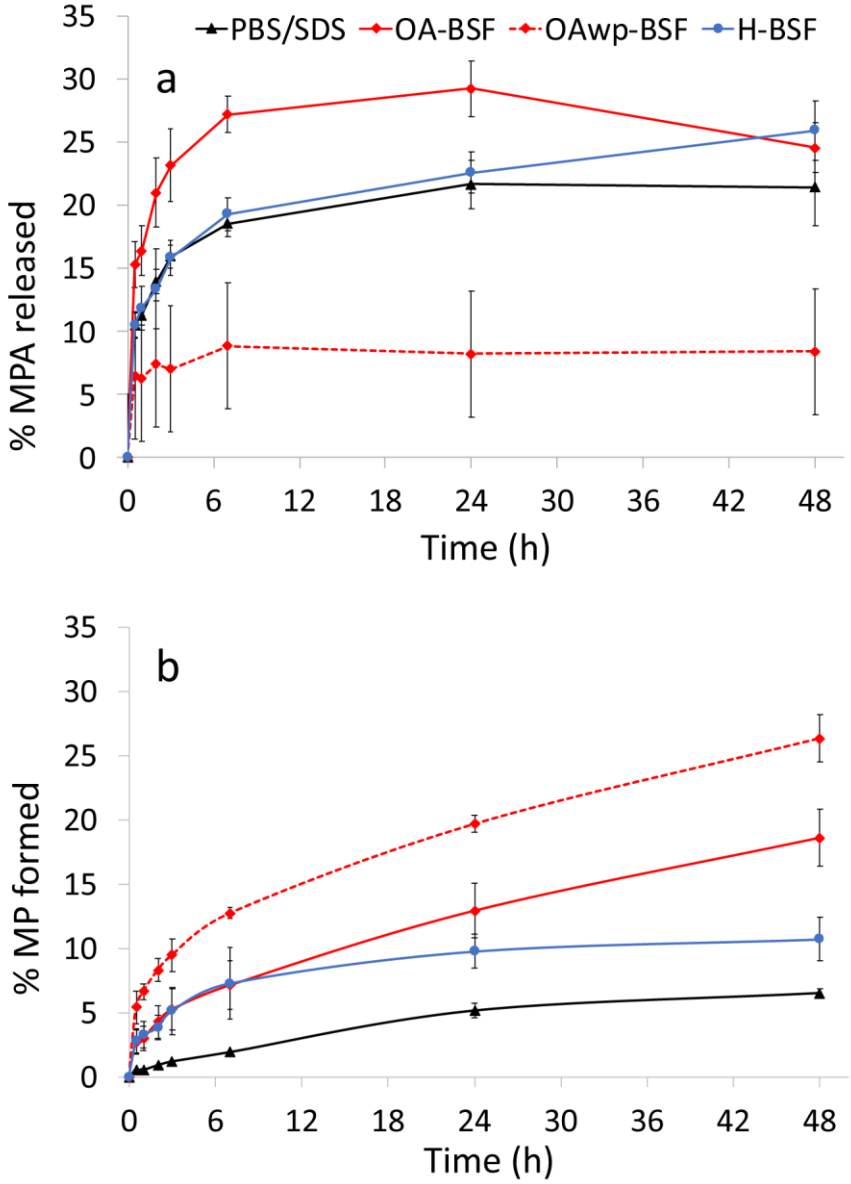
Figure 2



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582 Figure 3

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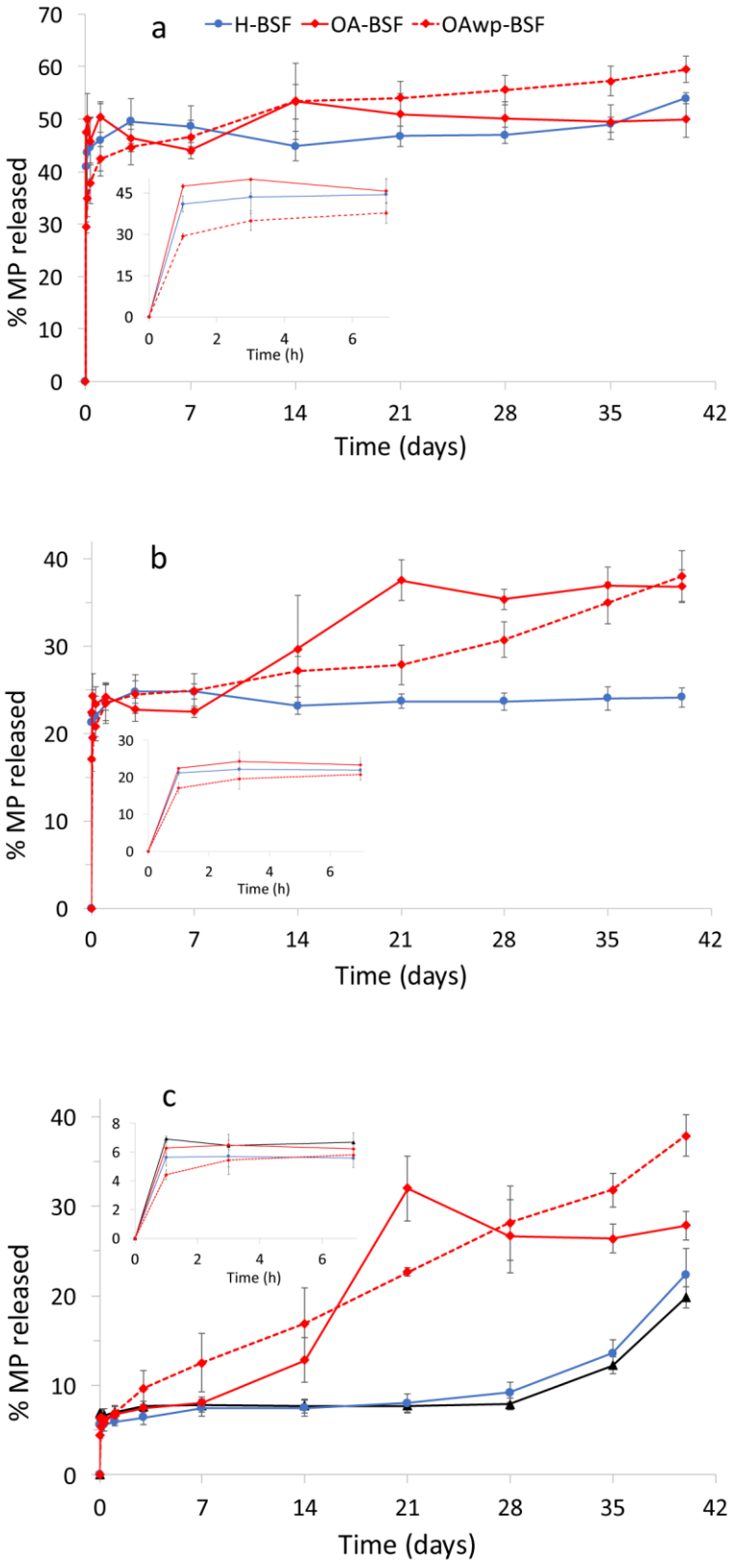

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585 Figure 4

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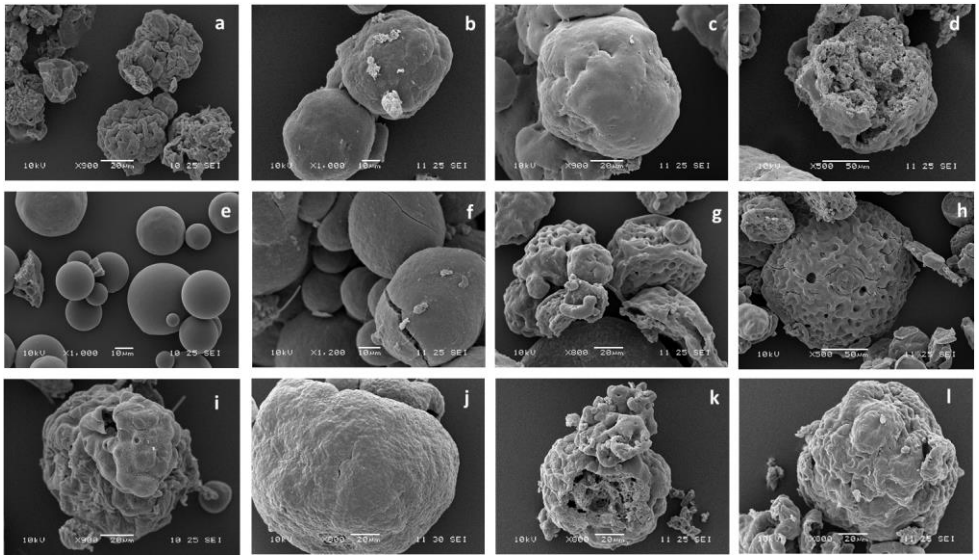

Figure 5

Supplementary material

Thermal analysis

Thermal analyses of raw polymers were performed using DSC 1 Star^e System (METTLER TOLEDO, CH). The instrument was calibrated with indium for melting point and heat of fusion. Samples of about 10 mg exactly weighted were crimped in 40 μL aluminium pan and scanned from room temperature to 90 °C at 20 K/min to erase polymers' thermal history. After a cooling step, samples were re-heated up to 60 °C at 10 K/min. Glass transition temperature were calculated on the second heat scan and reported as inflection point value. All the analyses were performed under inert atmospheres of nitrogen (80 mL/min) and using an empty aluminium pan as reference material.

Gel permeation chromatography

Molecular weight distribution of raw polymers were measured by gel permeation chromatography. Samples of about 5-6 mg were dissolved in dichloromethane and filtered through a 0.45 μm PTFE syringe filter prior the injection, to remove undissolved particles. Three columns (Phenogel™ 300x4.6 mm, Phenomenex, I) with gel pore size of 10^4 Å, 10^3 Å and 500 Å were connected in series. The mobile phase was dichloromethane at a flow rate of 0.35 mL/min at a temperature of 25.0±0.1 °C. An injection volume of 50 μL was used. The instrument was equipped with a double detector: refractive index detector and UV/visible detector set at λ=230 nm. The weight-average molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were calculated using monodisperse polystyrene standards with M_w ranging from 486 to 188,000 Da and a software to compute molecular weight distribution (Agilent, USA). Dispersity index (DI) resulted from the ratio between M_w and M_n.

Results

Table S1 enlists the main properties of the selected PLGAs. As expected, PLGA 75:25 had a higher glass transition temperature with a lower heat capacity associated with the glass-rubber transition, indicating a higher rigidity of the polymer chains compared to PLGA 50:50 [1]. Both polymers were quite homogenous in terms of molecular weight distribution, with a M_w of about 20 KDa.

Table S1- Main properties of the two types of PLGA selected to prepare the microspheres for this study.

PLGA	DL-lactide content*	T_g (°C)	ΔC_p (J/g·K)	M_w (KDa)	DI
50:50	47-53 %mol	36.5±0.1	0.602±0.027	20.4±0.4	1.5±0.0
75:25	72-78 %mol	37.7±0.1	0.458±0.021	23.6±0.3	1.7±0.2

*as reported by the supplier.

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