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1 **Vegetable Oil-based Hybrid Microparticles**
2 **as a Green and Biocompatible System for**
3 **Subcutaneous Drug Delivery**

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6 *Koceïla Doufène^a, Ilaria Basile^b, Aurélien Lebrun^c, Nelly Pirot^{d,e},*
7 *Aurélie Escande^f, Joël Chopineau^a, Jean-Marie Devoisselle^a, Nadir Bettache^g*
8 *and Anne Aubert-Pouëssel^{a*}*

9
10 ^a Institut Charles Gerhardt Montpellier (ICGM), Univ. Montpellier, CNRS, ENSCM, Montpellier, France.

11 ^b NanoMedSyn, Montpellier, France.

12 ^c Laboratoire des Mesures Physiques (LMP), Univ. Montpellier, CNRS, Montpellier, France.

13 ^d Institut de Recherche en Cancérologie de Montpellier (IRCM), Univ. Montpellier, ICM, INSERM, Montpellier,
14 France.

15 ^e BioCampus Montpellier (BCM), Univ. Montpellier, CNRS, INSERM, Montpellier, France.

16 ^f Hydrosiences Montpellier (HSM), Univ. Montpellier, CNRS, IRD, Montpellier, France.

17 ^g Institut des Biomolécules Max Mousseron (IBMM), Univ. Montpellier, CNRS, ENSCM, Montpellier, France.

18 * Email Address: anne.aubert@umontpellier.fr

19 **Abstract**

20 The aim of this study was to evidence the ability of vegetable oil-based hybrid microparticles
21 (HMP) to be an efficient and safe drug delivery system after subcutaneous administration.
22 The HMP resulted from combination of a thermostabilized emulsification process and a sol-
23 gel chemistry. First of all, castor oil was successfully silylated by means of (3-
24 Isocyanatopropyl)trimethoxysilane in solvent-free and catalyst-free conditions. Estradiol, as a
25 model drug, was dissolved in silylated castor oil (ICOm) prior to emulsification, and then an
26 optimal sol-gel crosslinking was achieved inside the ICOM microdroplets. The resulting
27 estradiol-loaded microparticles were around 80 μm in size and allowed to entrap 4 wt.%
28 estradiol. Their release kinetics in a PBS/octanol biphasic system exhibited a one-week
29 release profile, and the released estradiol was fully active on HeLa ERE-luciferase ER α cells.
30 The hybrid microparticles were cytocompatible during preliminary tests on NIH 3T3
31 fibroblasts (ISO 10993-5 standard) and they were fully biocompatible after subcutaneous
32 injection on mice (ISO 10993-6 standard) underlining their high potential as a safe and long-
33 acting subcutaneous drug delivery system.

34

35 **Keywords**

36 Vegetable oil; green process; sol-gel; estradiol; subcutaneous drug delivery; biocompatible

37

38 **Abbreviations**

39 API: active pharmaceutical ingredient = drug

40 CO: castor oil

41 CD: condensation degree

42 CY: condensation yield

43 EC₅₀: half-maximal effective concentration

44 ER α : estrogen receptor alpha

45 HELN: HeLa Estrogen Response Element (ERE)-luciferase

46 HES: hematoxylin-eosin-saffron

47 HMBC: heteronuclear multiple bond correlation

48 HMP: hybrid microparticles

49 HMPe / HMPm: hybrid microparticles made of ICOe or ICOM, respectively

50 HSQC: heteronuclear single quantum coherence

51 ICOe / ICOM: castor oil silylated by means of IPTES or by IPTMS, respectively

52 IPTES: (3-Isocyanatopropyl)triethoxysilane

53 IPTMS: (3-Isocyanatopropyl)trimethoxysilane

54 PBS: phosphate buffer saline

55 PLGA: poly(lactic-co-glycolic acid)

56 RS: red sirius

57 SC: subcutaneous

58 T^x: condensation state of the hydrolyzed trialkoxysilanes depending on the number of the

59 siloxane (Si-O-Si) generated bonds: (T⁰, T¹, T², and T³)

60 **1. Introduction**

61 The therapeutic management of chronic diseases is a tricky challenge for both healthcare
62 professionals and their patients. Although most of the drugs prescribed are orally
63 administered, some formulation constraints (e.g. pH-sensitive active pharmaceutical
64 ingredients (APIs)) and the non-compliance of some patients (e.g. mental disorders) may
65 require alternative routes of administration (Sav et al., 2015). In these cases, the subcutaneous
66 (SC) delivery of APIs offers a valuable alternative. Indeed, it is a relatively low-cost route
67 compared to the intravenous one, it is safe, effective, and it allows patient self-administration
68 (Jones et al., 2017). Nevertheless, the scientific community has agreed on the critical need for
69 the development of new technologies and systems in this area (Collins et al., 2020). Indeed,
70 the SC route is relatively unexplored and there is still plenty of room for improvement
71 through long-acting delivery systems (Chen et al., 2018) and systems dedicated to
72 biotherapeutics (e.g. antibodies, insulin and antibiotics) (Bittner et al., 2018; Hernández-Ruiz
73 et al., 2020; P.V. et al., 2017; Viola et al., 2018).

74 For a long time, injectable oils have been used to deliver APIs such as antipsychotics and
75 steroid hormones (Gao et al., 1995; Kalicharan et al., 2017; Vintiloiu and Leroux, 2008).
76 Among the used oils, castor oil (CO) and its derivatives (hydrogenated and polyoxylated CO)
77 have been widely introduced in parenteral formulations since their approval by the US-FDA
78 (Strickley, 2004). While the derivatives are used as hydrophilic surfactants in aqueous
79 formulations, native CO is used to formulate oily depots for the sustainable release of poorly
80 water-soluble APIs. Indeed, Riffkin et *al.* pointed out as early as the 1960s the interest of
81 using CO as a parenteral vehicle for steroids (Riffkin et al., 1964). This oil in particular offers
82 a greater API solubilization capacity and an improved safety. Furthermore, we highlighted in
83 a previous paper that silylated CO (ICO) synthesized with the aim of a further sol-gel
84 crosslinking kept its solubilizing capacity of poorly water-soluble APIs. ICO was able to

85 solubilize up to 160 mg of ibuprofen per milliliter and the hybrid microparticles (HMP)
86 obtained after crosslinking of the ICO exhibited an interesting sustained release of ibuprofen
87 in a subcutaneous simulated medium (Doufène et al., 2019).

88 In this present study, the chemistry of CO silylation was redesigned in order to improve the
89 "green" conditions of HMP synthesis, i.e. in solvent-free and catalyst-free conditions.
90 Estradiol (LogP = 4.01) was used as a model for poorly water-soluble APIs from the broad
91 chemical family of steroids that includes corticosteroids and contraceptives. A comprehensive
92 characterization of the new estradiol-loaded HMP is exposed with an emphasis on estradiol
93 release kinetics, the *in vitro* activity of the released estradiol, and the *in vivo* biocompatibility
94 of the HMP evaluated on mice.

95

96 **2. Materials and methods**

97 **2.1. Materials**

98 Pharmaceutical grade castor oil (CO, 934 g·mol⁻¹) was purchased from Cooper
99 Pharmaceutique. (3-Isocyanatopropyl)trimethoxysilane (IPTMS, 205 g·mol⁻¹), κ-carrageenan,
100 estradiol (272 g·mol⁻¹) and solvents (acetonitrile, acetic acid, methanol and octanol) were
101 supplied by Sigma-Aldrich.

102 HeLa ERE-luciferase (HELN) ERα cell line aimed to *in vitro* activity assays was kindly
103 donated by the team of Prof. P. Balaguer (Institut de Recherche en Cancérologie de
104 Montpellier - Inserm U1194), and NIH 3T3 fibroblast cell line aimed to cytocompatibility
105 assays was obtained from ATCC. Their culture media were purchased from Thermofisher.

106

107

108 **2.2. Synthesis and characterization of silylated castor oil**

109 **2.2.1. Synthesis of silylated castor oil**

110 IPTMS was grafted as a silylating agent on castor oil following an isocyanate-hydroxyl
111 reaction in solvent-free and catalyst-free conditions as follows: 15 g of castor oil (containing
112 one molar equivalent of hydroxyl groups (-OH)) were silylated with 7.58 g IPTMS
113 (containing 0.8 molar equivalent of isocyanate groups (-N=C=O)) during 72 h at 60 °C, under
114 nitrogen atmosphere. The silylation ratio 0.8 (i.e., ratio between the quantity of -N=C=O
115 groups in the silylating agent, and -OH groups in the oil) was selected from our previous
116 study (Doufène et al., 2019) because of the lowest toxicity of the resulting HMP on NIH 3T3
117 fibroblasts and their interesting pharmacotechnical qualities (i.e. hardness and flow properties
118 of the HMP powder). The IPTMS was chosen to replace the (3-
119 Isocyanatopropyl)triethoxysilane (IPTES) previously used to functionalize castor oil then to
120 obtain ICOe. Indeed, IPTMS is a better sol-gel reactive agent (Loy et al., 2000) that could
121 enhance the reactivity of the new silylated castor oil referred to as ICOM. In short, it allows
122 the synthesis of hybrid particles without the use of any metallic catalyst.

123 **2.2.2. Characterization of the silylated castor oil**

124 ¹H, ¹³C and ²⁹Si NMR analyses (Bruker Avance III 500 and 600 MHz equipped with
125 cryoprobes) were performed on ICOM in order to determine its structure. The methodology
126 was as follows: the NMR spectra were recorded at 298 K on a Bruker Avance III 600 MHz
127 NMR spectrometer, using TCI Cryoprobe Prodigy®. Chemical shift data were given in δ ppm
128 calibrated with residual protic solvent (e.g. CDCl₃: 7.26 ppm -¹H / 77.16 ppm - ¹³C). 2D
129 heteronuclear spectra ¹H-¹³C g-edited HSQC (Heteronuclear Single Quantum Coherence) and
130 ¹H-¹³C g-HMBC (Heteronuclear Multiple Bond Correlation) were acquired to assign the
131 compound (8 - 16 scans, 512 real (t1) × 2048 (t2) complex data points). 2D heteronuclear
132 spectra ¹H-²⁹Si g-HMBC (16 scans, 512 real (t1) × 2048 (t2) complex data points) was

133 obtained on a Bruker Avance III 500 MHz NMR spectrometer, using BroadBand Observable
134 helium cryoprobe in order to establish presence of transesterification on silicium positions.
135 Spectra were processed and visualized with Topspin 3.6.2 (Bruker Biospin) on a Linux
136 station. CDCl₃ was purchased from Aldrich. For the quantitative determination of x, y, and z
137 substituent ratios (explained in the result section), the various “CH” were identified by 2D
138 NMR g-edited HSQC ¹³C and g-HMBC ¹³C and then integrated on 1D ¹H NMR. The ICOM
139 was synthesized and analyzed in triplicate for the purpose.
140 Furthermore, the viscosity of ICOM was measured with a rotative rheometer (RM 200
141 Rheomat, Lamy Rheology) equipped with a DIN33 measuring system.

142

143 **2.3. Formulation and characterization of hybrid microparticles**

144 **2.3.1 Microparticle synthesis**

145 The ICOM-based hybrid microparticles (referred to as HMPm) were formulated following
146 the thermostabilized emulsion process we developed (Doufène et al., 2019). Estradiol, as a
147 representative API of the steroid class, was dissolved at 4 wt.% in the ICOM, An aqueous
148 phase composed of an acetate buffer (pH = 2.8) and 0.5 wt.% κ-carrageenan was prepared,
149 and the presence of the latter allows it to be liquid above 60 °C and gelled under 25 °C. The
150 oily solution was emulsified in the heated aqueous phase (60 °C) by means of a T 18 digital
151 Ultraturrax® (IKA) at 9000 rpm during 2 min. In order to induce the gelation of the aqueous
152 phase, the temperature was dropped in an ice bath for 5 min. The stabilized emulsion was kept
153 at room temperature for 8 days to allow the sol-gel crosslinking inside the oil microdroplets,
154 and the emulsion to turn into a suspension of HMPm. To recover the particles, the gelled
155 aqueous phase was liquefied by heating at 60 °C, and the resulting HMPm were washed by
156 means of milli-Q water then freeze-dried.

157

158 **2.3.2 Microparticle characterization**

159 The synthesized microparticles were characterized by complementary techniques to study
160 their morphology (i.e. granulometry using a Mastersizer 2000 from Malvern instruments),
161 their structure (i.e. solid-state ²⁹Si NMR using a Varian VNMRS 400 MHz [9.4T] NMR
162 spectrometer equipped with a 7.5 mm Varian T3 HX MAS probe spinning at 5 kHz;
163 thermogravimetric analysis using STA 6000 from PerkinElmer) and their effective loading of
164 estradiol. The theoretical loading of estradiol was 4 wt.%, i.e. the amount of estradiol
165 solubilized in ICOM, whereas the effective loading of estradiol was equal to the mass of
166 estradiol divided by the mass of the HMPm, and it was determined using a liquid
167 chromatography assay after estradiol extraction (LC-2010HT Shimadzu: static phase =
168 C18 Protonsil® column from Bischoff, mobile phase = acetonitrile/Milli-Q water, 40/60, v/v).
169 The reader is directed to the reference (Doufène et al., 2019) for further technical details.

170

171 **2.4. *In vitro* release of estradiol**

172 Release tests of estradiol from HMPm were carried out in a flow-through cell apparatus
173 and a biphasic system was chosen to mimic the *in vivo* release of estradiol after SC
174 administration, as depicted in Fig. 1. Indeed, the biphasic systems were extensively reported
175 in literature as of great interest in the study of the *in vitro* release of poorly water-soluble API
176 (Denninger et al., 2020; Phillips et al., 2012a, 2012b) and in the correlation with the *in vivo*
177 behavior of the drug delivery system (Al Durdunji et al., 2016). The used system consisted of
178 200 ml PBS buffer at pH = 7.4 and T = 37 °C as a biorelevant phase flowing at 4 ml·min⁻¹ in
179 contact with the sample (40 mg of estradiol-loaded HMPm, containing 5.874 μmol of
180 estradiol) that was entrapped in a flow-through cell. As a storage phase that entraps the
181 estradiol released from the HMPm, 40 ml of octanol at room temperature were used. The very
182 high affinity of estradiol for octanol rather than for water caused all the estradiol released in

183 PBS to be entrapped in the organic phase, and thus it ensured the sink conditions (LogP
184 (estradiol) = 4.01, meaning that the partition coefficient of estradiol in octanol : water mixture
185 is 10233 : 1, respectively). At determined intervals, samples of the organic phase were
186 analyzed by liquid chromatography as described above, and then a percentage curve of
187 released estradiol over time was drawn from triplicate tests.

188 **(Fig. 1)**

189

190 **2.5. *In vitro* activity assays of released estradiol**

191 **2.5.1. Cell line**

192 In order to check the activity of the estradiol released from HMPm and thus to ensure the
193 suitability of the process for drug loading, an already established cell model was used: HELN
194 cell line expressing the estrogen receptor alpha (ER α) (Bellet et al., 2012; Delfosse et al.,
195 2012) that exhibits estradiol-induced ER α activity (Fig. S.1). Briefly, HELN ER α cells were
196 obtained by stably expressing the ER α DNA binding domain in HELN cells.

197 The HELN ER α cells were cultured in Dulbecco's Modified Eagle Medium without phenol
198 red (DMEM/F-12) supplemented with 5 % steroid free foetal bovine serum, 1 g·ml⁻¹ glucose,
199 100 units·ml⁻¹ of penicillin, 100 μ g·ml⁻¹ of streptomycin, 0.5 μ g·ml⁻¹ puromycin and 1 mg·ml⁻¹
200 geneticin in a 5 % CO₂ humidified atmosphere at 37 °C.

201 **2.5.2. Extract preparation**

202 Unloaded and estradiol-loaded HMPm (freshly synthesized, or stored 12 months at 25 °C
203 and referred to as “old HMPm”) were suspended in culture medium (10 mg in 50 ml) and the
204 samples were gently shaken during 24 h at 37 °C. After centrifugation, the supernatants
205 (referred to as extracts) were analyzed by HPLC to determine the amount of released
206 estradiol. On the other hand, solutions of pure estradiol in dimethyl sulfoxide (10⁻⁵ to 10⁻¹² M)
207 were prepared and used as references.

208 **2.5.3. Transactivation experiments**

209 The cells were seeded at a density of 25000 cells per well in 96-well white opaque tissue
210 culture plates (Greiner CellStar) in the aforementioned medium and conditions. The extracts
211 were diluted in the culture medium according to defined concentrations of estradiol (10^{-5} to
212 10^{-12} M) and added to the cells. After 16 h of co-incubation, the diluted extracts were replaced
213 with test medium containing 0.3 mM luciferin. Luciferase activity was measured for 2 s in
214 intact living cells using a MicroBeta Wallac luminometer (PerkinElmer). Tests were
215 performed in quadruplicate in at least three independent experiments. Data were expressed as
216 a percentage of the theoretical maximal activity of pure estradiol and were given as the mean
217 \pm SD.

218 Agonistic activity of the ER α was tested in presence of increasing concentrations of extracted
219 estradiol. For each sample, the potency corresponding to the concentration yielding half-
220 maximal luciferase activity (EC₅₀ value) was determined using the dose/response fitting on
221 Origin[®] software.

222

223 **2.6. Cytocompatibility assays**

224 Cytocompatibility assays of unloaded HMPm were conducted according to the ISO 10993-
225 5 standard. Various concentrations of HMPm (0, 0.1, 1 and 10 mg·ml⁻¹) were infused in
226 DMEM culture medium during 48 h then centrifuged. The NIH 3T3 fibroblasts (cultured in
227 the same medium and seeded at 5000 cells per well in 96-well plate) were exposed to the
228 extracts, and the viability of the cells was checked after 24 and 48 h using a CellTiter 96[®] AQ
229 cell proliferation assay (Promega). This test was composed of a tetrazolium compound (3-
230 (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,
231 inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS), and it was
232 used as follows: 20 μ l of a mixture MTS-PMS was added in each well for 3 h reaction with

233 the cells, then the assay plate was read at 490 nm using a microplate reader (Multiskan Go,
234 Thermo Fisher Scientific). Finally, cell viability rates were normalized to the absorbance
235 values of the negative control cells (100 % viability), the results are given as the mean \pm SD
236 of three independent experiments and statistical analysis was performed using the Student's t-
237 test on Minitab[®] software.

238

239 **2.7. Biocompatibility assays**

240 Biocompatibility assays of unloaded HMPm were conducted according to the ISO 10993-6
241 standard.

242 **2.7.1. Treatment of the animals**

243 18 BALB/cAnNRj albino male mice (Janvier Labs, France) 10 weeks aged weighting
244 26.1 ± 1.4 g were used for the experiment. The mice were cared for in accordance with the
245 ethical guidelines and the standard protocol was approved by the Committee on the Ethics of
246 Animal Experiments of Languedoc-Roussillon C2EA-36 (reference: #23448-
247 2019122017024717). Before injection, the mice had back hairs shaved and were disinfected
248 with chlorhexidine. The mice were divided into 4 experimental groups with two injected
249 preparations (either the vehicle defined hereafter, or the unloaded HMPm suspended in the
250 vehicle), and with two experiment time-points (4 or 28 days).

251 The unloaded HMPm were sterilized through exposure to UVC rays (Bio-Link 254 nm
252 from Vilber Lourmat, France), then suspended at $25 \text{ mg}\cdot\text{ml}^{-1}$ in an apyrogenic, isotonic
253 aqueous solution containing 1.5 wt.% carboxymethylcellulose and 0.1 wt.%
254 polysorbate 80. A single dose containing 8 mg of HMPm was injected in the SC space
255 behind the neck of the mouse. The same preparation without HMPm, referred to as
256 “vehicle”, was used for the control group.

257

258 **2.7.2. Sample collection and data analysis**

259 At the two time-points (day 4 and day 28), mice were euthanized and their dorsal skin was
260 carefully resected in the injection site. The skin samples were fixed in 4 % formaldehyde
261 solution for 24 h at room temperature and embedded in paraffin. 3 μ m thick sections were cut
262 and stained with hematoxylin-eosin-saffron (HES) and red sirius (RS). The scoring criteria
263 permitted to evaluate local tissue effects according to the aforementioned ISO standard. The
264 grading system was performed by a veterinary pathologist as follows: 0 = none, 1 = mild, 2 =
265 moderate, 3 = marked, and 4 = severe.

266

267 **3. Results and discussion**

268 **3.1. Characterization of the silylated castor oil**

269 In order to elucidate the structure of the newly synthesized ICOM, a wide range of NMR
270 experiments were performed (see Fig. S.2 to S.6 in supplementary data). Four types of CH-O
271 were identified, and they were assigned to “CH-O” of the glycerol part, “CH-OH” of the non-
272 silylated branch, “CH-O-IPTMS” of the silylated branch, and the transesterified form “CH-O-
273 Si” (see Fig. S.7 and Table S.1 in supplementary data). Then the percentages x, y and z,
274 depicted in Fig. 2 were determined by integration on 1D 1 H NMR using the “CH-O” of the
275 glycerol part as reference, even if the measurement of x was slightly imprecise because of
276 overlapped signals on this area. The results were as follows: x = 9.7 % (\pm 0.6), y = 62 % (\pm
277 0.4) and z = 28.3 % (\pm 0.2).

278

(Fig. 2)

279

280 Overall, a complete silylation was reached and no early crosslinking was detected as
281 underlined by the absence of Si-O-Si bridge signals on Fig. S.6, but an unexpected reaction
282 between some methoxysilane groups of IPTMS and free hydroxyl groups of castor oil was

283 pointed out by a non-negligible z percentage, in contrast with the results previously obtained
284 during the silylation with IPTES. It can be explained by the high reactivity of the
285 methoxysilanes that are well-known to induce interfering reactions. However, the narrow
286 standard deviations on the triplicate demonstrate the reproducibility of the results and the
287 mastering of the silylation process.

288 To highlight the physical impact of the transesterification reaction, the viscosities of ICOM
289 and ICOe were compared. The viscosity of ICOM had a value of 3180 mPa·s while that of
290 ICOe was 764 mPa·s, showing a considerable increase of approximately 4-fold. In the
291 absence of early sol-gel crosslinking, the high viscosity value can only be explained by the
292 formation of ICOM oligomers, which are linked by the urethane group and by the Si-O-C
293 bridge.

294

295 **3.2. Physicochemical characterization of the hybrid microparticles**

296 The resulting HMPm exhibited a spherical morphology and a micrometric size. Their
297 volume median diameter was around 83 μm (span = 1.43) and was slightly higher than that of
298 HMPe (i.e. HMP made of ICOe, around 55 μm) as previously reported (Doufène et al., 2019).
299 This size increase was due to the higher viscosity of ICOM compared to ICOe. The tendency
300 was confirmed by the higher volume moment mean $D[4,3]$ (89 μm), whereas the surface-area
301 moment mean $D[3,2]$ was 38 μm reflecting a high number of small particles.

302 The organic/inorganic hybrid structure of HMPm was attested by a mineral residue of
303 approximately 6 % after heating up to 800 °C (Fig. S.8). The formation of siloxane bonds
304 between the silylated fatty acid chains by sol-gel reaction is behind this crosslinked structure.
305 The density of this hybrid structure was then investigated by solid-state ^{29}Si NMR. As
306 indicated in Table 1, the condensation yield (CY) and degree (CD) were 85 % and 79 %
307 respectively, and they were in the same range with those of HMPe at the same silylation ratio

308 (Doufène et al., 2019). However, the condensation behavior was different as evidenced by the
309 analysis of the various T^x , i.e. the condensation state of the hydrolyzed trialkoxysilanes,
310 depending on the number of siloxane (Si-O-Si) generated bonds (T^0 , T^1 , T^2 , and T^3). Indeed,
311 the amount of T^3 was slightly higher for HMPm (44 %) than for HMPe (36 %), meaning that a
312 higher amount of silicon parts fully crosslinked, and it was due to the better sol-gel reactivity
313 of trimethoxysilanes than triethoxysilanes. In fact, the former are more able to form multiple
314 siloxane bonds since the steric hindrance and the electro-donor inductive effect of the methyl
315 residue are lower on the silicium center (Si) than those of the ethyl one. Overall, a better sol-
316 gel crosslinking efficiency was ensured for these new particles despite the absence of a tin
317 catalyst, and a more sustained API release might be expected.

318 **(Table 1)**

319
320 Furthermore, the effective loading of estradiol within HMPm showed a value of 4.29 ± 0.18
321 wt.% slightly higher than the theoretical one (4 wt.%) pointing out a concentration effect of
322 estradiol inside HMPm that occurred during the formulation process. This phenomenon was
323 explained by methanol evaporation during the sol-gel hydrolysis reaction (Doufène et al.,
324 2019).

325

326 **3.3. *In vitro* release kinetics of estradiol**

327 **(Fig. 3)**

328

329 As depicted in Fig. 1, release kinetics of estradiol from HMPm were carried out in a
330 PBS/octanol biphasic system. The data depicted in Fig. 3 point out a “burst” stage during the
331 1st day leading to the release of 56 ± 6 % (3.29 ± 0.35 μmol) of the payload. This step was
332 followed by a sustained release of 391 nmol per day starting from day 1 until the release of

333 the entire payload after 7 days. Hence, HMPm seem to be adapted to the delivery of APIs that
334 require low plasma concentrations for their systemic activity, such as steroid hormones and
335 their analogs. However, a more sustained release would be interesting in order to reduce the
336 SC injection frequency. One could argue that it can be achieved by increasing the density of
337 the hybrid network since it have been shown that API release from HMP occurs by a diffusion
338 mechanism (Doufène et al., 2019). To set the context in the literature, two examples of
339 estradiol-loaded microparticles subcutaneously injected could be discussed: Guo et al. (Guo et
340 al., 2016) synthesized an estradiol-polyketal conjugate and formulated it into microparticles
341 that showed an interesting sustained release of estradiol over 20 weeks. However, the
342 synthesis and purification involved several steps and various organic solvents were used
343 (ether, toluene, tetrahydrofuran, chloroform, hexane, acetonitrile and methanol), and the
344 particles released simultaneously equivalent amounts of acetone and 1,4-
345 cyclohexanedimethanol from the conjugate. In another case, the researchers entrapped
346 estradiol in poly(lactic-co-glycolic acid) (PLGA) microparticles and the *in vitro* release in
347 mini dialysis device took place over 4 weeks. Here again, the same manufacturing
348 disadvantages were reported. Moreover, the degradation of PLGA *in vivo* is well-known to
349 acidify the surrounding cellular environment and may eventually alter the tissues.

350 Furthermore, it is well-known that the use of different *in vitro* release set-ups can significantly
351 affect the release kinetic profiles (Qureshi and Shabnam, 2001). Regarding the PBS/octanol
352 biphasic system used here, it appeared to be the most biorelevant set-up for such poorly
353 water-soluble API release and, to the best of our knowledge, no study on steroid release in a
354 biphasic system has already been described in the literature (Pestieau et al. 2017).

355

366 **3.4. *In vitro* activity of estradiol released from the hybrid microparticles**

357

358 **(Fig. 4)**

359 **(Table 2)**

360

361 The suitability of the formulation process was determined by assessing the *in vitro* activity
362 of estradiol released from 12 months old and freshly synthesized HMPm (Fig. 4). The two
363 formulations reached 100 % of the theoretical estradiol activity meaning that the intrinsic
364 activity of the released estradiol was fully preserved in both cases. Moreover, the EC₅₀
365 deduced from the dose-response curves (Table 2) were close to the reference attesting that the
366 affinity of the released estradiol to ER α was also fully maintained. Hence, the chemical
367 structure of the API was not affected by the formulation process, and the entrapped API was
368 stable over 12 months at room temperature highlighting a protective effect of HMPm.

369

370 **3.5. Cytocompatibility evaluation of the hybrid microparticles**

371 As a prerequisite for the *in vivo* study, the HMPm cytocompatibility was evaluated on NIH
372 3T3 cell line (Fig. 5). Almost all the cells survived (> 98 %) up to a concentration of 10
373 mg·ml⁻¹ after 24 h exposure to HMPm extracts. The viability decreased to 84 \pm 5 % with the
374 upper concentration of HMPm after 48 h exposure, but still being acceptable. This could be
375 explained by the presence of residual carrageenan on HMPm arising from the aqueous phase
376 after washing.

377 **(Fig. 5)**

378

379

380 **3.6. Biocompatibility evaluation of the hybrid microparticles**

381 For each animal, the one section out of four stained with HES that showed the most severe
382 changes was scored. After 4 days, the aqueous vehicle content (i.e. carboxymethylcellulose
383 and polysorbate) was evidenced inside moderate numbers of vacuolated macrophages in the
384 subcutaneous tissue. However, there was no evidence of vehicle-induced acute or subacute
385 inflammation. For animal treated with HMPm dispersed in the vehicle, the particles could be
386 seen as round imprints of variable sizes surrounded by macrophages, which were the main
387 cell type present to phagocytose them (Fig. 6). However, there was no inflammation as proved
388 by the absence of neutrophils and lymphocytes.

389 **(Fig. 6)**

390

391 After 28 days, the vehicle was still visible as small amounts of grey transparent amorphous
392 materials surrounded by large numbers of macrophages as seen after 4 days. Multifocally,
393 there was minimal to mild fibrosis confirmed on the section stained with RS (data not shown).
394 The sites injected with the HMPm were characterized by variable amounts of grey transparent
395 materials and several rounded clear spaces (imprints) surrounded by walls of vacuolated
396 macrophages and occasionally thin strands of collagen (consistent with minimal to mild
397 fibrosis) (Fig. 7).

398 **(Fig. 7)**

399

400 A global score was given to reflect the overall host tissue reaction based on the most marked
401 parameter (mainly macrophagic reaction and fibrosis, Table 3). At day 4, the severity was
402 deemed moderate for the vehicle alone and marked for the HMPm suspension. After 28 days,
403 there was no real difference between the vehicle and the HMPm suspension (both marked)
404 (Fig. 8).

405

406

(Fig. 8)

407

(Table 3)

408

409 Overall, the histopathologic examination showed that the local tissue reactions elicited by the
410 HMPm and the vehicle were very similar in nature and severity. They both were well
411 tolerated causing an expected and appropriate macrophagic reaction (phagocytosis), but no
412 acute or subacute reaction. The level of lymphocytic infiltration was extremely low. It can be
413 noticed that there was no evolution over time, and that the HMPm tend to migrate as there
414 was no structure, such as a fibrotic capsule, to hold the product in place.

415 When considering the *in vitro* release time of the entire estradiol payload (i.e. 7 days), the
416 persistence of HMPm after that in tissues would be irrelevant with this model API. Indeed, the
417 HMPm were still present in tissues even after 28 days because of their full biocompatibility.
418 Hence, this may suggest that either an improvement of the API retention would turn the
419 HMPm into a very long-acting drug delivery system, or an enhancement of the microparticle
420 degradability have to be studied. The HMPm could potentially degrade under the action of
421 enzymes and generate metabolizable fatty acids, glycerol, and small chains of siloxanes (less
422 than 7% of the entire mass). The latter compounds require longer degradation time lasting
423 several months by oxidation/hydrolysis mechanisms inside macrophages, as described by
424 Pfeiderer et al. (Pfeiderer et al., 1999). Indeed, they are uptaken by macrophages present in
425 all tissues, and under the action of reactive oxygen metabolites generated by macrophages,
426 they are biotransformed in functional groups containing silicon atoms bound to three or four
427 oxygens. Such metabolites are then transported to organs (i.e. liver, kidneys) and excreted. If
428 the microparticle biodegradation does not take place over a suitable period of time, their

429 elimination is consequently delayed. An alternative would be the development of monolithic
430 and removable drug delivery devices made of ICOM.

431

432 **4. Conclusion**

433 New hybrid microparticles were synthesized in two steps consisting of a silylation of
434 castor oil then an emulsification throughout a thermostabilized emulsion process. In order to
435 reduce the environmental impact, the green process requirements were fully complied here
436 since no catalyst and no solvents other than water were used, thanks to the high sol-gel
437 reactivity of ICOM. Resulting HMPm were 80 μm sized and presented high condensation
438 yield and degree reflecting the crosslinking quality, and when loaded with estradiol, they
439 showed a satisfying effective loading. Moreover, the HMPm demonstrated a complete
440 stability at room temperature, and the entrapped API was unaltered over 12 months. Release
441 kinetics in a PBS/octanol biphasic system exhibited a one-week release profile, and the
442 released estradiol was fully active on HELN ER α cells pointing out the suitability of the
443 formulation process. Finally, the HMP demonstrated their safety through their
444 cytocompatibility on NIH 3T3 fibroblasts (ISO 10993-5 standard) and their full
445 biocompatibility after subcutaneous injection on mice (ISO 10993-6 standard). Hence, HMPm
446 proved to be a promising green and biocompatible drug delivery system that can substantially
447 contribute in the area of long-acting and subcutaneously delivered systems.

448

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462

463

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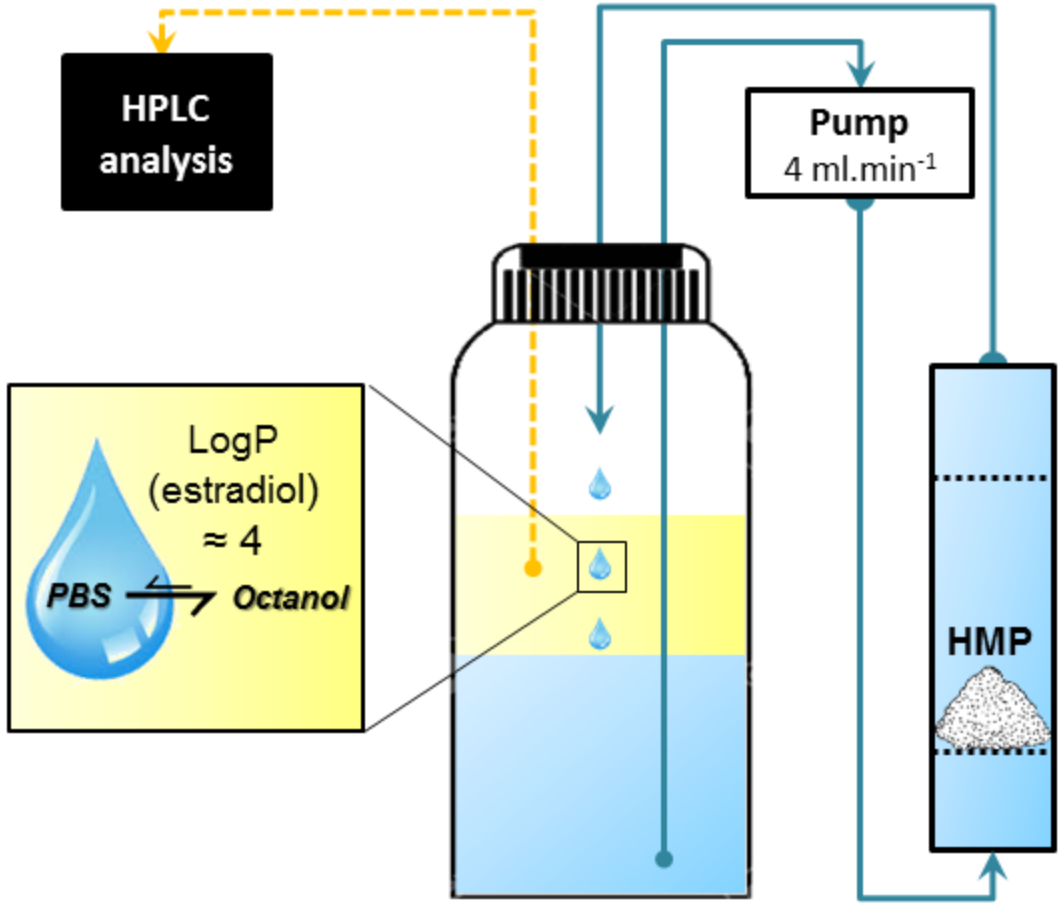
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analysis

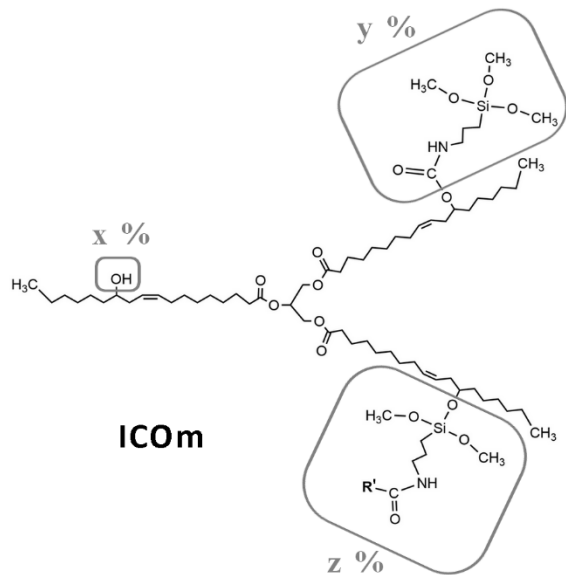
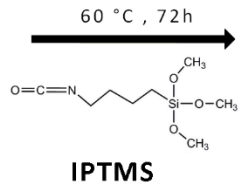
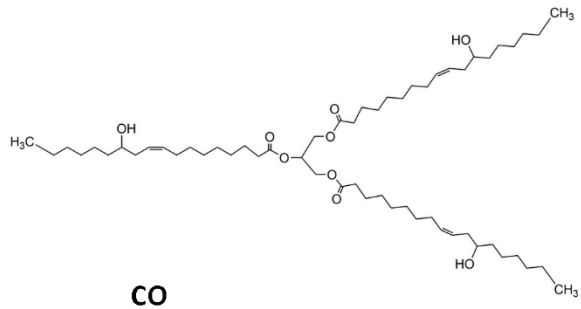
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4 ml.min⁻¹

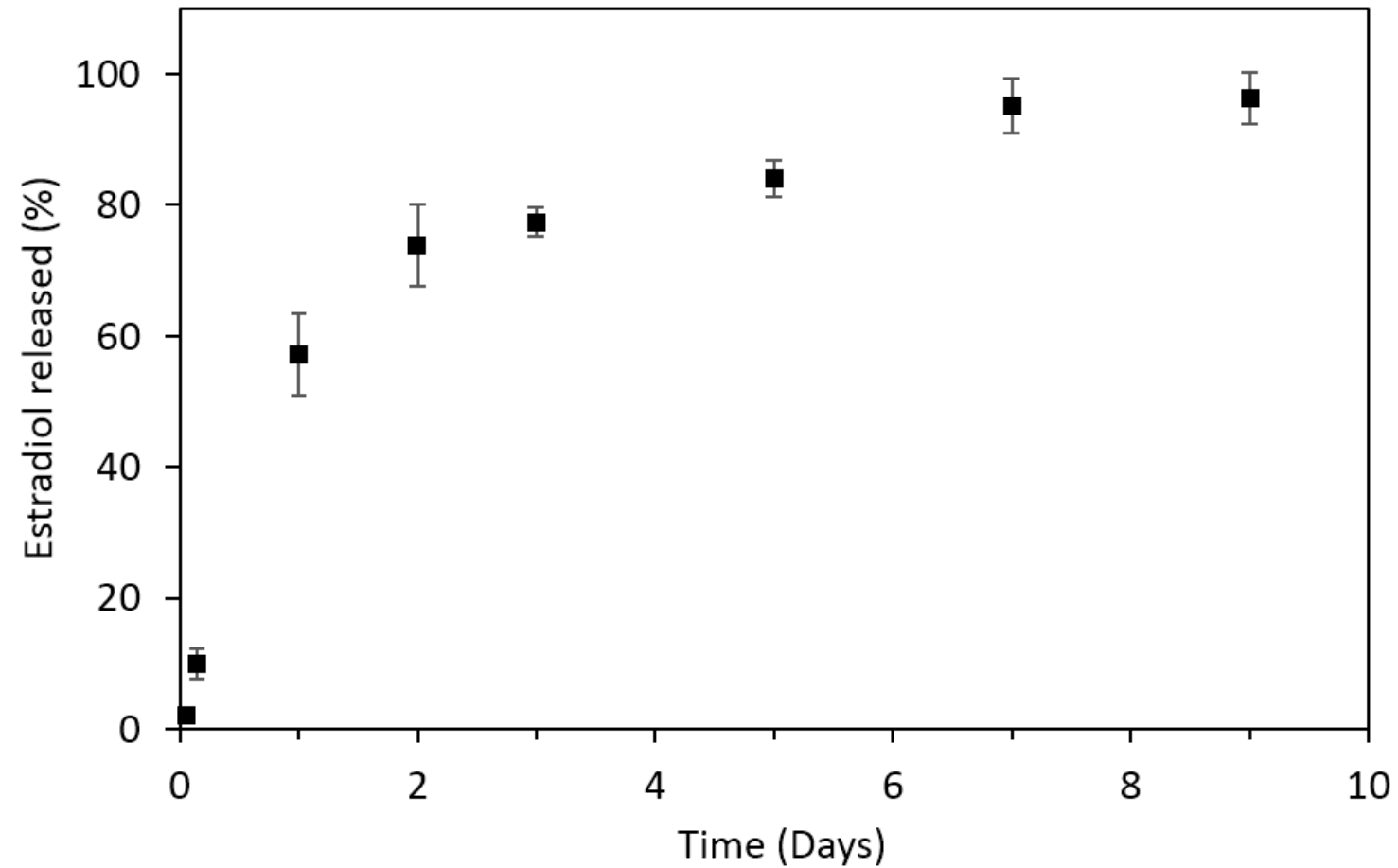
LogP
(estradiol)
≈ 4

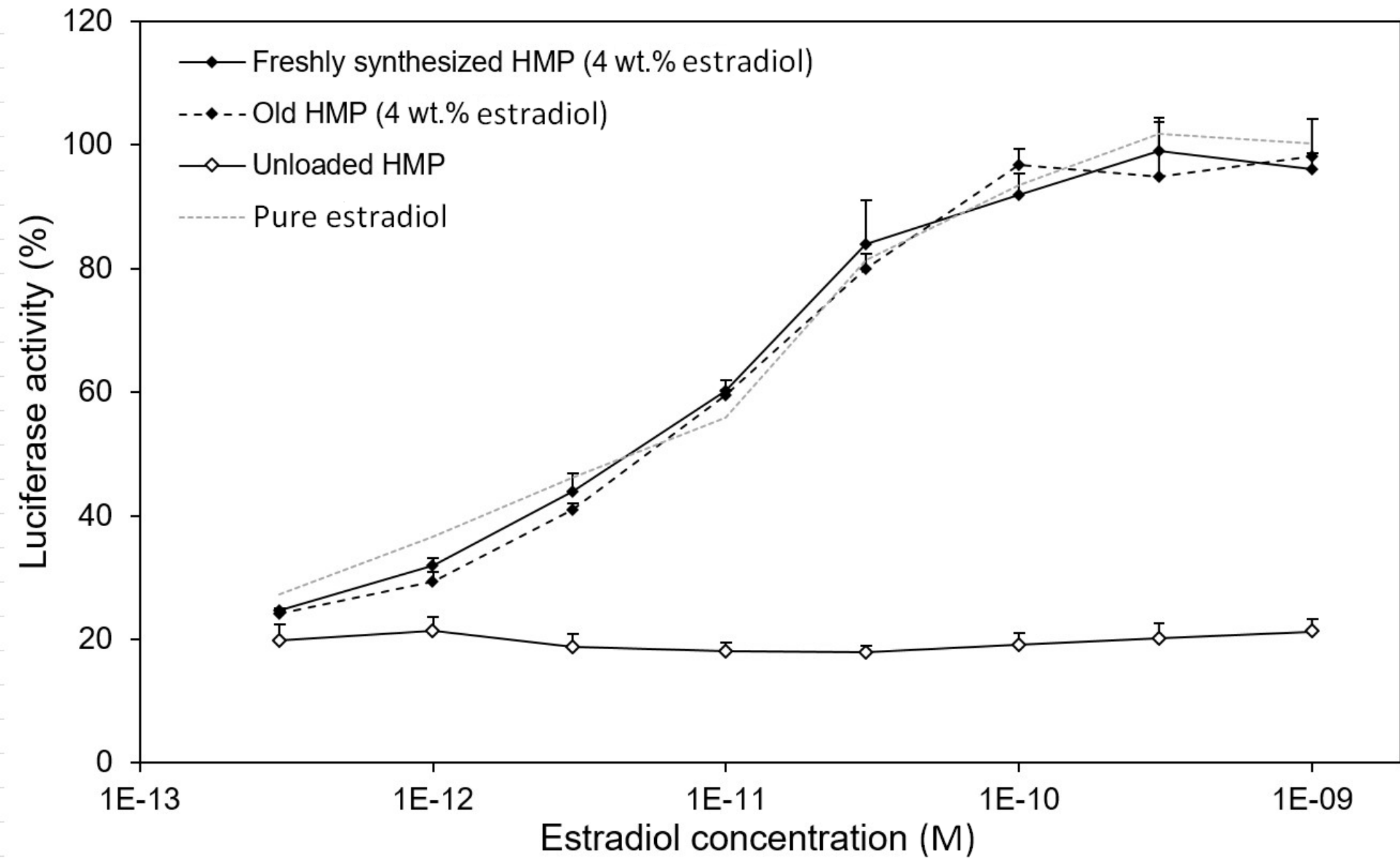
PBS ⇌ Octanol

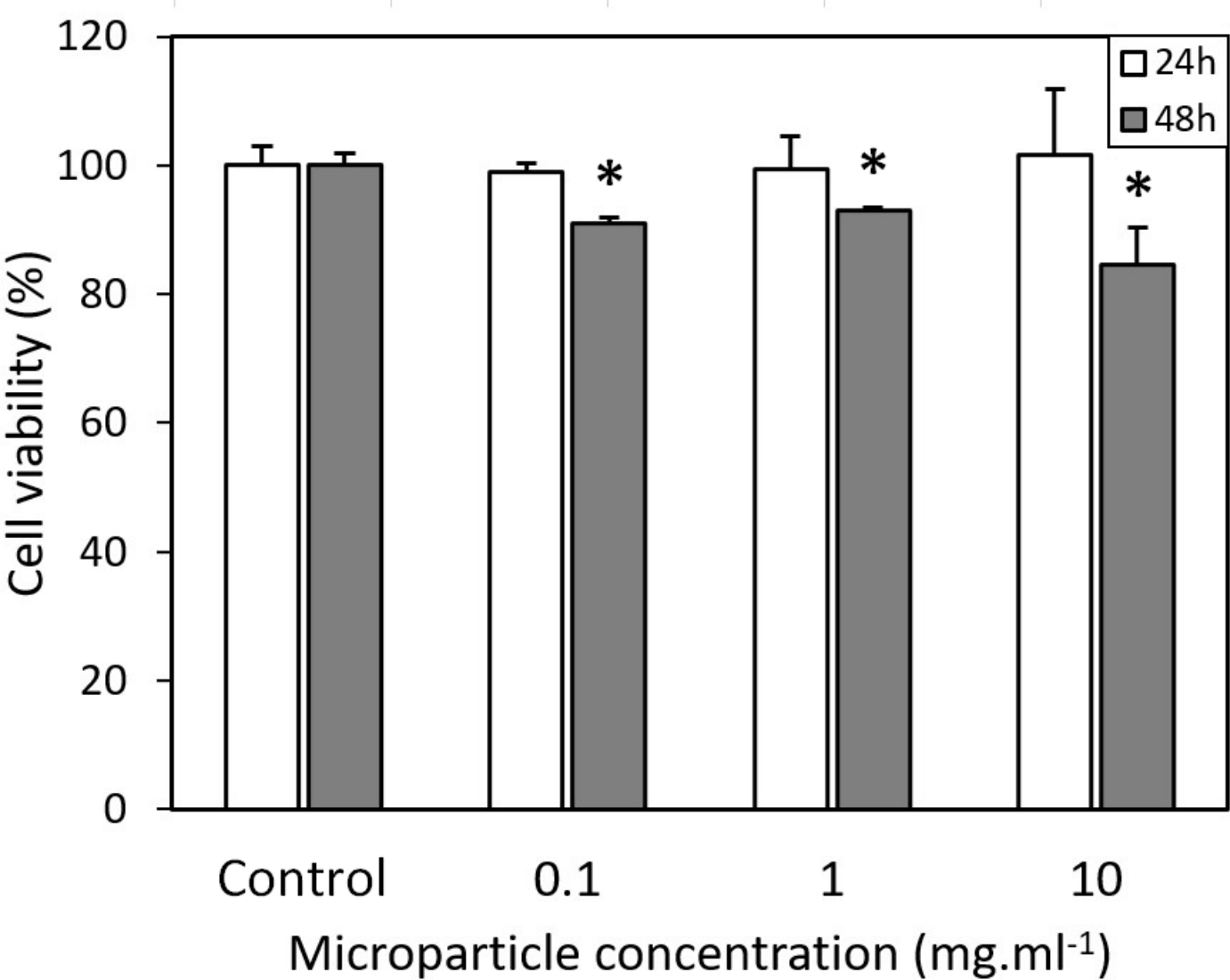
HMP

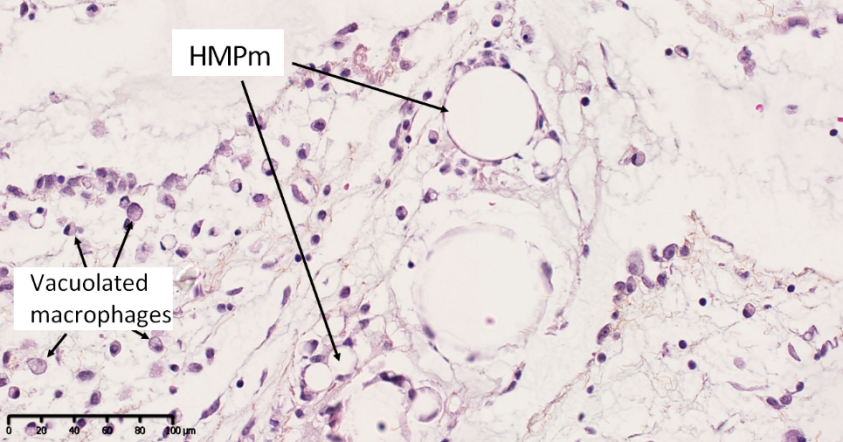


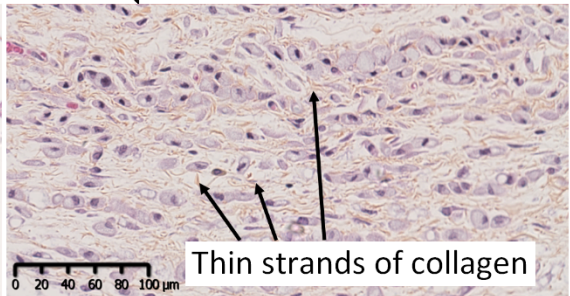
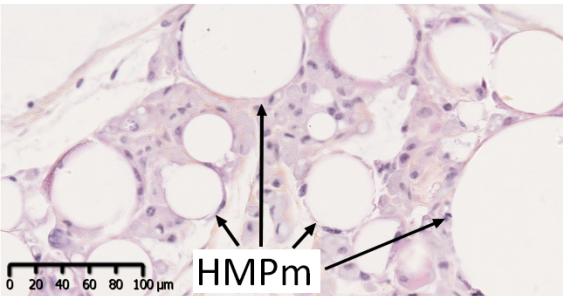
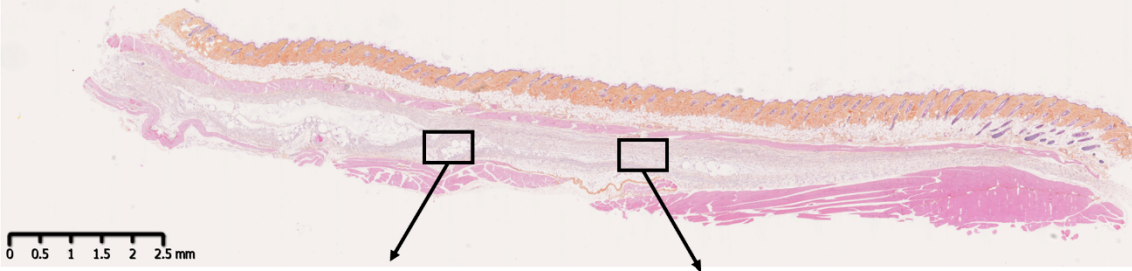








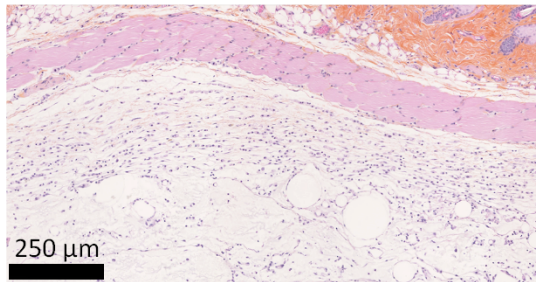
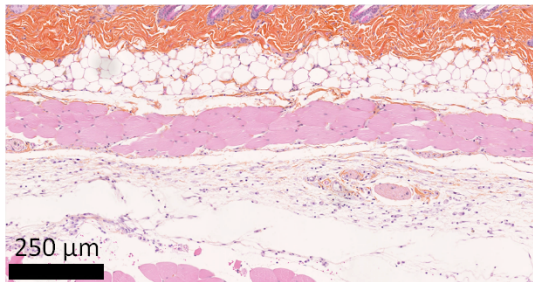




Vehicle

HMPm

Day 4



Day 28

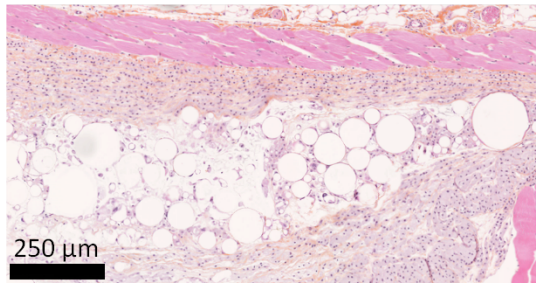
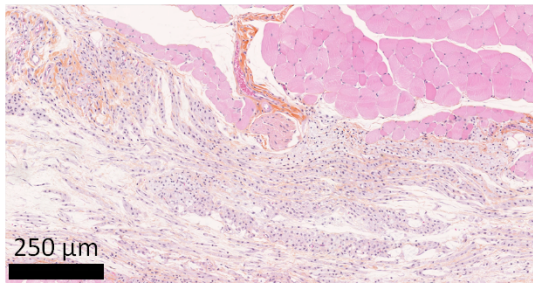


Table 1. Solid-state ^{29}Si NMR results of the HMPm sample analysis. CY and CD were calculated as

follows: $\text{CY} = 100\% - \text{LP}$; $\text{CD} = \frac{\text{T}^1 + 2 \cdot \text{T}^2 + 3 \cdot \text{T}^3}{3 \cdot \text{CY}}$. CD: condensation degree, CY: condensation yield,

LP: liquid peak.

^{29}Si signal (%)					CY (%)	CD (%)
Liquid peak	T ⁰	T ¹	T ²	T ³		
-45 ppm	-49 ppm	-54 ppm	-58 ppm	-67 ppm		
14.98	4.22	4.33	32.51	43.89	85	79

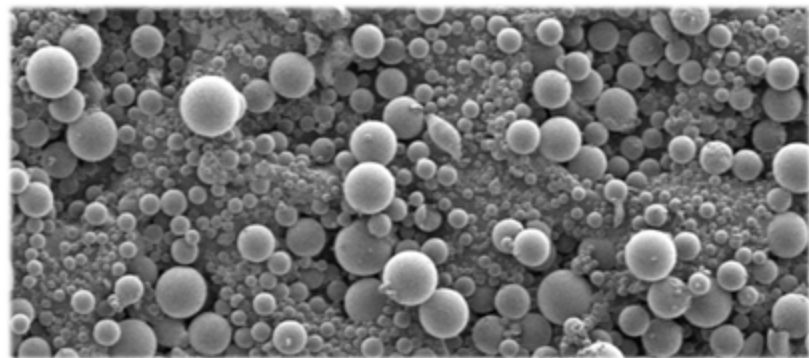
Table 2. EC₅₀ of pure estradiol and estradiol released from HMPm on HELN Er α cells.

Sample	EC ₅₀ ($\times 10^{-12}$ M)
Pure estradiol	7.3 \pm 0.07
Freshly synthesized HMPm (4 wt.% estradiol)	6.2 \pm 0.03
Old HMPm (4 wt.% estradiol)	6.2 \pm 0.03

Table 3. Summary of the histopathology scores (mean of n = 3 for vehicles and n = 6 for HMPm, NA:

not applicable).

Time period	Group	Neutrophils	Lymphocytes	Plasma cells	Macrophages	Giant cells	Necrosis	Fibrosis	Fibrin	Cell or tissue degeneration	Tissue integration	Tissue ingrowth	Global score
Day 4	Vehicle	0	0.3	0	2	0	0	0	0	0.3	NA	NA	2
	HMPm	0.1	1	0	3	0.4	0	0	0	0.3	NA	NA	3
Day 28	Vehicle	0	0.3	0	3	0	0	1	0	0	1	1	3
	HMPm	0	0.5	0	2.7	0.3	0	1	0	0	1	1	2.7



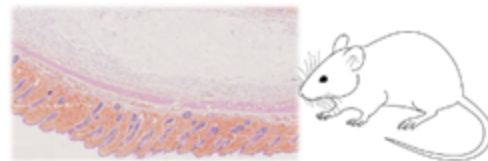
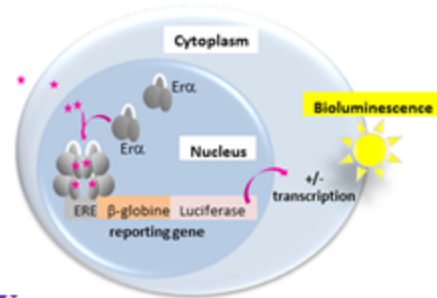
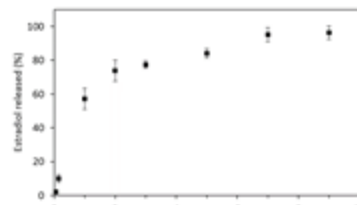
Hybrid microparticles

Estradiol loading

Sustained Drug Delivery

Drug Activity

Biocompatibility



Drug Delivery System for Subcutaneous Administration