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1 **Echogenicity enhancement by end-fluorinated polylactide perfluorohexane**
2 **nanocapsules: towards ultrasound-activable nanosystems**

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13

14 **Abstract**

15 Polylactide (PLA) polymers containing five distinct lengths of fluorinated (from C₃F₇ to C₁₃F₂₇)
16 and non-fluorinated (C₆H₁₃) end-groups were successfully synthesized by ring-opening
17 polymerization of D,L-lactide. Fluorination was expected to increase the encapsulation
18 efficiency of perfluorohexane (PFH). 150 nm nanocapsules were obtained and ¹⁹F nuclear
19 magnetic resonance revealed that nanocapsules formulated with fluorinated polymers
20 increased by 2-fold the encapsulation efficiency of PFH compared with non-fluorinated
21 derivatives, without any effect of fluorine chain length. Fluorination of the polymers did not
22 induce any specific *in vitro* cytotoxicity of nanocapsules towards HUVEC and J774.A1 cell
23 lines. The echogenicity of fluorinated-shelled nanocapsules was increased by 3-fold to 40-
24 fold compared to non-fluorinated nanocapsules or nanoparticles devoid of a perfluorohexane
25 core for both fundamental and harmonic ultrasound imaging modalities. In particular, an
26 enhanced echogenicity and harmonic response was observed as the fluorinated chain-length
27 increased, probably due to an increase of density and promotion of bubble nucleation. When
28 submitted to focused ultrasound, both intact and exploded nanocapsules could be observed,
29 also with end-group dependency, indicating that PFH was partly vaporized. These results
30 pave the way to the design of theranostic perfluorohexane nanocapsules co-encapsulating a
31 drug for precision delivery using focused ultrasound.

32 **Keywords:** Nanocapsules, perfluorohexane, fluorinated polymers, ultrasound imaging,
33 focused ultrasound, fluorous interactions; cell viability.

34 1. Introduction

35 Ultrasound contrast agents (UCAs) are efficient intravascular echo-enhancers
36 currently indispensable to safe and accurate diagnosis of many diseases, including kidney
37 cysts [1], acute myocardial ischemia [2] and solid tumors [3]. All the commercially available
38 UCAs, such as Definity® and Sonovue®, are microbubbles constituted by a perfluorinated
39 gaseous-core stabilized by a monolayer of phospholipids [4]. Unfortunately, the imaging and
40 therapeutic applications of such materials are severely limited by (1) the fast-diffusing gas
41 component, responsible for a relatively short distribution half-life in the bloodstream (~60s
42 after bolus injection) and an elimination half-life of about 6 minutes [5, 6], and (2) their
43 inherent micrometer size range that prevents extravasation into solid tumors.

44 To overcome these limitations, research has focused on nanosystems that can be
45 more stable and are able to diffuse beyond the vascular compartment. Since gas
46 nanobubbles are difficult to stabilize [7], the strategy has consisted in encapsulating liquid
47 perfluorocarbons (PFCs) into nanoemulsions using surfactants or in polymer nanocapsules
48 [8]. Although interesting, this approach leads to a significant reduction of the echogenicity.
49 Indeed, as the scattering cross section of a particle is defined according to equation 1, a
50 reduction of size and the replacement of the gas core by a liquid one, induce a decrease of
51 the scattering cross section and subsequently of echogenicity.

$$52 \quad SCS = \frac{4\pi}{9} k^4 R^6 \left[\left(\frac{\kappa_d - \kappa}{\kappa} \right)^2 + \frac{3}{4} \frac{(\rho_d - \rho)^2}{(\rho_d + \rho)^2} \right], \text{ Eq. 1}$$

53 where k is the wavenumber, R is the radius of the particle, κ_d and κ the
54 compressibilities of respectively the particle and the medium, ρ_d and ρ the densities of
55 respectively the particle and the medium [9].

56 To provide strong and long-lasting ultrasonic echoes, liquid PFCs of low boiling point,
57 such as perfluorohexane (PFH) or perfluoropentane, have been selected for their ability to
58 experience a liquid-to-gas transition once exposed to high acoustic pressures [10]. During
59 this phenomenon – known as acoustic drop vaporization (ADV) – the PFC vapor phase
60 undergoes oscillations around an equilibrium radius, which enhances the ultrasound
61 scattering intensity of nanometric systems by several orders of magnitude [11-16]. An
62 advantage of nanocapsules over microbubbles is their ability to co-encapsulate a drug for
63 further therapeutic use [17]. As previously reported, PFC-containing nanocapsules can
64 preserve their integrity and initial diameter after intravenous administration and passively
65 accumulate in tumor tissues through the enhanced permeability and retention effect (EPR)
66 [17-20] provided this effect occurs in patients [21]. After reaching tumor tissue, the release of

67 a co-encapsulated drug might be triggered by focused ultrasound (FUS), causing local PFC
68 cavitation/vaporization and may be followed by capsule shell rupture with subsequent drug
69 release [22]. This strategy is currently investigated to deliver higher drug concentrations in
70 the tumor vicinity and increase the chemotherapy efficacy while avoiding unwanted toxicity to
71 healthy cells [23]. As FUS is clinically employed to ablate and eradicate tumor cells,
72 particularly in prostate [24] and hepatocellular carcinomas [25], it represents an ideal trigger
73 for drug delivery because it is a non-invasive technique, safe to adjacent tissues and
74 provides a precise spatiotemporal control over the thermal and mechanical energy
75 dissipation [26].

76 Before pushing forward these theranostic applications, the efficient encapsulation of
77 low boiling point PFCs is required. Contrary to high boiling point PFCs such as
78 perfluorooctylbromide, the entrapment of low boiling point PFCs such as PFH into polymeric
79 nanosystems is a challenging process due to their fluorophilic character and high vapor
80 pressure [27, 28]. Perfluorinated compounds are usually immiscible with hydrophilic or
81 hydrophobic solvents due to favored interactions between fluorinated domains [29].
82 Accordingly, perfluorinated liquids tend to phase-separate, resulting in low encapsulation
83 efficiencies – from 3 to 9% in nano-sized formulations [30, 31] – which represents a limitation
84 for ultrasound imaging and ultrasound-triggered drug delivery. To promote better PFH
85 encapsulation into nanocapsules, we have synthesized polylactide (PLA) polymers
86 terminated by linear fluorinated end-groups of distinct lengths. The presence of the
87 fluorinated moiety is expected to increase PFH encapsulation efficiency by playing on
88 fluorous-fluorous interactions as it was observed for another PFC: perfluorooctyl bromide
89 [32]. Polymer synthesis, nanocapsule formulation and characterization are reported. Finally,
90 the nanocapsule echogenicity and ability to be destroyed by focused ultrasound is evaluated.

91 **2. Materials and Methods**

92 **2.1 Materials**

93 D,L-lactide was purchased from Polysciences (Germany) and perfluorohexane
94 (purity>98%) was acquired from Alfa Aesar (Germany). 1-hexanol from Acros Organics
95 (Belgium), 2,2,3,3,4,4-heptafluoro-1-butanol, 1H,1H perfluoro-1-heptanol, 1H,1H perfluoro-
96 1-nonanol, 1H,1H perfluoro-1-dodecanol and 1H,1H perfluoro-1-tetradecanol were acquired
97 from Fluorochem (United Kingdom). Acetone, tetrahydrofuran (THF) and dichloromethane
98 were purchased from Carlo Erba Reactifs (France), chloroform and diethyl ether from VWR
99 (France). Stannous octoate, dry toluene, D₂O, sodium cholate, trifluoroacetic acid (TFA)
100 and polyvinyl alcohol (PVA, 30-70kDa, 87-90% hydrolyzed) were provided by Sigma-Aldrich
101 (France). Deuterated solvents (CDCl₃ and acetone-d) were purchased from Eurisotop
102 (France), Cell culture reagents such as DMEM (Dulbecco's modified Eagle's medium),
103 RPMI 1640 (Roswell Park Memorial Institute medium), FBS (Fetal Bovine Serum), trypsin-
104 EDTA solution and PBS (Ca²⁺ and Mg²⁺ free phosphate buffer) were purchased from Sigma
105 Aldrich (France). The ultrapure water was produced by a Millipore Synergy 185 apparatus
106 coupled with a RiOs5™ (Millipore, France) with a resistivity of 18.2 MΩ.cm. The NMR
107 sample tubes and coaxial inserts were obtained from CortecNet (France).

108 **2.2 Polymer Synthesis**

109 All fluorinated (PLA-C₃F₇, PLA-C₆F₁₃, PLA-C₈F₁₇, PLA-C₁₁F₂₃ and PLA-C₁₃F₂₇) and
110 non-fluorinated (PLA-C₆H₁₃) derivatives of polylactide polymers were synthesized by ring
111 opening polymerization (ROP) with the presence of stannous octoate as catalyst [33] [34].
112 All glassware and stir bars were flame-dried and cooled under argon flow. Briefly, in a 10
113 mL schlenck tube equipped with a magnetic stir-bar, the D,L-lactide (10.4 mmol, 1.5g) and
114 corresponding initiator (0.075 mmol) – 1-hexanol for PLA-C₆H₁₃, 2,2,3,3,4,4-heptafluoro-1-
115 butanol for PLA-C₃F₇, 1H,1H perfluoro-1-heptanol for PLA-C₆F₁₃, 1H,1H perfluoro-1-
116 nonanol for PLA-C₈F₁₇, 1H,1H perfluoro-1-dodecanol for PLA-C₁₁F₂₃ or 1H,1Hp-1-
117 tetradecanol for PLA-C₁₃F₂₇ – were added to the flask under argon flow. The tube was
118 sealed with a rubber cap and a stannous octoate solution (0.05 mmol, 20 mg) dissolved in 2
119 mL of dried toluene was added through the septum. The tube was purged with argon for
120 0.5h and the polymerization reaction was conducted with continuous stirring at 130°C for 55
121 minutes in an oil bath under argon flow. The reaction was quenched by immersing the flask
122 in a cold water bath. Afterwards, the solvent was evaporated under reduced pressure for 1h
123 and the material was dissolved in 5 mL of chloroform. The product was purified by
124 precipitation as previously described [20]: all polymers were precipitated into cold diethyl
125 ether (80 mL), next, PLA-C₆H₁₃ was dissolved into THF (5mL), whereas the fluorinated

126 polymers were dissolved in acetone (20 mL) and precipitated again in ultrapure water (150
127 mL). The samples were freeze-dried for 24h and a white powder was obtained. Lactide
128 conversion $\geq 95\%$ ($^1\text{H-NMR}$). $^1\text{H-NMR}$ [400 MHz, CDCl_3 , 25°C] **PLA-C₆H₁₃**: δ_{H} 5.10-5.28
129 (PLA-CHCH₃COO-), 1.52-1.61 (-CCH₃), 0.88-0.92 (-CH₂CH₃). **PLA-C₃F₇**, **PLA-C₆F₁₃**, **PLA-**
130 **C₈F₁₇**, **PLA-C₁₁F₂₃** and **PLA-C₁₃F₂₇**: δ_{H} 5.10-5.28 (PLA-CHCH₃COO-), 4.50-4.70 (-
131 OCH₂CF₂-), 1.52-1.61 (-CCH₃). $^{19}\text{F-NMR}$ [200 MHz, CDCl_3 , 25°C] **PLA-C₃F₇**: δ_{F} -78.9 to -
132 79.05 (-CF₃), -118.5 to -119 (-CH₂CF₂-) and -125.7 to 125.8 (-CF₂CF₂CF₃); **PLA-C₆F₁₃**,
133 **PLA-C₈F₁₇**, **PLA-C₁₁F₂₃** and **PLA-C₁₃F₂₇**: $^{19}\text{F-NMR}$ [400 MHz, CDCl_3 , 25°C]: δ_{F} -78.8 to -
134 79.05 (-CF₃), -117.6 to -117.9 (-CH₂CF₂), -120.0 to -120.6 (-CF₂CF₂CF₃), -120.7 to -121.2 (-
135 CH₂CF₂CF₂), -121.2 to -121.6 (-CH₂CF₂CF₂CF₂) and -124.0 to -124.5 (-CF₂CF₃).

136 **2.3 Characterization**

137 All polymers were characterized by size exclusion chromatography (SEC) in
138 chloroform at 30°C with a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$ by two columns (PL-gel $5\mu\text{m}$ MIXED-D
139 $300 \times 7.5 \text{ mm}$) calibrated against a curve based on poly(methyl methacrylate) standards
140 (PMMA, Polymer Laboratories, Varian Inc.). The system was coupled to a refractive index
141 detector (Spectrasystem RI-150, Thermo Electro Corp.). ^1H and $^{19}\text{F-NMR}$ spectra were
142 obtained on a Bruker Advance 400 MHz in CDCl_3 at room temperature.

143 **2.4 Differential Scanning Calorimetry (DSC)**

144 All polymers were sealed in aluminum pans ($50 \mu\text{L}$) and placed in a DSC apparatus
145 (DSC Q1000, TA Instruments). All analyses were conducted under nitrogen flow
146 ($20 \text{ mL}\cdot\text{min}^{-1}$) in a temperature range between 20 and 70°C at a heat and cooling rate of
147 $20^\circ\text{C}\cdot\text{min}^{-1}$. The glass transition temperature was determined during the second heating
148 run.

149 **2.5 Nanocapsules Formulation**

150 Nanocapsules (NCs) were prepared by the emulsion-evaporation technique, as
151 previously described with modifications[19]. Briefly, 50 mg of polymer was dissolved in 2
152 mL of chloroform in a 50 mL glass flask that was sealed with a rubber cap. Next, $30 \mu\text{L}$ of
153 PFH was introduced through the septum with constant stirring, and the media was
154 emulsified with 10 mL of sodium cholate 1.5% (w/w) at 4°C in a vortex for 1 min . The
155 mixture was then submitted to probe sonication (Branson digital sonifier, France) at 30% of
156 maximal power for 1 min over ice. The solvent was evaporated by magnetic stirring at 300
157 RPM during 4 h in a thermostated bath at 20°C . The samples were then filtered on $0.45 \mu\text{m}$
158 PVDF filters (Whatman) and incubated with PVA 1% w/w for 5 days at 4°C . Finally, they
159 were centrifuged at 27.240 g (Optima LE-80K Ultracentrifuge Beckman Coulter, France)

160 during 1h at 4°C and the pellet was resuspended in 5 mL of ultrapure water to yield a final
161 polymer concentration of 25 mg.mL⁻¹.

162

163 **2.6 Size and ζ -potential**

164 The hydrodynamic diameter, polydispersity index (Pdl) and zeta potential of
165 nanocapsules were measured using a Zetasizer Nano ZS (Malvern, France) at 20°C. All
166 suspensions were diluted 1:10 in ultrapure water for size measurements and 1 mM NaCl for
167 zeta potential measurements.

168 **2.7 Electron Microscopy**

169 Scanning electron microscopy was performed using a Merlin 6040 (Carl Zeiss,
170 Germany) operating at 3 kV. The nanocapsules were deposited on carbon conductive
171 double-sided tape (Euro-medex, France) and dried at room temperature. Afterwards, they
172 were coated with a palladium–platinum layer of about 3 nm using a Cressington sputter-
173 coater 208HR with a rotary-planetary-tilt stage, fitted with an MTM-20 thickness controller.
174 Transmission electron microscopy (TEM) images were obtained in a JEOL 1400 (Jeol Ltd,
175 USA) operating at 80 kV. All nanocapsules suspensions were diluted to a final
176 concentration of 1 mg/mL polymer in ultrapure water and a 5 μ L droplet was deposited on a
177 glow discharged carbon-coated 200 mesh grid. Negative staining was performed using 2%
178 phosphotungstic acid (w/v). All images were acquired with an Orius camera (Gatan Inc,
179 USA).

180 Cryo-Transmission Electron Microscopy (Cryo-TEM) images were performed using
181 a JEOL 1400 TEM/STEM operating at 120 kV. NC suspensions at 50 mg.mL⁻¹ polymer
182 were deposited (5 μ L) on a glow discharged carbon grid (Lacey 200 mesh), automatically
183 blotted for 5 s and plunged into liquid ethane at -175°C by a grid plunge freezer (Leica EM
184 GP). The samples were kept in liquid nitrogen until analysis. Next, they were transferred to
185 a pre-cooled cryo-TEM holder (Gatan 914) and observed under 10 pA/cm² of luminosity, 2
186 s of exposition and -2 μ m of defocus. All images were acquired with a US1000XP camera
187 (Gatan Inc, USA).

188 **2.8 PFH encapsulation efficiency**

189 For PFH quantification, 500 μ L of NC suspension freshly prepared was transferred
190 to NMR tubes loaded with a stem coaxial insert containing TFA in D₂O (12 μ mol.mL⁻¹) as
191 external standard [32, 35]. Freeze-dried samples were also analyzed: 1 mL of freshly

192 prepared nanocapsule suspension was freeze-dried during 24h employing an Alpha-1-2 LD
193 apparatus (Christ, France). Afterwards, they were hermetically sealed with a rubber cap
194 and stored at -20°C until analysis. A total volume of 1 mL of chloroform then was introduced
195 into the sealed tube by a needle, the suspension was vortexed (5 cycles of 30 seconds)
196 and centrifuged at 0°C for 10 minutes at 1000 RPM. The organic solution was collected in a
197 cold room at 4°C and introduced into an NMR tube loaded with the stem coaxial insert
198 containing TFA in D₂O (12 μmol.mL⁻¹). All spectra were obtained by a Bruker Avance 300
199 (400 MHz) operating at 4°C. The total amount of encapsulated PFH was determined based
200 on a PFH calibration curve. For each NC sample, the integration of the TFA peak at -76.5
201 ppm was determined as 3 and the resulting integration of the PFH CF₃ at -81.2 ppm was
202 used to calculate the PFH concentration in the NMR tube based to the equation of the
203 calibration curve.

204 **2.9 Cell viability**

205 The cytotoxicity of all NCs was evaluated in two distinct cell lines obtained from ATCC
206 (USA). Human endothelial umbilical vein cells (HUVEC) were cultivated in DMEM
207 supplemented with 50 U.mL⁻¹ of penicillin, 50 U.mL⁻¹ of streptomycin and 10% FBS. Murine
208 macrophage-monocytes (J774.A1 cell line) were cultivated in RPMI 1640 medium
209 supplemented with 50 U.mL⁻¹ of penicillin, 50 U.mL⁻¹ of streptomycin and 10% heat
210 inactivated FBS. All cells were cultivated at 37°C at 5% CO₂ in a humidified atmosphere;
211 they were splitted twice a week by trypsinisation for HUVEC and scraping for J774.A1. To
212 assess the *in vitro* cytotoxicity, the MTT assay that evaluates the mitochondrial activity was
213 employed [36]. A total volume of 100 μL of the cells suspension was seeded into 96 well
214 plates (TPP, Switzerland) at a concentration of 8x10³ cells/well or 2x10³ cells/well for 24
215 and 72h of incubation, respectively. The cells were pre-incubated for 24h and 100 μL of a
216 serial dilution of NCs (1.0x10⁻² to 10 mg.mL⁻¹) was added to the medium. After 24 or 72h of
217 incubation, 20 μL of MTT solution at 5mg/mL(3-(4,5-dimethylthiazol-2-yl)-2,5-
218 diphenyltetrazolium bromide) (Sigma, France) was added to each well. After 1h of
219 incubation, the medium was then aspirated and replaced by 200 μL of mL dimethylsulfoxide
220 (ACS grade, BioBasic Inc, France). The absorbance [A] was measured with a microplate
221 reader (LAB Systems Original Multiscan MS, Finland) at 570 nm and the cell viability was
222 calculated according to Eq. 2, where [A]_{control} corresponds to the absorbance of untreated
223 control cells:

$$224 \quad \frac{[A]_{test}}{[A]_{control}} \times 100 \text{ Eq. 2}$$

225

226

227 **2.10 *In vitro* Ultrasound Characterization**

228 Ultrasound images were acquired in a degassed water bath maintained at 37°C
229 equipped with acoustic absorbing rubber. All NC were previously degassed and 0.8 mL
230 were transferred to a silicone tube to a final 50 mg.mL⁻¹ suspension. Suspensions of
231 Sonovue[®] 0.1%, PVA 0.7% and nanoparticles (NP) produced with PLA-C₈F₁₇ (without PFH)
232 were used as controls. *In vitro* ultrasound imaging experiments were performed using an
233 ultrafast programmable ultrasound scanner (Aixplorer, Supersonic Imagine, Aix-En-
234 Provence, France). The scanner was equipped with a 5 MHz linear array transducer (128
235 elements, 0.2 mm pitch) able to emit and accumulate a series of plane waves. The US
236 focus was placed at the center of the silicone tube relative to the transducer at 3 cm depth.
237 The pulse repetition frequencies used in this experiment was 50 Hz. The images were
238 obtained using a plane-wave B-mode sequence [37] at 0.1 MPa peak negative pressure.
239 Conventional focused B-mode (0.1 MPa) and contrast specific mode (~0.2 MPa, executed
240 by pulse inversion and amplitude modulation) images were initially performed for each
241 sample independently. Imaging and monitoring pulses were 1 cycle long at 5 MHz. Finally, a
242 disruption pulse was emitted (~5 MPa peak negative pressure, 100 cycles long, which
243 corresponds to 20 μs) and the imaging process was repeated, with 65 B-mode images for
244 the total 140 ms pulse sequence. Plane-wave images were acquired at a frame rate of 100
245 Hz and all data was transferred to a computer for analysis at Matlab (Mathworks, Natick,
246 MA, USA). The backscattered intensity of the samples was obtained by averaging the
247 power of the first and last image of the video in the region-of-interest (the silicone tube).
248 The resulting intensities were subtracted by the backscattered intensity obtained with the
249 tube filled with Milli-Q water.

250 Insert Figure 1 here

251 **2.11 *Focused Ultrasound***

252 Focused ultrasound experiments were performed to assess the ability to destroy NCs.
253 100 μL of 5 mg.mL⁻¹ NC suspension were transferred to a 96 well microplate (OptiCell[®])
254 that was placed in a support positioned at the center of the chamber. A 2.25 MHz
255 transducer (Imasonics, Voray-sur-l'Ognon, France) was focused (38 mm f/d=1) separately at
256 each microplate well and single bursts of 5 cycles were transmitted by a waveform
257 generator and amplified to 12.3 MPa peak-negative pressure by a radio frequency
258 amplifier[26].

259

260 **2.12 Statistical analysis**

261 The statistical analysis was performed using Statistica™ 8.0 software (StatSoft Inc., Tulsa,
262 USA). The data was analyzed by two-sided Student's *t*-test and a statistical significance
263 was set at the level of $p < 0.05$.

264 3. Results and discussion

265 3.1 Synthesis and characterization of fluorinated polymers

266 Polylactide polymers functionalized with either alkyl or fluorinated end-groups of five
267 distinct lengths were synthesized by ring opening polymerization of D,L-lactide as described
268 previously [32]. The end-groups are composed of linear carbon chains substituted by
269 fluorine or hydrogen atoms; henceforth, the synthesized polymers were designated
270 according to the terminal group chemistry and length of fluorinated unit as PLA-C₆H₁₃, PLA-
271 C₃F₇, PLA-C₆F₁₃, PLA-C₈F₁₇, PLA-C₁₁F₂₃ and PLA-C₁₃F₂₇.

272 All synthesis products were initially characterized by the presence of the lactide
273 signals at 1.60 and 5.23 ppm and the new ester linkage performed with the fluorinated or
274 alkylated alcohol initiators by ¹H-NMR (**Figure 2A**). As previously observed by Böhmer *et*
275 *al.*, all the fluorinated polymers exhibited a significant shift of the –CH₂O– signal compared
276 with the correspondent initiator from 4.1 ppm to 4.55 and 4.74 ppm (Supplementary
277 information, Figures S1 and S2), indicating that the chemical environment of those groups
278 were severely influenced by the PLA backbone as well as by the adjacent CF₂ units [38].
279 Accordingly, the –CH₂O– intensity was splitted into two signals with multiplicity of two
280 triplets due to the strong coupling with both fluorine atoms and the geminal proton in the
281 same order of magnitude (~13 Hz), which confirmed the synthesis of fluorinated PLA
282 polymers. Also, the fluorinated derivatives exhibited a shift of the –CF₂CH₂– signals while
283 other CF₂ and CF₃ (-78.9 to -79.0 ppm) resonances were similar to each specific precursor
284 as displayed in the ¹⁹F spectra (**Figure 2B**). The formation of the non-fluorinated derivative,
285 PLA-C₆H₁₃, was verified by the –CH₂O– signal shift from 3.60 to 4.14 ppm of the initiator.

286 As shown in **Table 1**, a relative good agreement between the experimental molar
287 masses determined by SEC and the molar masses targeted at 20 000 g.mol⁻¹ for all the
288 derivatives was observed (Supplementary information Figure S3). The *M_n* was also
289 determined by ¹H-NMR analysis on the basis of the integration ratio of peak at 5.23 ppm
290 (methine proton of lactide) and 4.55 - 4.74 ppm (methylene protons adjacent to the CF
291 chain) (**Figure 2A**). Slightly lower *M_n* were calculated than using SEC, around 18 000 g.mol⁻¹,
292 which may be attributed to the difference of polymer conformation with poly(methyl
293 methacrylate) standards.

294 The end-modified polymers were also characterized by DSC. They all displayed the
295 same glass-transition temperature (*T_g*), from 50 to 53°C, similar to unmodified PLA
296 polymers of similar molar mass (**Table 1**)[34, 39]. Hence, the small molecular weight end-
297 group did not impact the *T_g* of high molar mass polylactide.

298 Insert Figure 2 here

299 Insert Table 1 here

300 **3.2 PFH Nanocapsules**

301 Fully fluorinated liquid perfluorocarbons usually display high vapor pressure values –
302 29.1 kPa at 25°C for PFH – and restricted solubility in organic solvents due to their
303 fluorophilic character [40]. As a result, they tend either to phase separate or to evaporate
304 during the formulation of nano or microcapsules by solvent emulsion-evaporation, reducing
305 the PFC encapsulation efficacy [27, 28]. Therefore, fluorinated end-groups with distinct
306 lengths were introduced into the PLA structure to promote “fluorous – fluorous” interactions
307 between the polymer and liquid perfluorocarbons and enhance PFH encapsulation into
308 nanocapsules. This strategy was shown efficient recently for a high-boiling point PFC:
309 perfluorooctyl bromide [32]. The features of NCs formulated with fluorinated polymers, such
310 as morphology, size, surface charge and PFH encapsulation efficiency were compared with
311 samples produced with the non-fluorinated polymer PLA-C₆H₁₃.

312 Initial characterization demonstrated that all NC were spherical with smooth surfaces as
313 shown by TEM and SEM images (**Figure 3**). The Z-average size was around 150 nm – in
314 agreement with electron microscopy observations – with a negative ζ -potential around -8
315 mV and a low polydispersity below 0.15, regardless of end-group chemistry or fluorinated
316 length as displayed in **Table 2**.

317 Insert Table 2 here

318 Insert Figure 3 here

319 Encapsulated PFH was effectively visualized by Cryo-TEM images, revealing spherical
320 core-shell morphologies constituted by thin polymeric layers surrounding the electron-dense
321 perfluorocarbon as shown in **Figure 4** for PLA-C₆H₁₃, PLA-C₃F₇, PLA-C₈F₁₇ and PLA-
322 C₁₃F₂₇. All samples displayed capsules containing distinct amounts of PFH, exhibiting dark
323 cores with a wide intensity range and also presented apparently unloaded polymeric
324 nanoparticles, an indication of inhomogeneous encapsulation. From these images, no
325 morphological distinction among polymer end-group chemistry or F-length was observed.

326 Some capsules also presented a predominant lighter core or bright spots within the
327 perfluorocarbon phase (white arrows in **Figure 4**). Such areas might arise from the freezing
328 process: as PFH retracts when it solidifies, low PFH densities regions appear in the
329 capsules with probable coexistence of solid PFH (dark) with gaseous PFH (light). After

330 exposure to the electron beam radiation, partial melting of the solid PFH occurs and light
331 regions becomes darker (Supplementary Information, Figure S4). Those observations are
332 consistent with Lattin *et al.* that reported the increased frequency of bright nanoemulsion
333 droplets for the lower boiling point perfluoropentane (Bp=29°C) in comparison with
334 perfluorohexane (Bp=56°C) and perfluoroheptane (Bp=82°C) [41] and from our own
335 observations with PFOB [32, 42].

336 Insert Figure 4 here

337 The total encapsulated PFH was then quantified by ¹⁹F-NMR for NC suspensions
338 either freshly prepared or after freeze-drying. The samples prepared with the non-
339 fluorinated polymer (PLA-C₆H₁₃) exhibited an encapsulation efficiency of 9.2±0.1% for
340 freshly prepared suspensions and 4.0±0.5% after freeze drying, in agreement with results
341 obtained for nanocapsules formulated with regular PLGA polymers [30]. All NC formulated
342 with F-polymers showed a 2-fold increase in the total PFH encapsulation compared to PLA-
343 C₆H₁₃ as exhibited in **Figure 5**. A maximum encapsulation efficiency of 15±1.0% for
344 suspensions and a 9.5±0.8% plateau for freeze-dried samples was reached, although
345 without significant difference as a function of fluorinated chain length. Obviously, the freeze-
346 drying process induced the loss of non-encapsulated PFH droplets, as previously observed
347 for PFOB NCs [42], although no free PFH could be observed by cryo-TEM. These results
348 suggest a preferential interaction between fluorinated polymers and PFH, independently of
349 the fluorinated end-chain length, reducing the PFH loss during solvent evaporation and
350 increasing the final encapsulation efficiency. Nonetheless, the PFH solubility and volatility
351 still prevents higher encapsulation. Comparatively, mPEG-polycaprolactone polymers
352 containing terminal C₃F₇ groups were also successful in enhancing of PFH entrapment into
353 NCs around ~15% as reported by Li *et al.* [31].

354 Insert Figure 5 here

355 **3.3 *In vitro* cytotoxicity of NCs**

356 The use of fluorinated-compounds for biomedical applications is usually limited
357 because of their persistence and consequent toxicity in the organism [43]. Indeed, the
358 stable C-F bond hinders an effective enzymatic or metabolic degradation that results in low
359 excretion profiles, especially for linear fluorinated compounds longer than C₇F₁₅ [44, 45]. To
360 assess the *in vitro* impact of fluorinated end-groups with distinct lengths on cellular viability,
361 NCs formulated with fluorinated polymers were incubated with two distinct cell lines, human
362 umbilical vein endothelial (HUVEC) and murine macrophage-like (J774.A1) during 24h or
363 72h. The MTT assays showed that NCs did not inhibit HUVEC growth after 24h or 72h,

364 independently of NC concentration, end-group chemistry or fluorinated end-group lengths
365 (**Figure 6**). For J774.A1, an inhibition down to 75% was observed for 10 mg/mL NCs after
366 24h of exposure. After 72h exposure, cell viability decreased down to 75 and 50% of
367 controls at NC concentrations of 4 and 10 mg/mL, respectively (**Figure 6**). For both cell
368 lines, no specific toxicity was induced due to the presence of the fluorinated groups. One
369 should note that the reduction of cell viability of J774.A1 cells was only observed for very
370 high NC concentrations. In addition, the distinct profiles for the two cell lines arise from the
371 ability of J774.A1 to internalize faster and to a higher extent NCs than HUVEC [46].
372 Although only a limited *in vitro* cytotoxicity was observed, additional *in vivo* studies are
373 required to investigate the effects resulting from the prolonged exposure to NCs.

374 Insert Figure 6 here

375 **3.4 *In vitro* Ultrasound imaging**

376 The imaging ability of NCs was evaluated *in vitro* using a plane-wave B-mode
377 imaging system based on compounded plane-wave ultrasound transmissions[47]. All
378 images were obtained with fixed 50 mg.mL⁻¹ concentrations of NC at 37°C and compared to
379 the background gray-scale level produced by ultrapure water. At this temperature, the PFH
380 vapor pressure increases to 48.6 kPa – against 29.1 kPa at 25°C – and potentially favors a
381 gas-phase nucleation. Ultrasound images obtained in the conventional focused B-mode
382 displayed a significant contrast enhancement ($p < 0.001$) arising from NC formulated with
383 fluorinated polymers (from 1.5×10^4 until 5.5×10^4 arbitrary units, a.u.) compared to those
384 produced with PLA-C₆H₁₃ (0.5×10^4 a.u.) (**Figure 7**). Moreover, significant differences were
385 observed between fluorinated samples ($p < 0.001$). Additionally, the fluorinated NCs
386 generated contrast specific echoes, enhancing the backscattering intensities from 10-fold to
387 40-fold higher than PLA-C₆H₁₃. Again, significant differences were observed between
388 fluorinated samples except for PLA-C₁₁F₂₃ and PLA-C₁₃F₂₇ which are not statistically
389 different. For both ultrasound-imaging modalities, all samples exhibited long-lasting
390 echogenicity for more than 10 minutes. As expected, Sonovue[®] greatly increased the
391 ultrasound contrast compared to all NCs (from 12-fold to 35-fold) in the conventional
392 focused B-mode and contrast specific mode due to its micrometer size and its gaseous
393 core. In absence of PFH, the PLA-C₈F₁₇ nanoparticles (NP in Figure 7) and PVA solution
394 did not yield any significant signal enhancement.

395 Insert Figure 7 here

396 The acoustic enhancement observed for samples formulated with fluorinated
397 polymers indicates that the total amount of PFH is a key parameter required to modulate

398 the echogenicity of NCs, given these possess similar sizes. Interestingly, polymers
399 containing longer F-units, such as PLA-C₁₁F₂₃ and PLA-C₁₃F₂₇, exhibited higher
400 backscattering compared to shorter or non-fluorinated NCs. Given NC sizes are similar,
401 such variation of echogenicity with F-length might be related to an increase of NC density
402 that further affects the scattering cross section of the NCs as described in the introduction
403 [9]. The fluorinated initiator density increases from 1.6 g.cm⁻³ to 1.73 g.cm⁻³ for C₃F₇ to
404 C₁₃F₂₇ (as provided by the supplier) whereas the density of PLA is close to the one of PLGA
405 (1.28 g.cm⁻³)[48]. In addition, as we expect fluorinated moieties to position at the
406 PFH/polymer interface, they might provide nuclei for PFH cavitation/bubble nucleation [49].
407 The presence of these bubbles could also explain the harmonic response of fluorinated
408 NCs as no contrast specific signal can be observed for non-fluorinated ones (obtained with
409 PLA-C₆H₁₃) or with nanoparticles (NP, Figure 7).

410 At higher acoustic pressures (peak negative pressure ~5 MPa), a signal
411 enhancement is observed after 20 ms for all NCs formulated with fluorinated polymers,
412 most probably indicating that the shell was disrupted from a certain population of capsules
413 (as illustrated for PLA-C₈F₁₇ in **Figure 8**). For these samples, the PFH is possibly
414 completely vaporized causing the polymeric shell to shatter and allow the gas-phase to
415 freely vibrate, which momentarily increases the echo. This transitory higher echogenicity
416 then decreases back to the initial echogenicity as the gas bubble is dissolved or destroyed
417 in a similar profile as observed for microbubbles [47].

418 Insert Figure 8 here

419 **3.5 PFH vaporization by focused ultrasound**

420 PFH-loaded NCs present an interesting potential to release chemotherapeutics
421 inside solid tumors after local accumulation driven by the EPR effect, provided they are
422 PEGylated and can long circulate in the blood stream. Their great advantage resides on the
423 acoustic-sensitivity of the low boiling point PFH that can be remotely induced to vaporize by
424 focused ultrasound (FUS) to trigger the release of co-encapsulated drug locally. To
425 evaluate the ability of PFH to undergo acoustic drop vaporization under FUS, NCs were
426 exposed to FUS acoustic pulses and analyzed morphologically by cryo-TEM.

427 All samples displayed a predominant population of spherical and intact NCs with
428 core-shell morphology (**Figure 9 top**). However, a distinct population of capsules that
429 exhibited deformed structures according to polymer end-chemistry were also visualized:
430 samples produced with PLA-C₆H₁₃ exhibited some buckled NCs, suggesting that a partial
431 PFH vaporization occurred and produced only a localized distortion of the polymeric shell.

432 For NCs prepared with fluorinated polymers – PLA-C₃F₇, PLA-C₈F₁₇ and PLA-C₁₃F₂₇ – more
433 intense shell deformations led to a complete morphological change that culminated, in
434 many cases, in capsule disruption and observation of debris, some of them at the
435 micrometric size range (white arrows) (**Figure 9 bottom**). It is important to emphasize that
436 the focus of the ultrasonic beam is small (less than 1 μ L or 1mm³) compared to the total
437 volume of the samples, a factor that contributed to reduce the number of viewable debris
438 during cryo-TEM. The presence of PFH inside several capsules indicates that longer FUS
439 exposition time intervals are still required for homogeneous NC disruption.

440 Insert Figure 9 here

441 Comparatively, corresponding samples were submitted to heating at 60°C for 10
442 minutes in an oil bath – above the PFH boiling point (56°C). The results showed no
443 morphological discrepancy or size variation compared to the control group (Supplementary
444 information, **Figures S5 and S6**), thus suggesting that the ultrasound mechanical effects
445 are required in addition to the thermal ones to promote a more effective vaporization as
446 previously reported [50]. Additionally, as the temperature in focal area of FUS might
447 become higher than 65°C within 1s [25], the polymer shell might be above its T_g of 52°C
448 (**Table 2**) – therefore making polymer shell deformation easier.

449

450 **4. Conclusion**

451 We have successfully synthesized polylactide polymers containing fluorinated end-groups
452 of different lengths, from C₃F₇ until C₁₃F₂₇ that were formulated into nanocapsules
453 containing a perfluorohexane core. The employment of fluorinated polymers increased by
454 2-fold the encapsulation efficiency of perfluorohexane into nanocapsules, although no effect
455 of fluorine chain length was observed. Fluorination of the polymers did not induce any
456 specific *in vitro* cytotoxicity towards HUVEC and J774.A1 cell lines. Nanocapsules
457 formulated with fluorinated polymers enhanced their acoustic response compared to PLA-
458 C₆H₁₃ in both fundamental and harmonic ultrasound imaging modalities. An increased
459 echogenicity and harmonic response was observed as the fluorinated chain-length
460 increased, probably due to an increase of density and promotion of bubble nucleation.
461 When submitted to focused ultrasound, both intact and exploded nanocapsules could be
462 observed, also with end-group dependency, indicating that the perfluorocarbon was partly
463 vaporized. These results pave the way to theranostic perfluorohexane nanocapsules co-
464 encapsulating a drug for precision delivery using focused ultrasound.

465

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478

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610

611

612 Tables and Figures captions

613 Table 1. Number-average molar masses (M_n) obtained by SEC/ $^1\text{H-NMR}$, dispersity and glass-
614 transition temperature (T_g) of all synthesized polymers.

615

616 Table 2. Z-average size, polydispersity index and ζ -potential for nanocapsules formulated with
617 fluorinated and non-fluorinated polymers. Data are presented as mean \pm SD (n=3).

618

619 Figure 1: Schematic representation (A) and real view (B) of the US set-up.

620

621 Figure 2. $^1\text{H-NMR}$ spectra of all synthesized polymers with enlargement of the corresponding
622 lactide and ester linkage signals (A). Magnified CF_3 and CF_2 regions in the ^{19}F spectra for PLA-C3F7,
623 PLA-C6F13, PLA-C8F17, PLA-C11F23 and PLA-C13F27 (B). The ^1H and ^{19}F peak assignments are
624 indicated in the insert. All samples were dissolved in CDCl_3 .

625

626 Figure 3. Transmission (left) and scanning electron microscopy images (right) for nanocapsules
627 formulated with fluorinated and non-fluorinated polymers.

628

629 Figure 4. Cryo-TEM images of nanocapsules formulated with fluorinated PLA-C3F7, PLA-C8F17 and
630 PLA-C13F27 as well as non-fluorinated PLA-C6H13 polymers. The white arrows show the bright
631 regions within the nanocapsules.

632

633 Figure 5. PFH encapsulation efficiency for NCs freshly prepared suspension (red bars) or submitted
634 to freeze-drying (blue bars).

635

636 Figure 6. Cell viability as a function of NC concentration for HUVEC (top) and J774.A1 (bottom) at
637 24h (left) and 72h (right). All results are presented as mean \pm SD (N=2).

638

639 Figure 7. Ultrasound backscattered intensity at the conventional focused B-mode (black
640 symbols) and contrast specific (red symbols) modalities for PVA, nanoparticles of PLA-
641 C_8F_{17} (NP) and all formulated nanocapsules. The error bars represent the ultrasound signal
642 variability between the echo-pulses for each sample (about 5% error). Differences between
643 NC formulated with fluorinated polymers and those produced with PLA- C_6H_{13} were
644 statistically significant in both modes. Moreover, significant differences were observed

645 between fluorinated samples in conventional mode ($p < 0.001$). In contrast specific imaging
646 Again, significant differences were observed between fluorinated samples except for PLA-
647 C₁₁F₂₃ and PLA-C₁₃F₂₇ which are not statistically different.

648

649

650 Figure 8. Sequential ultrasonic images of PLA-C8F17 nanocapsules prior (-200 ms) and after
651 exposure to FUS (from 20 to 1000 ms). The average amplitude signal is presented for each image
652 (bottom). The scale bar corresponds to 1 mm.

653

654 Figure 9. Cryo-TEM images of nanocapsules after focused ultrasound exposure. Intact/spherical
655 (top) and buckled or debris of nanocapsules (bottom) were observed for all samples. All scale bars
656 represent 100 nm.

657