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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_3 , 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 confort 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 oxyde) (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 synthetized 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[®] 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[®] 4-88) was supplied by Kuraray Specialities Europe (Frankfurt, Germany).

101 Tin(II) 2-ethylhexanoate (Sn(Oct)₂, 95%), dichloromethane (DCM), diethyl ether,
102 tetrahydrofuran (THF), chloroform (CHCl₃) 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 HO(C₂H₄O)₈₀(C₃H₆O)₂₇(C₂H₄O)₈₀H
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 (1H-NMR spectroscopy) at room temperature with an AMX300
117 Bruker® 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®, Japan) using two mixed medium columns PLgel 5 µm MIXED-C (300
120 × 7.8 mm), a Shimadzu® RI detector 20-A and a Shimadzu® 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 ($M_n=8,400$ g/mol) and two segments of PLGA ($M_n=40,000$ g/mol each) at the ends,
127 the copolymer average molecular mass (M_n) was 88,400 g/mol ($\text{Đ}=1.72$). The penta-block
128 copolymer 50P20 was composed of one central segment of P188 ($M_n=8,400$ g/mol) and
129 two segments of PLGA ($M_n=20,000$ g/mol each) at the ends, the copolymer average
130 molecular mass was (M_n) 48,400 g/mol ($\text{Đ}=1.51$).

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[®] Multisizer (Coultronics,
168 Margency, France). Microspheres were dispersed in an isotonic saline solution prior to be
169 analyzed (Isoton[®] 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[®] (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_g).

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 μ 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 μ 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_g) 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_g 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_g 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_g were mainly due to plasticizing effects
234 provided by the PEO block [26].

235 Besides, T_g 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_g
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_g values of microspheres,
241 the effect being more pronounced for 50P20-MS where T_g 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_g

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 μm [38.7, 41.2] and 38.3 μm [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 μm ; (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 75th percentile (upper horizontal line), median (black bold horizontal
286 line), and the 25th (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 μm). 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 μm 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 μm); (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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