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

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15

16 **Abstract** (100-200 words)

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

31

32 **Keywords** (5-10 words)

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

35

36 **1. Introduction**

37 Corticosteroids locally administered by intra-articular injections represent one of the major treatment
38 for arthritis, osteoarthritis or musculoskeletal disorders to reduce pain and inflammation, facilitate
39 motion and function [1]. Due to lymph drainage of synovial fluids, the drug residence into the joint
40 is very short even when prodrugs are used [2,3] and, thus, systemic side effects have been frequently
41 reported [2]. To overcome this limitation, the controlled release of drugs loaded in microspheres made
42 of poly(lactide-*co*-glycolide) [PLGA] have been proposed [4,5] and the efficacy and bioavailability
43 of methylprednisolone loaded in PLGA matrix has been demonstrated in an animal model [6]. The
44 optimization of these drug delivery systems is challenging, as compendial *in vitro* drug release tests
45 are not described in the main Pharmacopoeias. The sample-and-separate or modified USP 4 apparatus
46 methods using a buffer as release medium and sink conditions have been proposed in the literature
47 for the screening of different PLGA-based microspheres formulations and the evaluation of batch-to-
48 batch variability [7]. An *in vitro* release experimental set-up reflecting the *in vivo* conditions, which
49 would assist in formulation development and prediction of the *in vivo* performance, is missing. For
50 example, sink conditions which are generally applied in quality control testing are not bio-relevant in
51 some anatomic sites, such as in the sub-cutaneous or the intra-muscular environment [8,9]. Moreover,
52 simple buffers do not reflect the composition of physiological fluids in healthy or disease states. In
53 the case of joints synovial fluids of healthy subjects and osteoarthritic patients, it has been
54 demonstrated that these fluids significantly differ qualitatively and quantitatively in their composition
55 [10], and they present different physicochemical properties, such as viscosity, osmolarity, surface
56 tension and pH [11]. The simulation of the synovial fluid in both healthy and disease states can play
57 a crucial role in the development of *in vitro* release/dissolution testing for intra-articular formulations.
58 Up to date, there are no synovial fluid-simulating media approved by Regulatory Agencies, and
59 limited information on the impact of their composition on the drug release and dissolution are
60 reported. For instance, the addition of hyaluronic acid in a buffer system is the main focus of the
61 published simulated synovial fluids used as release media [12–15]. Recently, bio-relevant synovial

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

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

74 2. Materials and Methods

75 1.1. Materials

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

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

92 1.2. Methods

93 1.2.1. Bio-relevant synovial fluids preparation

94 Bio-relevant synovial fluids (BSF) were prepared according to the compositions and the protocols
95 reported by Nikolettos I. [16]. BSF reflecting healthy (H-BSF) and osteoarthritic (OA-BSF)
96 conditions contained physiologically relevant amounts of HA and PC with their ratio being 1:1.7 and
97 1:0.6 for OA and H conditions, respectively, γ -globulin and BSA. The pH of H-BSF was 7.4 and the
98 pH of OA-BSF was 8. The OA-BSF was also prepared without BSA and γ -globulin [OAwP-BSF] in
99 order to investigate the influence of the presence of proteins on the drug solubility and its release
100 from the formulations under test.

101 1.2.2. Bio-relevant synovial fluid-sample treatment

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

108 1.2.3. Solubility study

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

118 1.2.4. Preparation of drug loaded microspheres

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

130 1.2.5. Determination of polymer molecular weight

131 Molecular weight distribution of raw polymers and loaded microspheres before and after the 40-day
132 release studies were measured by gel permeation chromatography (GPC). Samples of about 5-6 mg
133 were dissolved in dichloromethane and filtered through a 0.45 µm PTFE syringe filter prior the
134 injection, to remove the undissolved particles. The instrument was equipped with a G1379A degasser,
135 a G1310A isocratic pump, a G1313A auto-sampler, a G1316A thermostated column compartment
136 and double detector: refractive index detector G1362A and UV/visible detector (G1314A) set at
137 $\lambda=230$ nm. Three columns (Phenogel™ 300x4.6 mm, Phenomenex, I) with gel pore size of 10^4 Å, 10^3
138 Å and 500 Å were connected in series. The mobile phase was dichloromethane at a flow rate of 0.35
139 mL/min at a temperature of 25.0 ± 0.1 °C. An injection volume of 70 µL was used. The weight-average
140 molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were calculated
141 using monodisperse polystyrene standards with M_w ranging from 486 to 188,000 Da and a GPC-
142 Addon HP ChemStation software (Hewlett-Packard Co., USA) to compute molecular weight
143 distribution. Dispersity index (DI) was calculated by the ratio between M_w and M_n .

144 1.2.6. Microspheres size distribution and morphology

145 The mean particle size and the size distribution of microspheres suspended in 0.05% polysorbate 80
146 solution were evaluated by the single particle optical sensing (SPOS) technique, using an Accusizer
147 770 (PSS Inc. USA). The results were expressed as undersize cumulative percentages and the
148 dispersion of the size distribution as Span [Eq. (1)].

149
$$Span = \frac{d_{90} - d_{10}}{d_{50}} \quad \text{Eq. (1)}$$

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

152 All the samples were analysed in triplicate and the results reported as mean \pm SD.

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

159 1.2.7. MP content in the microsphere formulations

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

166

$$167 \text{ Experimental MP loading \%} = \frac{\text{amount of MP entrapped in microspheres}}{\text{mass of microspheres}} \times 100 \quad \text{Eq. (2)}$$

168

$$169 \text{ EE \%} = \frac{\text{amount of MP entrapped in microspheres}}{\text{theoretical amount of MP}} \times 100 \quad \text{Eq. (3)}$$

170 1.2.8. ATR-FTIR spectroscopy

171 IR spectra were recorded using a SpectrumTM One spectrophotometer (PerkinElmer, USA) equipped
172 with a diamond crystal mounted in a ATR cell (PerkinElmer, USA). Samples of MP and drug loaded
173 microspheres were scanned with a resolution of 4 cm^{-1} over a wavenumber region between 4000 and
174 650 cm^{-1} . 64 scans for each sample were collected. Baseline and ATR correction were performed on
175 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 $2.7\ \mu\text{m}$ 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

$$187 \quad Y = Y_{\text{max}}(1 - e^{-k_{\text{MP}}t}) \quad \text{Eq. (4)}$$

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**).

201

$$202 \quad \frac{M_t}{M_\infty} = kt^{0.5} \quad \text{Eq. (5)}$$

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

205

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

211 1.2.10. HPLC analysis

212 MP and MPA were quantified by HPLC, using an Agilent HP1200 series (Agilent, UK) equipped
213 with a G1312A binary pump, a G1329A auto-sampler, a G1316A thermostated column compartment
214 and a G1315D UV detector. A Phenomenex[®] Inertsil ODS-2 (C18, 250 x 4.60 mm, 5 μ m) column
215 was used for the reversed phase chromatography [18]. The mobile phase was a mixture of water and
216 methanol in the volume ratio of 70:30, at the flow rate of 1 mL/min. The injection volume was 50 μ L
217 and the temperature 35 $^\circ$ C. The detection of MP and MPA was performed at 247 nm. For the BSF
218 samples, a gradient method was applied after the isocratic elution of MP and MPA, increasing the
219 water content up to 90% over 12 min. MP and MPA calibration curves in the range of 1-10 μ g/mL
220 were freshly prepared in each medium prior each experiment ($R^2 > 0.99$). Standard solutions of MP
221 and MPA in BSF were prepared by adding the relative amount of MP or MPA working solution at
222 20 μ g/mL (in PBS) to the BSF and treating them as reported previously (section 1.2.2).

223 1.2.11. Statistical analysis

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

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

230 3. Results and discussion

231 3.1. Solubility study

232 The equilibrium solubility of MP in healthy and disease states BSF (with and without proteins) was
233 reached after 24h and the results are summarized in **Table 2**. Among the different BSF studied, the
234 highest value of MP solubility was found in the osteoarthritic medium with proteins ($p<0.05$ in all
235 the cases); while the lowest in the same medium without proteins (OAwP-BSF vs H-BSF $p=0.035$
236 and OAwP-BSF vs OA-BSF $p<0.01$). The MP solubility in OAwP-BSF was similar to the one in
237 PBS/SDS ($p=0.190$), in accordance also with the value reported in literature [19]. The lower solubility
238 values in the media without proteins compared to the ones where they were present can be attributed
239 to the fact that particularly BSA can bind molecules through hydrophobic and electrostatic
240 interactions [20] and act like a solubilizing agent, as in the case of ketoconazole, danazole, felodipine
241 [21], itraconazole [22] and cholesterol [23]. Considering that MP has an albumin binding of
242 approximately 78% [24], BSA can act similarly in the tested BSF influencing its solubility, as also
243 revealed by the higher value resulted in H-BSF compared to PBS/SDS (H-BSF vs PBS/SDS $p=0.011$,
244 **Table 2**). Being a surfactant [25], PC impacts MP solubility, even though in a lower extent, as
245 demonstrated by the comparison of the values in OA-BSF and H-BSF (**Table 2**).

246 3.2. Microsphere formulations: preparation and characterization

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

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

255 Experimental MP loading and encapsulation efficiency were slightly higher when PLGA 5050 was
256 used (formulation MS 50-23) instead of PLGA 7525 (formulation MS 75-23, **Table 1**), probably due
257 to the lower rigidity of PLGA with a similar content between lactic and glycolic acids (**Table S1**).

258 The increase of MP amount led to a higher loading of the drug in the microspheres with PLGA 5050
259 (MS 50-30 *vs* MS 50-23), with an encapsulation efficiency comparable to the one obtained for
260 formulation MS 75-23. The GPC data confirmed that the use of an ultrasound probe to prepare
261 microspheres did not have a detrimental effect on polymers, since the M_w calculated for all the
262 microsphere formulations were superimposable at time 0 (**Table 4**). Additionally, the ultrasounds or
263 the evaporation of the solvent during microspheres' formation did not change the solid state of MP
264 that was encapsulated as Form I. This is evidenced by the three strong absorption bands between 1800
265 and 1580 cm^{-1} of the ATR-FITR spectrum that were attributed to the stretching of carboxylic acid
266 and ketone groups (1650 and 1720 cm^{-1} , respectively) and to the aromatic bending (1592 cm^{-1}) [**Fig.**
267 **2**] [26].

268 3.3. *In vitro* release studies

269 3.3.1. Marketed formulation of methylprednisolone acetate

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

289 3.3.2. Methylprednisolone loaded PLGA microspheres

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

292 spectrum of this formulation in which the high intensities of MP bands at 1592 and 1650 cm^{-1} was
293 attributed to the high amount of surface-associated drug particles (**Fig. 2**). Moreover, the high
294 discontinuity of PLGA matrix at microspheres' surface (**Fig. 1**) allowed the medium to enter quickly,
295 fill the empty channels and dissolve the drug that then diffused out [27]. The burst effect of MS 50-
296 30 was less pronounced in OAwp-BSF (MP released in 1h in OA-BSF=47.5±0.7% and in OAwp-
297 BSF=29.4±1.1%, $p<0.05$), suggesting that the presence of proteins in the release medium influenced
298 not only the drug solubility, but also the wettability of the matrix, as revealed by the SEM (**Fig. 5a-**
299 **d**). After the burst effect, MP release reached a plateau after 3 days only in H-BSF and OA-BSF
300 (49.6±4.3% and 46.3±2.5% in H-BSF and OA-BSF, respectively). On the contrary, a constant release
301 occurred in OAwp-BSF (k_{50-30_OAwp} : 0.032±0.002 $\text{days}^{-0.5}$; $R^2=0.96\pm0.03$). In this medium, a
302 59.4±2.6% MP was released after 40 days, that is significantly higher than the % MP released in the
303 other media (OAwp-BSF vs H-BSF $p=0.004$, OAwp-BSF vs OA-BSF $p=0.048$), revealing the effect
304 of proteins on the amount of drug released from MS 50-30.

305 Concerning the formulation MS 75-23, only the burst MP release of around 20% after the first hour
306 was seen in the H-BSF (**Fig. 4b**). The SE micrograph of the microspheres recovered after 40 days in
307 this medium showed that the particles had a completely smooth surface, called the “skin” type
308 structure (**Fig. 5e**), probably due to a remodelling/healing process occurred in the plasticized particles
309 over time [28]. This phenomenon determined the occlusion of the pores and the inability of the drug
310 to be continuously released out of the particles. MS 75-23 behaved differently in OA-BSF as a typical
311 tri-phasic release was noticed, with a burst effect similar to the other two media (22.4±0.2 % at $t=1\text{h}$,
312 $p>0.05$), a lag-phase of about 7 days and a second release phase which fitted the Higuchi model (k_{75-}
313 $23_OA=0.051\pm0.024 \text{ days}^{-0.5}$; $R^2=0.90\pm0.04$) [**Fig 4b**]. After day 21, a plateau on MP release was
314 reached (37.5±2.3 %). In absence of proteins (OAwp-BSF), a completely different shape of the MP
315 release profile was obtained: after the burst effect, a continuous release of MP without lag phase was
316 obtained and the release data was well characterized by the Higuchi model ($k_{75-23_OAwp}=0.025\pm0.002$
317 $\text{days}^{-0.5}$; $R^2=0.84\pm0.11$, $p>0.05$ compared to k_{75-23_OA}), indicating that the MP release was mostly

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

323 Also in the case of MS 50-23, the MP release was dependent on the composition of the release
324 medium (**Fig. 4c**), and a prolonged and controlled MP release was reached. The burst effect of about
325 6% at 1h was similar in H-BSF, OA-BSF and PBS/SDS ($p>0.05$). The % MP released from these
326 microspheres in the first hour and after day 1 were lower than the corresponding ones from the MS
327 50-30 and MS 75-23. This difference can be attributed to the different distribution of the drug within
328 the polymer matrix during the microsphere preparation when PLGA 5050 and the lowest theoretical
329 drug loading was used (MS 50-23) [29]. In H-BSF and PBS/SDS, MP release started after a lag phase
330 of about 28 days, revealing that for releasing the drug it was necessary that polymer chains
331 degradation reached a certain critical PLGA M_w . According to the SEM of the recovered
332 microspheres after 40 days (**Fig. 5i and l**), a bulk-erosion controlled release was observed, that is
333 typical of microspheres made of PLGA with relatively low molecular weight (as PLGA 5050 used in
334 this study) and encapsulating poorly soluble small molecules [30]. Despite the similarity in the release
335 profiles, the microspheres did not behave in the same way in PBS/SDS and H-BSF, with microspheres
336 recovered from H-BSF having a wrinkled surface compared to the ones from PBS/SDS (**Fig. 5i and**
337 **l**). GPC data confirmed that MS 50-23 underwent a greater degradation in H-BSF than in the
338 PBS/SDS, with a molecular weight loss of about 74% and 37%, respectively (**Table 4**). In the case
339 of the MP release in BSF mimicking the disease state (OA-BSF), even though the burst effect was
340 similar to the one observed in the simulated healthy conditions (H-BSF), MP release started after day
341 7, reaching a 27.8 ± 1.6 % MP released at day 40, indicating that the simulation of disease state had an
342 impact on the release. The absence of proteins resulted in a decrease of the burst effect, as a burst of
343 4.4% was measured in OAwp-BSF ($p=0.004$). Furthermore, in the same BSF medium, there was no

344 lag phase and the MP release was characterised by the Higuchi model ($k_{50-23_OAwp}=0.050\pm0.004$ days⁻
345 ^{0.5}; $R^2=0.95\pm0.05$, **Fig. 4c**), with about 37% of MP released after 40 days. Conversely, the MP
346 released from MS 50-23 in OA-BSF seemed to follow the tri-phasic release: burst effect, lag phase
347 of about 1 week and second pulse zero-order release from day 7 to day 21, fitting the Higuchi model
348 with $k_{50-23_OA}=0.119\pm0.014$ days^{-0.5} ($R^2=0.84\pm0.11$). Afterwards, a plateau was reached (27.8 ± 1.6 %
349 after 40 days) probably due to the occurrence of healing processes, as evident in the SE micrograph
350 (**Fig. 5j**). No significant differences in terms of M_w were detected in the microspheres recovered from
351 OA-BSF and OAwp-BSF after 40 days (**Table 4**).

352 The overall release data from the performed studies clearly indicated that the presence of proteins
353 influenced significantly the drug release, both in terms of the amount released and the release
354 mechanism of MP from all types of microspheres. The presence of proteins in the bio-relevant
355 synovial fluid has to be carefully considered. Their interactions with other components of the synovial
356 fluid are not completely understood, but they affect properties of the synovial fluid, such as the
357 surface tension, and consequently the performance of a drug delivery system inside the joint [31,32].
358 For these reasons, the disease state BSF was prepared with and without the proteins. Proteins interact
359 with hydrophobic polymers, such as PLGA, and they can be adsorbed in a selective and a competitive
360 manner onto the surface of nanoparticulate PLGA systems, forming the so called “protein corona”
361 [33]. Among them, BSA, which has a good sequence identity with human serum albumin, is adsorbed
362 onto PLGA nanoparticles better than other proteins, such as γ -globulin [34,35]. Based on these
363 considerations, it can be assumed that similar interactions could also occur in the release studies
364 carried out in BSF, determining the different mechanisms in MP release from the different
365 microsphere formulations. A tri-phasic MP release in OA-BSF was observed from formulations MS
366 75-23 and MS 50-23, reaching a plateau at day 21 probably due to a polymer remodelling that closed
367 the surface pores of microspheres, as previously discussed (**Fig. 5f and j**). These formulations after
368 40 days of incubation in OAwp-BSF presented a sponge-like structure (**Fig. 5g, h and k**) which
369 favours the release of MP according to a drug diffusion mechanism. For all the formulations, the

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

372 Both types of PLGA used in the microspheres (MS 50-23 and MS 50-23 prepared with PLGA 5050
373 and MS 75-23 prepared with PLGA 7525) underwent a more pronounced degradation after incubation
374 in H-BSF compared to the other media. The hydrolysis of PLGA ester bonds starts immediately upon
375 contact with the release medium and the acidic degradation by-products accumulate within
376 microsphere until a critical M_w is reached. As a result, the drop of micro-environmental pH catalyses
377 the hydrolysis reaction, causing in some cases a heterogeneous degradation inside PLGA matrices
378 [36,37]. The high viscosity of H-BSF could slow down the diffusion of PLGA oligomers allowing
379 the establishment of the auto-catalysis phenomenon which accelerated PLGA degradation.
380 Afterwards, the diffusion of PLGA degradation by-products outside microspheres determined the
381 acidification of the H-BSF medium, which presents a low buffering capacity. On the other hand, in
382 both the osteoarthritic media (OA-BSF and OAwp-BSF) the lower viscosity and the basic pH allowed
383 a better oligomers' diffusion out of microspheres and their further neutralization. This hypothesis is
384 supported by the pH of the BSF measured after the 40-day release (**Table 4**). The massive degradation
385 occurred in H-BSF determined the greater drop in the pH value compared to the osteoarthritic media.

386 **3. Conclusions**

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

395 the release from both the marketed and microsphere formulations was affected by the medium
396 composition, with a significant impact by the presence of proteins on the release mechanism and the
397 hydrolysis rate. Furthermore, the proposed bio-relevant conditions permitted to discriminate among
398 all formulations and individuate a possible candidate able to control MP prolonged release over a 30
399 day-period.
400

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 at 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

423 **References**

424

425 [1] W. Lavelle, E.D. Lavelle, L. Lavelle, Intra-articular injections., *Med. Clin. North Am.* 91
426 (2007) 241–50. doi:10.1016/j.mcna.2006.12.002.

427 [2] H. Derendorf, H. Möllmann, A. Grüner, D. Haack, G. Gyselby, Pharmacokinetics and
428 pharmacodynamics of glucocorticoid suspensions after intra-articular administration, *Clin.*
429 *Pharmacol. Ther.* 39 (1986) 313–317. doi:10.1038/clpt.1986.45.

430 [3] A. Panusa, L. Regazzoni, G. Aldini, M. Orioli, A. Giombini, P. Minghetti, C. Tranquilli, M.
431 Carini, Urinary profile of methylprednisolone acetate metabolites in patients following intra-
432 articular and intramuscular administration, *Anal. Bioanal. Chem.* 400 (2011) 255–267.
433 doi:10.1007/s00216-011-4744-6.

434 [4] J.H. Ratcliffe, I.M. Hunneyball, A. Smith, C.G. Wilson, S.S. Davis, Preparation and
435 evaluation of biodegradable polymeric systems for the intra-articular delivery of drugs, *J.*
436 *Pharm. Pharmacol.* 36 (1984). doi:10.1111/j.2042-7158.1984.tb04419.x.

437 [5] F. Cilurzo, F. Selmin, P. Minghetti, L. Montanari, Design of methylprednisolone
438 biodegradable microspheres intended for intra-articular administration., *AAPS*
439 *PharmSciTech.* 9 (2008) 1136–1142. doi:10.1208/s12249-008-9158-1.

440 [6] A. Panusa, F. Selmin, G. Rossoni, M. Carini, F. Cilurzo, G. Aldini, Methylprednisolone-
441 loaded PLGA microspheres: A new formulation for sustained release via intra-articular
442 administration. A comparison study with methylprednisolone acetate in rats, *J. Pharm. Sci.*
443 100 (2011) 4580–4586. doi:10.1002/jps.22722.

444 [7] C. Larsen, S.W. Larsen, H. Jensen, A. Yaghmur, J. Ostergaard, Role of in vitro release
445 models in formulation development and quality control of parenteral depots, *Expert Opin.*
446 *Drug Deliv.* 6 (2009) 1283–1295. doi:10.1517/17425240903307431 PM - 19941410 M4 -
447 Citavi.

448 [8] D. Burgess, A. Hussain, T. Ingallinera, M.L. Chen, Assuring quality and performance of

- 449 sustained and controlled release parenterals: Workshop report, *AAPS J.* 4 (2002) 13–23.
450 doi:10.1208/PS040205.
- 451 [9] C.K. Brown, H.D. Friedel, A.R. Barker, L.F. Buhse, S. Keitel, T.L. Cecil, J. Kraemer, J.M.
452 Morris, C. Reppas, M.P. Stickelmeyer, C. Yomota, V.P. Shah, FIP/AAPS Joint Workshop
453 Report: Dissolution/In Vitro Release Testing of Novel/Special Dosage Forms, *AAPS*
454 *PharmSciTech.* 12 (2011) 782–794. doi:10.1208/s12249-011-9634-x.
- 455 [10] I. Kushner, J.A. Somerville, Permeability of human synovial membrane to plasma proteins.
456 Relationship to molecular size and inflammation, *Arthritis Rheum.* 14 (1971) 560–570.
457 doi:10.1002/art.1780140503.
- 458 [11] I. Nikolettos, O. Savvidou, P. Katsibri, N. Fotaki, Characterisation and drug solubility studies
459 in Rheumatoid Arthritis and Osteoarthritis synovial fluid towards the development of in vitro
460 disease state biorelevant media, in: *AAPS Annu. Meet.* 2016, Denver, Colorado (USA),
461 2016.
- 462 [12] M.R.C. Marques, R. Loebenberg, M. Almukainzi, Simulated biological fluids with possible
463 application in dissolution testing, *Dissolution Technol.* 18 (2011) 15–28.
464 doi:10.14227/DT180311P15.
- 465 [13] K.M.N. Oates, W.E. Krause, R.L. Jones, R.H. Colby, Rheopexy of synovial fluid and protein
466 aggregation., *J. R. Soc. Interface.* 3 (2006) 167–74. doi:10.1098/rsif.2005.0086.
- 467 [14] A.M. Smith, L. Fleming, U. Wudebwe, J. Bowen, L.M. Grover, Development of a synovial
468 fluid analogue with bio-relevant rheology for wear testing of orthopaedic implants, *J. Mech.*
469 *Behav. Biomed. Mater.* 32 (2014) 177–184. doi:10.1016/j.jmbbm.2013.12.009.
- 470 [15] B. Sterner, M. Harms, M. Weigandt, M. Windbergs, C.M. Lehr, Crystal suspensions of
471 poorly soluble peptides for intra-articular application: A novel approach for biorelevant
472 assessment of their in vitro release, *Int. J. Pharm.* 461 (2014) 46–53.
473 doi:10.1016/j.ijpharm.2013.11.031.
- 474 [16] I. Nikolettos, Predictive models for intra-articular drug delivery, Thesis (Ph.D.) - University

- 475 of Bath, 2018, 2018.
- 476 [17] E. Sottofattori, M. Anzaldi, L. Ottonello, HPLC determination of adenosine in human
477 synovial fluid, in: *J. Pharm. Biomed. Anal.*, 2001: pp. 1143–1146. doi:10.1016/S0731-
478 7085(00)00574-4.
- 479 [18] N.K. Hopkins, C.M. Wagner, J. Brisson, T.E. Addison, Validation of the simultaneous
480 determination of methylprednisolone and methylprednisolone acetate in human plasma by
481 high-performance liquid chromatography, *J. Chromatogr. B Biomed. Sci. Appl.* 577 (1992)
482 87–93. doi:10.1016/0378-4347(92)80601-L.
- 483 [19] R. Dannenfelser, S.H. Yalkowsky, Database for aqueous solubility of nonelectrolytes,
484 *Bioinformatics.* 5 (1989) 235–236. doi:10.1093/bioinformatics/5.3.235.
- 485 [20] P. Macheras, C. Reppas, Dissolution and in vitro permeation behaviours of dicumarol
486 nitrofurantoin and sulfamethizole in the presence of protein, *Int. J. Pharm.* 37 (1987) 103–
487 112. doi:10.1016/0378-5173(87)90014-7.
- 488 [21] M. Vertzoni, A. Diakidou, M. Chatziliadis, E. Söderlind, B. Abrahamsson, J.B. Dressman, C.
489 Reppas, Biorelevant media to simulate fluids in the ascending colon of humans and their
490 usefulness in predicting intracolonic drug solubility, *Pharm. Res.* 27 (2010) 2187–2196.
491 doi:10.1007/s11095-010-0223-6.
- 492 [22] H.S. Ghazal, A.M. Dyas, J.L. Ford, G.A. Hutcheon, In vitro evaluation of the dissolution
493 behaviour of itraconazole in bio-relevant media, *Int. J. Pharm.* 366 (2009) 117–123.
494 doi:10.1016/j.ijpharm.2008.09.003.
- 495 [23] K. Fukudome, K. Chijiwa, T. Furusawa, F. Nakayama, Effect of albumin on the solubility of
496 cholesterol in bile, *Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab.* 922 (1987) 155–161.
497 doi:10.1016/0005-2760(87)90149-4.
- 498 [24] S. Rohatagi, G. Hochhaus, H. Mollmann, J. Barth, H. Derendorf, Pharmacokinetic interaction
499 between endogenous cortisol and exogenous corticosteroids, *Pharmazie.* 50 (1995) 610–613.
- 500 [25] R.G. Strickley, Solubilizing Excipients in Oral and Injectable Formulations, *Pharm. Res.* 21

- 501 (2004) 201–230. doi:10.1023/B:PHAM.0000016235.32639.23.
- 502 [26] W.I. Higuchi, W.E. Hamlin, S.C. Mehta, Infrared attenuated total reflectance (ATR) method
503 for observing the water-mediated surface phase reversion of methylprednisolone II to I
504 during dissolution, *J. Pharm. Sci.* 58 (1969) 1145–1146. doi:10.1002/jps.2600580926.
- 505 [27] S. Fredenberg, M. Wahlgren, M. Reslow, A. Axelsson, The mechanisms of drug release in
506 poly(lactic-co-glycolic acid)-based drug delivery systems - A review, *Int. J. Pharm.* 415
507 (2011) 34–52. doi:10.1016/j.ijpharm.2011.05.049.
- 508 [28] J. Wang, B.M. Wang, S.P. Schwendeman, Characterization of the initial burst release of a
509 model peptide from poly(D,L-lactide-co-glycolide) microspheres, *J. Control. Release.* 82
510 (2002) 289–307. doi:10.1016/S0168-3659(02)00137-2.
- 511 [29] C. Berkland, K. Kim, D.W. Pack, PLG microsphere size controls drug release rate through
512 several competing factors, *Pharm. Res.* 20 (2003) 1055–1062.
513 doi:10.1023/A:1024466407849.
- 514 [30] C. Wischke, S. P. Schwendeman, Principles of encapsulating hydrophobic drugs in
515 PLA/PLGA microparticles, *Int. J. Pharm.* 364 (2008) 298–327.
- 516 [31] H. Fam, J.T. Bryant, M. Kontopoulou, Rheological properties of synovial fluids.,
517 *Biorheology.* 44 (2007) 59–74.
- 518 [32] D. Tercic, B. Bozic, The basis of the synovial fluid analysis., *Clin. Chem. Lab. Med.* 39
519 (2001) 1221–6. doi:10.1515/CCLM.2001.196.
- 520 [33] S. Tenzer, D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D.
521 Fischer, K. Kiouptsi, C. Reinhardt, K. Landfester, H. Schild, M. Maskos, S.K. Knauer, R.H.
522 Stauber, Rapid formation of plasma protein corona critically affects nanoparticle
523 pathophysiology, *Nat. Nanotechnol.* 8 (2013) 772–781. doi:10.1038/nnano.2013.181.
- 524 [34] C. Fornaguera, G. Calderó, M. Mitjans, M.P. Vinardell, C. Solans, C. Vauthier, Interactions
525 of PLGA nanoparticles with blood components: Protein adsorption, coagulation, activation
526 of the complement system and hemolysis studies, *Nanoscale.* 7 (2015) 6045–6058.

- 527 doi:10.1039/c5nr00733j.
- 528 [35] Y.M. Thasneem, S. Sajeesh, C.P. Sharma, Effect of thiol functionalization on the hemo-
529 compatibility of PLGA nanoparticles, *J. Biomed. Mater. Res. - Part A*. 99 A (2011) 607–617.
530 doi:10.1002/jbm.a.33220.
- 531 [36] A. Shenderova, T.G. Burke, S.P. Schwendeman, The Acidic Microclimate in Poly(lactide-
532 co-glycolide) Microspheres Stabilizes Camptothecins, *Pharm. Res.* 16 (1999) 241–248.
533 doi:Doi 10.1023/A:1018876308346.
- 534 [37] S. Li, S. McCarthy, Further investigations on the hydrolytic degradation of poly (DL-lactide),
535 *Biomaterials*. 20 (1999) 35–44. doi:10.1016/S0142-9612(97)00226-3.
- 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 \pm 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 \pm 0.3	81.7 \pm 1.2	11.1 \pm 1.2	14.4 \pm 1.7	31.6 \pm 3.7	1.2 \pm 0.1
MS 75-23	75:25	23	17.0 \pm 0.4	73.5 \pm 1.9	10.7 \pm 1.0	23.1 \pm 2.8	42.5 \pm 3.7	1.4 \pm 0.1
MS 50-30	50:50	30	22.1 \pm 0.5	73.8 \pm 1.8	9.6 \pm 0.2	18.6 \pm 0.5	36.4 \pm 2.4	1.4 \pm 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.

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546
547

Table 2 –MP solubility in different media after 24h, expressed as mean \pm SD (n=3).

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

553 **Table 3** – Maximum % MPA released from Depo-Medrone[®] corrected for hydrolysis and goodness
 554 (adjusted R² (Adj R²) and residual sum of square (RSQ)) of the first order fitting of the MP formation
 555 (k_{MP}). Results are reported as mean ± SD (n=3).
 556

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

557 Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwP-BSF]; healthy state BSF [H-BSF]; PBS with
 558 0.02% w/v SDS [PBS/SDS].
 559

560

561 **Table 4** – GPC data of PLGA microspheres after preparation (t=0 day) and after the 40-day release
 562 in different media. Molecular weight distribution is reported as weight average molecular weight
 563 (M_w) and polydispersity index (DI). The pH value of the release medium was measured after the 40
 564 day-release and reported as mean \pm SD (n=3).
 565

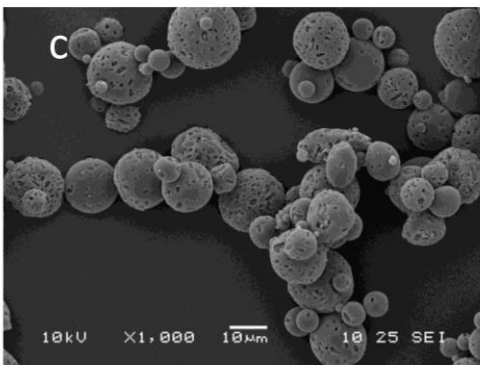
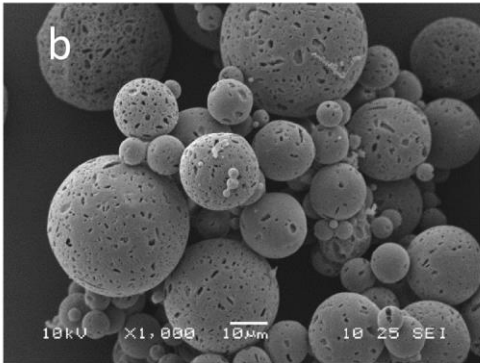
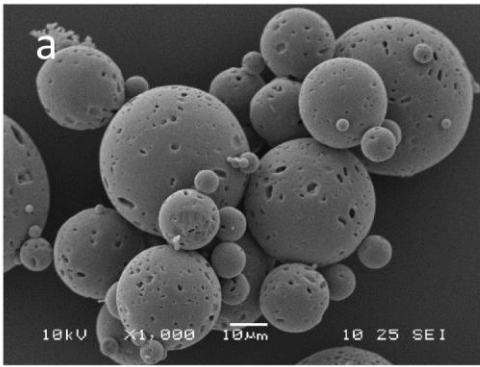
Release medium	Time (days)	MS 50-23			MS 75-23			MS 50-30		
		M_w (KDa)	DI	pH	M_w (KDa)	DI	pH	M_w (KDa)	DI	pH
--	0	20.1	1.6	--	22.2	1.6	--	20.2	1.5	--
PBS/SDS	40	12.6	1.4	7.23 \pm 0.03	n.d.	--	n.d	n.d	--	n.d
OAwP-BSF	40	11.8	1.7	7.93 \pm 0.04	15.1	1.7	7.98 \pm 0.04	14.1	1.7	7.98 \pm 0.02
OA-BSF	40	10.5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

566 Osteoarthritic BSF [OA-BSF]; osteoarthritic BSF without proteins [OAwP-BSF]; healthy state BSF [H-BSF]; PBS with
 567 0.02% w/v SDS [PBS/SDS].
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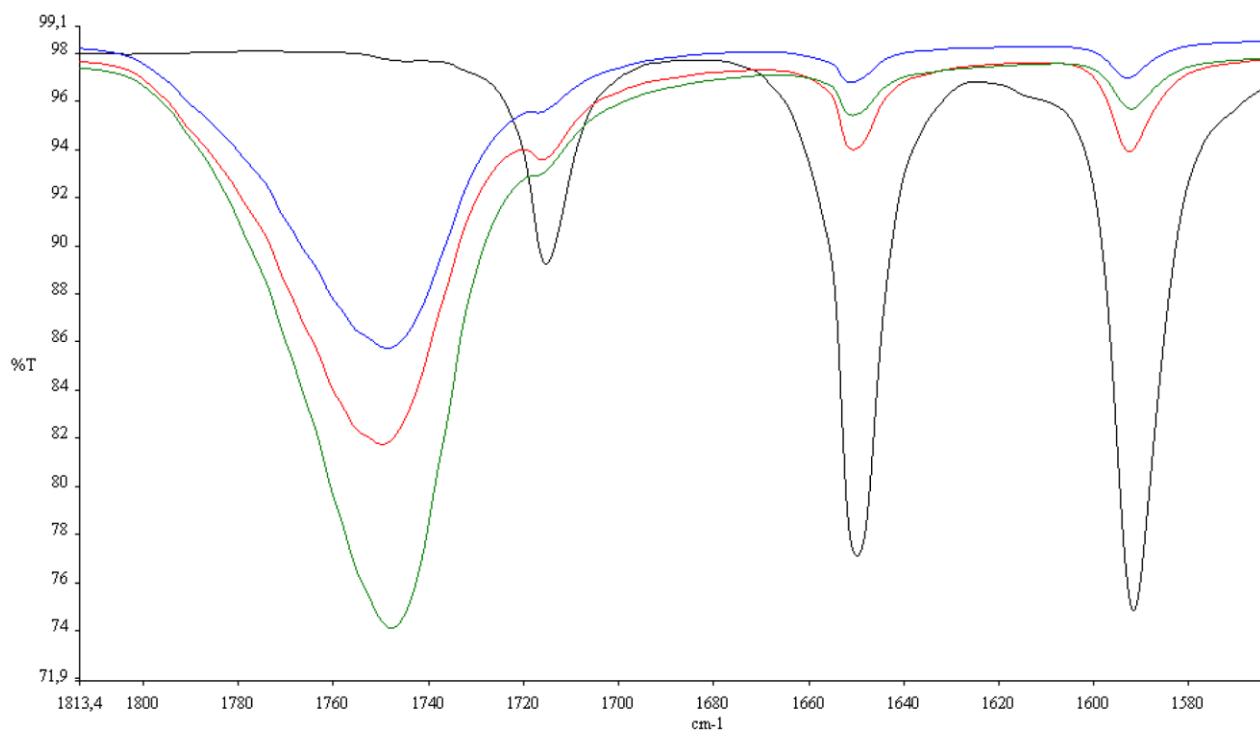
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574 Figure 1

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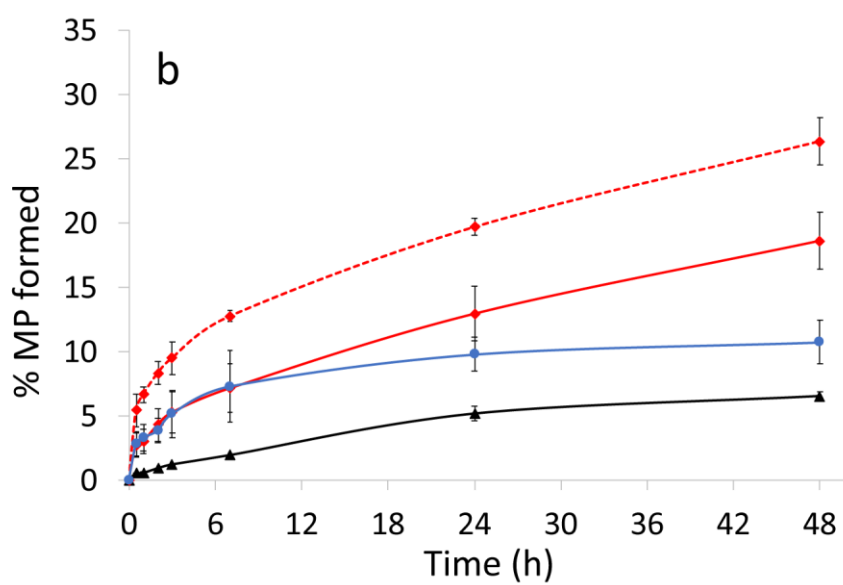
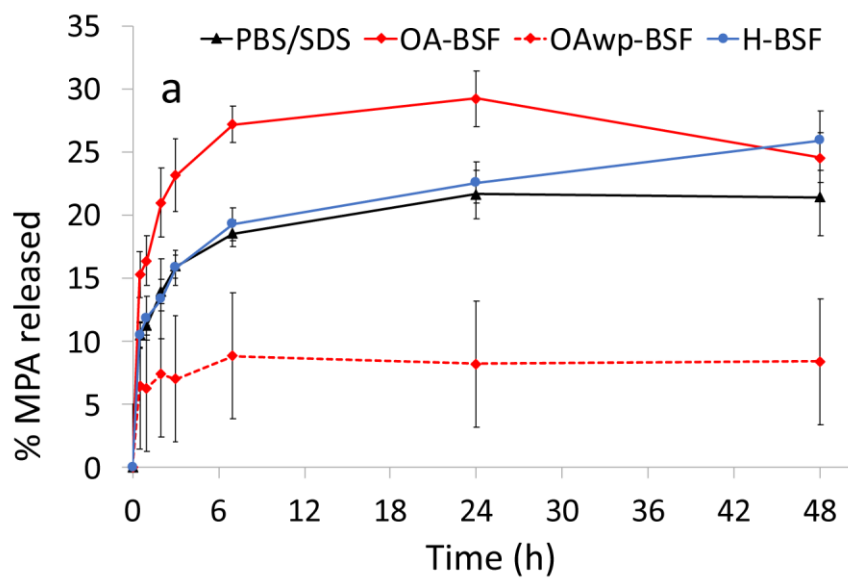


577

578 Figure 2

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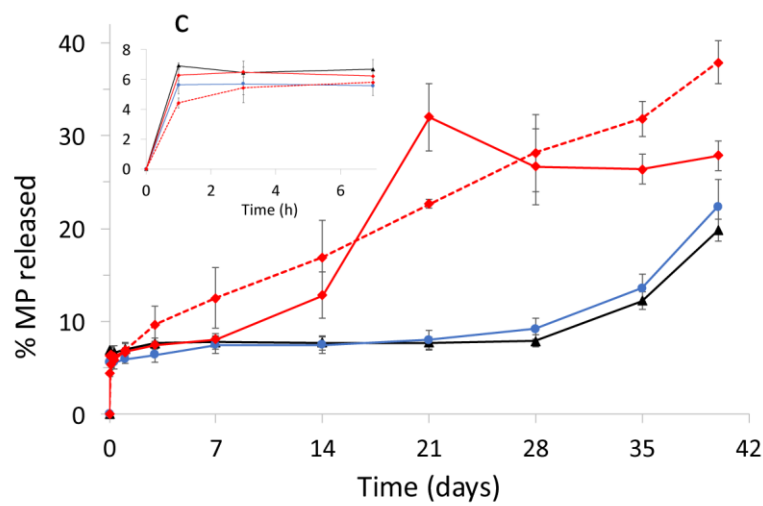
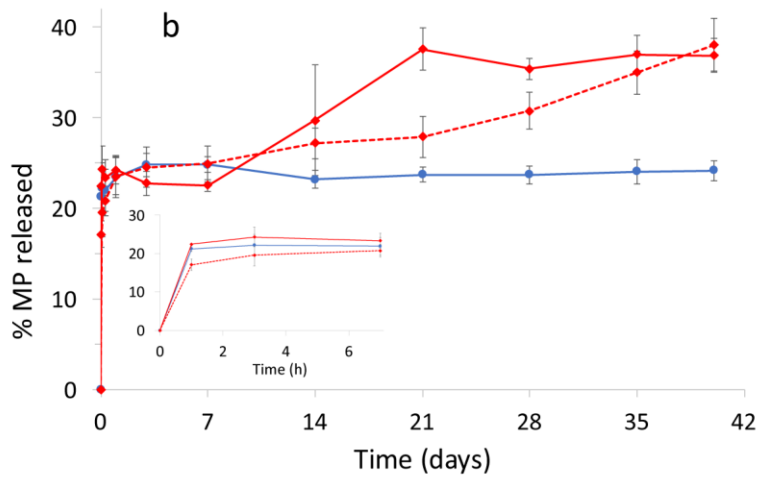
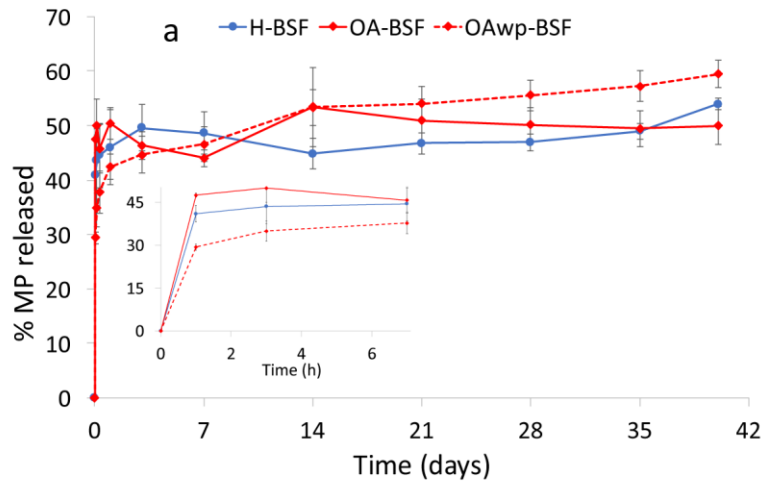
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581

582 Figure 3

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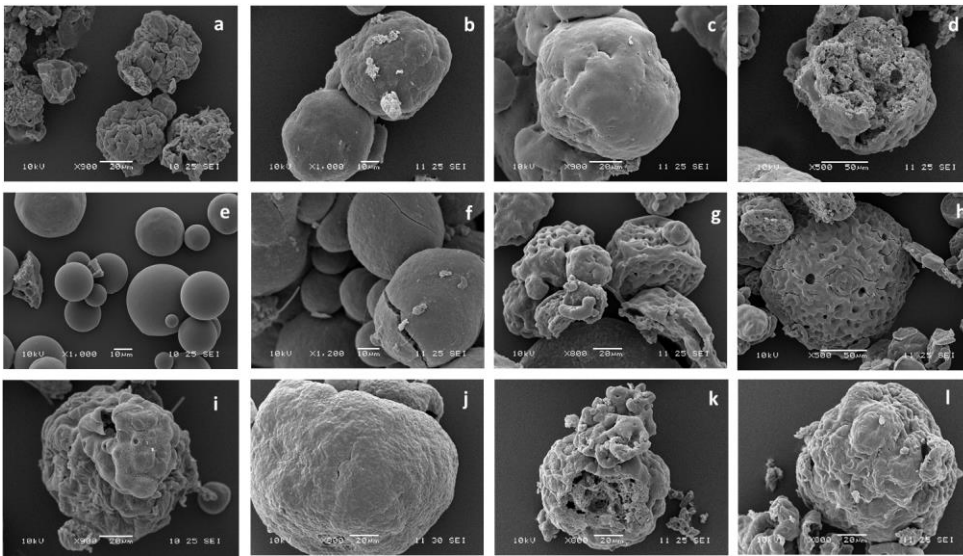


584

585 Figure 4

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589

590 Figure 5

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594 **Supplementary material**

595

596 **Thermal analysis**

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

605

606 **Gel permeation chromatography**

607 Molecular weight distribution of raw polymers were measured by gel permeation chromatography.
608 Samples of about 5-6 mg were dissolved in dichloromethane and filtered through a 0.45 μ m PTFE
609 syringe filter prior the injection, to remove undissolved particles. Three columns (PhenogelTM
610 300x4.6 mm, Phenomenex, I) with gel pore size of 10^4 Å, 10^3 Å and 500 Å were connected in
611 series. The mobile phase was dichloromethane at a flow rate of 0.35 mL/min at a temperature of
612 25.0 ± 0.1 °C. An injection volume of 50 μ L was used. The instrument was equipped with a double
613 detector: refractive index detector and UV/visible detector set at $\lambda=230$ nm. The weight-average
614 molecular weight (M_w) and the number-weight molecular weight (M_n) of each sample were
615 calculated using monodisperse polystyrene standards with M_w ranging from 486 to 188,000 Da and
616 a software to compute molecular weight distribution (Agilent, USA). Dispersity index (DI) resulted
617 from the ratio between M_w and M_n .

618

619 **Results**

620

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

625

626 **Table S1-** Main properties of the two types of PLGA selected to prepare the microspheres for this
627 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

628 *as reported by the supplier.

629

630 **References**

631 [1] J.H. Gibbs, E.A. DiMarzio, Nature of the glass transition and the glassy state, J. Chem. Phys.
632 28 (1958) 373–383. doi:10.1063/1.1744141.

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