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1 **Research article**

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4 **MODULATION OF PROTEIN RELEASE FROM PENTA-**  
5 **BLOCK COPOLYMER MICROSPHERES**

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25 **ABSTRACT**

26 Releasing a protein according to a zero-order profile without protein denaturation during  
27 the polymeric microparticle degradation process is very challenging. The aim of the  
28 current study was to develop protein-loaded microspheres with new PLGA based penta-  
29 block copolymers for a linear sustained protein release. Lysozyme was chosen as model  
30 protein and 40 µm microspheres were prepared using the solid-in-oil-in-water solvent  
31 extraction/evaporation process. Two types of PLGA-P188-PLGA penta-block copolymers  
32 were synthesized with two PLGA-segments molecular weight (20 kDa or 40 kDa). The  
33 resulting microspheres (50P20-MS and 50P40-MS) had the same size, an encapsulation  
34 efficiency around 50-60% but different porosities. Their protein release profiles were  
35 complementary: linear but non complete for 50P40-MS, non linear but complete for  
36 50P20-MS. Two strategies, polymer blending and microsphere mixing, were considered to  
37 match the release to the desired profile. The (1:1) microsphere mixture was successful. It  
38 induced a bi-phasic release with a moderate initial burst (around 15%) followed by a  
39 nearly complete linear release for 8 weeks. This study highlighted the potential of this  
40 penta-block polymer where the PEO block mass ratio influence clearly the Tg and  
41 consequently the microsphere structure and the release behavior at 37°C. The (1:1)  
42 mixture was a starting point but could be finely tuned to control the protein release.

43 **Keywords:** protein sustained release, penta-block copolymer, microsphere,  
44 microencapsulation, release modulation.

45 **Abbreviations:** PLGA, poly(D,L-lactic-co-glycolic acid); PLA, Polylactic acid; MS,  
46 microsphere; PEO, poly(ethylene oxide); PPO, poly(propylene oxide); S/O/W, solid-in-  
47 oil-in-water emulsion; DMF, dimethylformamide; DCM, methylene chloride; Tris,  
48 trizma base; P188, Poloxamer 188; THF, tetrahydrofuran; CHCl<sub>3</sub>, chloroform; DMSO,  
49 Dimethyl sulfoxide; NMR, nuclear magnetic resonance; PVA, Poly(vinyl alcohol); SEM,  
50 scanning electron microscopy; DSC, differential scanning calorimetry; Tg, glass transition  
51 temperature; EE, encapsulation efficiency; BSA, Bovine serum albumin; IQR,  
52 interquartile range; PEO, polyethylene oxide.

53

## 54 1. INTRODUCTION

55 During the past two decades, there has been a great interest in controlled release of drugs  
56 particularly for therapeutic proteins. Proteins are usually injected so it is crucial to reduce  
57 the frequency of the injections, to increase the comfort and patient compliance [1][2][3].  
58 Many approaches have been proposed to carry and deliver protein following continuous  
59 and sustained manners. Among those, protein encapsulation in microspheres using  
60 biodegradable and biocompatible polymers such as poly(D,L lactide-co-glycolide)  
61 (PLGA) [4] were often used to control the release over several weeks.

62 However protein sustained delivery from PLGA-based microspheres (MS) is still a  
63 challenge due to the variety of protein native structures, their instability particularly during  
64 the polymer degradation [5], and their very low diffusivity into PLGA matrixes [6]. To  
65 limit this drawback, a central hydrophilic segment such as poly(ethylene oxide) (PEO)  
66 was polymerized with PLGA to obtain a polymer triblock [7][8][9][10][11]. The protein  
67 release from A-B-A type triblock was enhanced as the presence of hydrophilic segments in  
68 multi-block copolymers facilitated the microsphere swelling during the release and  
69 provided protein diffusion pathways [7][16]. But an incomplete release was still observed  
70 due to the degradation of the entrapped protein into the microsphere core [12]. In addition,  
71 it was shown that by adding free poloxamer in the formulation, the protein degradation  
72 [Ref to add] and its adsorption on PLGA were reduced [14][15]. In that respect, to  
73 modulate both the hydrophilic/hydrophobic balance of the polymer and improve the  
74 protein stability, a penta-block was synthesized by copolymerizing PLGA with  
75 amphiphilic triblock polymers such as poloxamers [13][17].

76 In the literature, many efforts have been made to develop different strategies to modulate  
77 the protein release profile. Most of them relied on polymer blend strategies  
78 [18][19][20][21], whereas the strategy of mixing different types of PLGA-based  
79 microspheres was rarely addressed. Nevertheless, releasing a protein according to a zero-  
80 order profile without protein denaturation during the polymer degradation process is still  
81 very challenging.

82 The aim of the current study was to modulate the protein release from the penta-block  
83 copolymer microspheres. Lysozyme was chosen as a model protein [22] and 40 µm  
84 microspheres were prepared using the solid-in-oil-in-water (S/O/W) solvent  
85 extraction/evaporation process [13]. Penta-block copolymers were synthesized with two  
86 PLGA-segments molecular weight (20 kDa or 40 kDa). Various strategies (i.e. polymer  
87 blending, microsphere mixing) were also considered to modify the release profile while  
88 maintaining the completion of the protein release over 8 weeks. The proposal of a strategy  
89 to achieve a complete active protein release from microspheres with the desired profile  
90 was the main contribution of this study.

91

## 92 **2. MATERIALS AND METHODS**

93

### 94 **2.1. Materials**

95 Lysozyme (chicken egg white) and its substrate *Micrococcus lysodeikticus*, glycofurool,  
96 dimethylformamide (DMF), methylene chloride (DCM), acetone, and trizma base (Tris)  
97 were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Poloxamer (P188,  
98 Pluronic<sup>®</sup> F68) was obtained from BASF (Levallois-Perret, France). D,L-lactide and  
99 glycolide were purchased from Corbion (Gorinchem, The Netherlands). Polyvinyl alcohol  
100 (Mowiol<sup>®</sup> 4-88) was supplied by Kuraray Specialities Europe (Frankfurt, Germany).

101 Tin(II) 2-ethylhexanoate ( $\text{Sn}(\text{Oct})_2$ , 95%), dichloromethane (DCM), diethyl ether,  
102 tetrahydrofuran (THF), chloroform ( $\text{CHCl}_3$ ) were purchased from Sigma-Aldrich (St-  
103 Quentin Fallavier, France).

104 The poloxamer 188 (P188) is a commercial tri-block copolymer of PEO and  
105 poly(propylene oxide) (PPO) with general formula  $\text{HO}(\text{C}_2\text{H}_4\text{O})_{80}(\text{C}_3\text{H}_6\text{O})_{27}(\text{C}_2\text{H}_4\text{O})_{80}\text{H}$   
106 [23]. Two penta-block copolymers (named 50P20 and 50P40) were synthesized by ring-  
107 opening polymerization of D,L lactide and glycolide from P188 as initiator. Typically, 5g  
108 of P188 and various amounts of D,L-lactide and glycolide (respectively 25 and 20.1 g for

109 the 50P40 and 13 and 10.1 g for the 50P20) were introduced into two flasks, then were  
110 vacuum dried for 24h. Tin (II)-2 ethylhexanoate (0.1% of the number of hydroxyl  
111 functions of P188) was added in dried polymerization flasks. After degassing, the flask  
112 was sealed under vacuum and polymerization was carried out at 130°C for 5 days. The  
113 copolymers were recovered by dissolution in dichloromethane and precipitated in cold  
114 ethanol. The precipitated copolymers were filtered and dried under reduced pressure up to  
115 constant weight. The composition of copolymers was investigated by nuclear magnetic  
116 resonance spectroscopy (<sup>1</sup>H-NMR spectroscopy) at room temperature with an AMX300  
117 Bruker<sup>®</sup> spectrometer (300 MHz), using DMSO as a solvent and trimethylsilane as the  
118 internal standard. The dispersity (Đ) was determined by size exclusion chromatography  
119 (SEC, Shimadzu<sup>®</sup>, Japan) using two mixed medium columns PLgel 5 μm MIXED-C (300  
120 × 7.8 mm), a Shimadzu<sup>®</sup> RI detector 20-A and a Shimadzu<sup>®</sup> UV detector SPD-20A (270  
121 nm) (40°C thermostatic analysis cells). Tetrahydrofuran (THF) was the mobile phase with  
122 1 mL/mn flow at 30°C (column temperature).

123 The general formula of the resulting penta-block copolymer is (PLGA-PEO-PPO-PEO-  
124 PLGA). The PLGA segments contained 25% D-lactic units, 25% L-lactic units and 50%  
125 glycolic units. The penta-block copolymer 50P40 was composed of one central segment of  
126 P188 (Mn=8,400 g/mol) and two segments of PLGA (Mn=40,000 g/mol each) at the ends,  
127 the copolymer average molecular mass (Mn) was 88,400 g/mol (Đ=1.72). The penta-block  
128 copolymer 50P20 was composed of one central segment of P188 (Mn=8,400 g/mol) and  
129 two segments of PLGA (Mn=20,000 g/mol each) at the ends, the copolymer average  
130 molecular mass was (Mn) 48,400 g/mol (Đ=1.51).

131

## 132 **2.2. Microsphere preparation**

### 133 **2.2.1. Preparation of 50P40-MS or 50P20-MS**

134 Copolymer microspheres (50P40-MS and 50P20-MS) were prepared as described  
135 previously (called thereafter the standard procedure) [12]. The theoretical protein loading  
136 was 0.6% (w/w). Briefly, lysozyme and P188 (ratio lysozyme/P188 1/10 (w/w)) were

137 dissolved in water. Then 3.12 g of glycofurol were introduced in the solution to form a  
138 suspension. After an incubation at 4°C for 30 min, the nanoprecipitated protein was  
139 recovered by centrifugation (10,000 g, 4°C, 30 min). The precipitated protein was  
140 dispersed in 2 mL solution of dichloromethane/acetone 3/1 (v/v) containing 150 mg of  
141 penta-block copolymer. The suspension was emulsified in 90 mL PVA (6% w/v) at 1°C  
142 and mechanically stirred at 1,000 rpm for 1 min in a glass vessel (4.5 cm inside diameter)  
143 with a dual wall to control the temperature. 100 mL of cold deionized water were then  
144 added and the emulsion was stirred for 10 min more. Then, the emulsion was poured into a  
145 second glass vessel (10 cm inside diameter, with a dual wall) containing 500 mL of  
146 deionized water (1°C) and stirred continuously at 550 rpm during 20 min to extract the  
147 solvent. In all steps, an overhead stirrer with a 4 blades propeller was used (Heidolph®  
148 RZR 2041, Schwabach, Germany). Finally, microspheres were recovered by filtration on a  
149 5 µm filter (HVLP type, Millipore SA, Guyancourt, France), washed, freeze-dried and  
150 stored at -20°C.

### 151 **2.2.2. Preparation of microspheres using polymer blend**

152 The formulation process was similar to the standard procedure describe above. The  
153 mixture of lysozyme and P188 was nanoprecipitated in glycofurol using the same  
154 protocol. In this case, a physical blend of 50P40 and 50P20 copolymers (with a weight  
155 ratio 1/1) was dissolved in DCM/acetone (3/1, v/v). The nanoprecipitated protein was  
156 dispersed in a 2 mL solvent mixture containing 150 mg of the copolymer blend. Later on,  
157 the emulsification and solvent extraction/evaporation steps were performed using the  
158 standard procedure described above.

### 159 **2.2.3. Strategy of batch mixing following the preparation of microspheres**

160 The 50P40-MS and 50P20-MS were prepared separately following the standard procedure.  
161 Then, equal amounts of 50P40-MS and 50P20-MS (15 mg) were suspended in 1 mL of  
162 water. The mixture was gently mixed using the vortex prior to be freeze-dried. The  
163 resulting microsphere mixture (50P40-MS/50P20-MS 1/1, w/w) was stored at -20°C for  
164 further study.

## 165 **2.3. Microsphere characterization**

### 166 **2.3.1. Microsphere morphology and mean size**

167 The microsphere mean size was measured using a Coulter<sup>®</sup> Multisizer (Coultronics,  
168 Margency, France). Microspheres were dispersed in an isotonic saline solution prior to be  
169 analyzed (Isoton<sup>®</sup> II solution, Coultronics, Margency, France).

170 Microsphere surface morphology was observed using scanning electron microscopy -  
171 SEM (JSM 6310F, JEOL, Paris, France). Freeze-dried microspheres were mounted onto  
172 metal stubs using double-sided adhesive tape, vacuum-coated with a film of carbon using  
173 a MED 020 (Bal-Tec, Balzers, Lichtenstein) before being analyzed. The microsphere  
174 internal morphology was studied using the following process [13]. An appropriate amount  
175 of microspheres was dispersed into 1 mL of Tissue-Tek<sup>®</sup> (Sakura Finetek, USA) and  
176 freeze-d (-20°C, 1 hour). Resulting blocks were cut into slices (20 µm-thickness) at -15°C  
177 using a micro-cutting device (Leica, Nanterre, France). Slices were rinsed three times with  
178 cold water (1°C) before being freeze-dried. Samples were then analyzed by SEM as  
179 described above.

### 180 **2.3.2. Glass transition temperature of the copolymers**

181 To measure the glass transition of raw copolymers and microspheres, differential scanning  
182 calorimetry (DSC) analysis was carried out. DSC measurements were performed under  
183 nitrogen on a Perkin-Elmer Instrument DSC 6000 thermal analyzer. Samples were subject  
184 to a first heating ramp from -50°C to 200°C (10°C/min), followed by a cooling step  
185 (10°C/min) and finally a second heating ramp from -50°C to 200°C (10°C/min) was  
186 performed to measure the glass transition temperature (T<sub>g</sub>).

### 187 **2.3.3. Protein encapsulation efficiency**

188 The amount of entrapped lysozyme was determined by dissolving 5 mg of microspheres in  
189 0.9 mL DMF in a silanized glass tube at room temperature under agitation (1 hour). Then,  
190 3 mL of a Tris solution (Tris 0.05 M buffer and 0.09% w/v NaCl, pH 7.4) was added and  
191 the agitation was carried on for 1 hour more. The resulting solution was introduced into a



192 *Micrococcus lysodeikticus* test for active lysozyme quantification as described previously  
193 [13]. The protein encapsulation efficiency (EE) was determined in triplicate.

#### 194 **2.3.4. *In vitro* release of active lysozyme from microspheres**

195 5 mg of lysozyme-loaded microspheres were dispersed in 375  $\mu$ L of buffer solution (Tris  
196 0.05 M buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl) [24] using 1.5 mL  
197 polyethylene microtubes (Eppendorf type). The suspension was incubated in a water bath  
198 at 37°C and oscillated on a rack at 125 rpm. At defined time intervals, tubes were removed  
199 and centrifuged for 5 min at 3,000 g. The supernatant was collected, tested for active  
200 lysozyme quantification and replaced by fresh buffer. Release profiles were determined on  
201 at least 3 different microsphere batches and for each one, at least 3 experiments were  
202 carried out.

203 To investigate the effect of the release temperature on the microsphere morphology,  
204 release tests were carried out in a similar way but the water bath was controlled at 22°C or  
205 placed in a 4°C cold chamber.

#### 206 **2.3.5. Microsphere morphology change during the release test**

207 15 mg of lysozyme-loaded microspheres were dispersed into 1,050  $\mu$ L of buffer solution  
208 (Tris 0.05 M buffer, pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl) in 1.5 mL  
209 polyethylene microtubes (Eppendorf type). The suspension was incubated in a water bath  
210 at 37°C and oscillated on a rack at 125 rpm. After a defined duration, tubes were  
211 centrifuged for 5 min at 3,000 g. The supernatant was removed and the remaining particles  
212 were washed 3 times with cold water (1°C) before being freeze-dried. For microsphere  
213 cross-sectional observations, microspheres were cut and analysed with SEM as described  
214 above.

215

#### 216 **2.4. Statistical Analysis**

217 The quantitative variables were described using the mean +/- 95% confident interval when  
218 variable distribution was normal, and otherwise using median and Inter-Quartile Range.

219 Both batches and samples were taken into account to assess the global variability of the  
220 process. In view of the observation, a non-parametric Mann-Whitney test was performed  
221 when necessary.

222

## 223 **3. RESULTS AND DISCUSSION**

224

### 225 **3.1 Glass transition temperature**

226 The glass transition temperature (T<sub>g</sub>) of raw copolymers (table 1) was 24.3°C for 50P40  
227 and 9.6°C for 50P20. Both values were smaller than the ones reported in the literature for  
228 bulk PLGA where T<sub>g</sub> varies from around 50°C for 40kDa to around 40°C for 20kDa  
229 PLGA [25]. In a previous study, it was shown that when PLGA blocks were  
230 copolymerized with a central PEO blocks, the resulting triblock T<sub>g</sub> decreased noticeably  
231 as the relative amount of the hydrophilic block was increased [12]. This was also observed  
232 in the present study where the relative amount of PEO increased from 9.5% (w/w) in  
233 50P40 to 17% (w/w) in 50P20. Decreases of T<sub>g</sub> were mainly due to plasticizing effects  
234 provided by the PEO block [26].

235 Besides, T<sub>g</sub> values of uncharged lyophilized microspheres were very closed to those of  
236 raw copolymers. It is known from the literature that the surfactant, especially PVA due to  
237 its hydroxyl groups [27], and residual solvents like DCM [28] can significantly impact T<sub>g</sub>  
238 values. Then, it can be assumed a good elimination of PVA and residual solvents during  
239 the process.

240 On the other hand, the co-encapsulated poloxamer lowered the T<sub>g</sub> values of microspheres,  
241 the effect being more pronounced for 50P20-MS where T<sub>g</sub> fell from 9.1°C to 5.5°C.  
242 Incorporating the lysozyme enhanced these phenomena. Plasticizing effects of additives  
243 like pegylated compounds or proteins have been widely reported in the literature [29][30].

244 These observations were important to explain the morphology of microspheres formulated  
245 at 1°C and their behaviors during the protein release process at 37°C. In this work, T<sub>g</sub>

246 were measured on dry (lyophilized) products. But it is known that hydrated products,  
247 especially porous ones, display lower Tg [31]. Any how it was reasonable to assume that  
248 hydration should not modify our conclusions as the Tg of hydrated 50P20-MS should still  
249 be lower than the one of hydrated 50P40-MS.

250 **[Table 1] Glass transition temperatures of different copolymers.**

251

### 252 **3.2. Morphology, mean size and encapsulation efficiency**

253 Both types of microspheres were formulated below the glass transition temperature of  
254 their constitutive copolymer. For 50P40, the microsphere formation took place at 5%  
255 below Tg. They exhibited smooth surface with no visible pores. Their internal structure  
256 was porous with closed small pores distributed uniformly in the volume (Figure 1A). For  
257 50P20, the microsphere formation took place at 1% below Tg. They were highly porous  
258 with numerous interconnected pores visible on the surface as well as in the interior (Figure  
259 1B).

260 During the solvent/cosolvent extraction step, the phase separation occurs into the initial  
261 polymer solution droplets with coexisting polymer rich and polymer poor phases. The  
262 further removal of solvent induces the glass transition in the polymer rich phase, and the  
263 microsphere solidification comes to an end. The phase transition kinetics becomes arrested  
264 as the glassy state is reached in the dense polymer phase while the polymer poor phase  
265 will form the future pores in the microsphere structure. The competition between the  
266 ongoing phase separation and the appearance of the glassy state has been described and  
267 modeled in the literature [32][33] and could explain the structural differences observed.  
268 50P40-MS reached the glassy state faster than 50P20-MS and consequently developed less  
269 porous structures compared to 50P20-MS.

270

271 The median particle size and the interquartile range [IQR] of 50P40-MS were respectively  
272 39.0  $\mu\text{m}$  [38.7, 41.2] and 38.3  $\mu\text{m}$  [37.6, 41.5] for 50P20-MS (Figure 1C). Encapsulation  
273 efficiency (EE) values ranged from 52 to 61% regardless the copolymers used (Figure  
274 1C). Both types of microspheres were not significantly different considering their size ( $p =$

275 0.4681) and their encapsulation efficiency ( $p = 0.6501$ ). This result may be due to a  
276 statistical lack of power (the sampling was too small) or to the absence of real differences  
277 in the size or EE of 50P20-MS and 50P40-MS.

278 The rather low encapsulation yield can be attributed to the leakage of the protein into the  
279 external aqueous phase during the microparticle formation [34][35].

280 **[Figure 1]** Characterisation of MS produced using copolymers: (A) external/internal  
281 structure of 50P40-MS; (B) external/internal structure of 50P20-MS. In both cases, white  
282 scale bars represents 10  $\mu\text{m}$ ; (C) particle mean size ( $n=5$ ) and encapsulation efficiency  
283 ( $n=5$  for 50P40-MS and  $n=10$  for 50P20-MS) as function of copolymers. Grey plot aims  
284 to compare the distribution of both interest variable as function of copolymers types. The  
285 boxes indicate the 75<sup>th</sup> percentile (upper horizontal line), median (black bold horizontal  
286 line), and the 25<sup>th</sup> (lower horizontal line) percentiles of the distribution. Surrounding the  
287 boxed (shaded area) on each side is a rotated kernel density plot.

288

### 289 3.3. Protein release profiles

290 Drug release from PLGA-based microspheres can follow mono-, bi- or tri-phasic profiles  
291 depending on various factors including the hydrophilic balance or their morphology [29].  
292 Polymer characteristics ( $T_g$ ), porosity and mean particle size are recognized as critical  
293 factors for the protein release profile from PLGA-based microspheres [37][38]. In the  
294 present study, the microsphere diameter was kept constant to avoid its impact on the  
295 profile. The active protein release from 50P40-MS (dense surface/porous interior; Figure  
296 1A) was carried out during 8 weeks and compare to 50P20-MS (porous surface/porous  
297 interior; Figure 1B).

298 **[Figure 2]** Average protein release profiles from microspheres of copolymer 50P20  
299 (triangles) and copolymer 50P40 (squares). Error bars represent 95% confident intervals of  
300 mean values ( $n=24$  for 50P20 and  $n=10$  for 50P40). The dashed line is a guide to the eyes,  
301 it figures out the ideal desired profile.

302 Figure 2 shows release profiles for both 50P40-MS and 50P20-MS. Lysozyme release  
303 from 50P40-MS complied with a bi-phasic profile with a moderate burst ( $11.2\% \pm 1.3\%$   
304 released after 24 hours). The release was then sustained and linear until Day 56 but the  
305 cumulated amount was  $53.8\% \pm 6.2\%$  only. Despite a highly porous surface and a porous  
306 internal structure, 50P20-MS exhibited a biphasic protein release with also a moderate  
307 burst after 24 hours ( $15.2\% \pm 1.1\%$ ), followed by a sustained and complete release  
308 (ending-up with  $93.4\% \pm 3.1\%$  of active lysozyme released) but not linear as emphasized  
309 by the dashed line in figure 2.

310 For protein loaded microspheres, a bi-phasic release profile is commonly achieved with  
311 porous microspheres [38][39] or with microspheres having non-porous surface but porous  
312 internal structure [40]. The mechanism of drug release from PLGA is impacted by both the  
313 diffusion process and the polymer erosion [41][42]. However, due to the very low  
314 diffusion coefficient of a protein embedded in a PLGA matrix, its transport through the  
315 pores was proposed as the main release mechanism [6].

316

### 317 **3.4. Microsphere size and morphology change during the release test**

318 To clarify involved mechanisms, particle sizes and morphologies were monitored during  
319 the release (Figure 3 and Figure 4). After 2 days, 50P40-MS showed the appearance of  
320 few pores on their surface with an increase of the pore density in the peripheral structure  
321 (Figure 4-A2). These morphology changes were along with a 12% size increase (Figure 3).  
322 This phenomena was more marked on Day 10 with a stabilised size until Day 28 (Figure  
323 4-A3).

324 **[Figure 3]** Evolution of microsphere mean sizes as a function of the release time for  
325 50P20 (triangles) and 50P40 (squares). Error bars represent 95% confident intervals of the  
326 mean value ( $n=6$  for 50P20 and 50P40).

327 A delayed polymer degradation and erosion were previously observed for porous PLGA-  
328 based microspheres and have been explained by facilitated interchanges between the

329 internal environment and the external buffer medium. This reduced the acidification and  
330 autocatalysis phenomena [43][44] prolonging the drug release duration [45].

331 **[Figure 4]** Morphological changes of 50P40-MS (left column) and 50P20-MS (right  
332 column) during the release test (white scale bars represent 10  $\mu\text{m}$ ). After Day 28 MS could  
333 not be observed by SEM.

334 The active protein release was incomplete and a plateau around 54% was observed from  
335 Day 56 up to Day 70 (data not shown,  $n=3$ ). This phenomenon has already been observed  
336 for 60  $\mu\text{m}$  PLGA-PEG-PLGA microspheres and it was demonstrated that the protein was  
337 entrapped and degraded within the dense microsphere core [12].

338 The scenario was totally different for 50P20-MS which contained a higher amount of  
339 hydrophilic segments than 50P40-MS (17% vs 9.5% w/w respectively) with a lower Tg  
340 (3.5°C vs 15.9°C respectively, see Table 1).

341 The 50P20-MS size decreased continuously from the beginning to Day 28 (Figure 3). It  
342 appeared clearly from the SEM images that a pore-closing process occurred during the  
343 first 2 days. It was accompanied by a complete reorganization of the internal structure.  
344 Microsphere structure became homogeneous with no visible pores neither on the surface  
345 nor in the interior. This phenomenon could explain the reduced burst while a significant  
346 one is classically expected for microsphere showing an initial porous surface [46][47].

347 From Day 10, microspheres were no longer spherical and became brittle due to the  
348 polymer degradation/erosion.

349 To explain the drastic change observed at Day 2 for 50P20-MS, their morphology was  
350 investigated for various temperature of the release media, 3°C, 22°C, 37°C (Figure 5).  
351 These temperatures corresponded to three different relative distances to the dry  
352 microsphere Tg (-0.2%, +6.7%, +12.1% respectively). From figure 5, it was clearly  
353 evidenced that no reorganisation occurred at 3°C. A moderate one can be seen at 22°C and  
354 a complete one with, a pore closing process, was observed at 37°C. This demonstrated that  
355 the mobility of the polymer chains was a key parameter to explain this reorganization.

356 **[Figure 5]** Internal structure at day 2 of 50P20-MS incubated into the release medium at  
357 different temperature : (A): 3°C; (B): 22°C; (C): 37°C. White scale bars represent 10 µm.

358 50P20-MS have shown a nearly complete active protein release after 8 weeks ( $93 \pm 3\%$ )  
359 with a moderate burst but a non constant release rate (See the ideal expected profile in  
360 figure 2), while 50P40-MS had a constant rate after a moderate burst, but did not achieve  
361 a complete release ( $54 \pm 7\%$ ).

362 Both kinds of delivery system were good candidates for further optimizations using  
363 combination strategies. Indeed, a complete protein release with a moderate burst and a  
364 constant rate could ideally be wanted for some given therapeutic applications.

365

### 366 **3.5. Adjustment of protein release profile**

367 We have tried to design an optimal protein delivery system using two strategies: either a  
368 polymer blending or a microsphere mixing.

#### 369 **3.5.1. Release profile from polymer blending microspheres**

370 The mean size ( $39.9 \pm 3.4 \mu\text{m}$ ) and encapsulation yield ( $45.9 \pm 1.9\%$ ) ( $n=3$ ) of  
371 microsphere from polymer blending (hereafter called blend-microspheres) were rather  
372 similar to those of 50P40-MS and 50P20-MS. Blend-microspheres exhibited numerous  
373 pores on the surface and in the internal structure (Figure 6-A1). Regarding the release of  
374 the protein (Figure 6-A2), the initial burst ( $20.4 \pm 1.0\%$ ) was higher than previously  
375 observed. Then the subsequent protein release was rather linear from Day 1 to Day 42  
376 ( $R^2 = 0.9026$ ) and reached a plateau ( $69.1 \pm 4.9\%$  on Day 56). This kind of strategy has  
377 already been used with more or less success for PLA and PLGA microspheres [18][21].

378 In the present study, the polymer blend strategy was not an improvement.

379 **[Figure 6]** (A1) Initial morphology of blend-MS (white scale bars represent 10 µm); (A2)  
380 average protein release profile from microspheres of a copolymer blend 50P20 and 50P40  
381 (see text for details). Error bars represent 95% confident intervals of mean values ( $n=13$ );  
382 (B1) Initial morphology of mix-MS, porous particles represent 50P20-MS while non-

383 porous surface particles correspond to 50P40-MS (white scale bar represents 10  $\mu$ m); (B2)  
384 Average protein release profile from a mixture of microspheres of copolymer 50P20 and  
385 50P40 (see text for details). Error bars represent 95% confident intervals of mean values  
386 ( $n=8$ ). The red straight line represents a linear fit to the data ( $R^2 = 0.9795$ ) from Day 1 to  
387 Day 56.

### 388 **3.5.2. Release profile from a mixture of microspheres**

389 Concerning the microsphere mixing strategy, SEM picture (Figure 6 - B1) clearly revealed  
390 the two different types of microspheres (hereafter called Mix-microspheres). The initial  
391 burst was not significantly modified ( $15.2 \pm 4.0\%$ ). Then the subsequent protein release  
392 was linear from Day 1 to Day 49 ( $R^2 = 0.9915$ ) and reached a plateau ( $82.3 \pm 2.6\%$  on Day  
393 56) (Figure 6 - B2). This was an improvement in the protein release control compared to  
394 the non linear profile of 50P20-MS.

395 Hickey and co-workers [48] developed PLGA-MS for continuous delivery of  
396 dexamethasone over a month. Authors proposed to use a physical mixture of fresh and  
397 predegraded PLGA-MS. The resultant mixture exhibited an interesting profile with a  
398 moderated initial burst (from 15% to 20%) followed by a linear release but end up at  
399 around 60% after Day 3. Duvvuri and co-workers [49] prepared a physical mixture of two  
400 types of microspheres (containing ganciclovir) and dispersed the mixture into a  
401 thermogelling polymer. The resultant mixture exhibited an intermediate release profile  
402 compared to individual ones. In spite of a nearly 100% ganciclovir released after 25 days,  
403 the profile was tri-phasic with an initial massive burst. Herein, we successfully prepared a  
404 mixture of protein-loaded microspheres with the desired release profile: bi-phasic with a  
405 moderate burst, followed by a nearly complete linear release. The current achievement  
406 could be considered as a promising protein sustained release system.

407

## 408 **4. CONCLUSION**

409 The protein release profile from penta-block copolymer microspheres was successfully  
410 modulated using a mixture strategy based on 50P20-MS and 50P40-MS. Both had the



411 same size, a moderate burst and complementary profiles (linear but non complete for  
412 50P40-MS; non linear but complete for 50P20-MS) The microsphere mixture (1/1, w/w)  
413 induced a bi-phasic protein release profile with a moderate initial release followed by a  
414 nearly complete linear protein release over 8 weeks. This study highlighted the potential of  
415 this kind of penta-block polymers where the mass ratio of PEO blocks influenced clearly  
416 the Tg and consequently the release behavior at 37°C associated with the microsphere  
417 mixture strategy. The 1:1 mass ratio was used as a starting point but could be finely tune  
418 to control the protein release.

419

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425

## 426 **Declaration of Interest**

427 The authors declare no conflict of interest.

428

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