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## Comb-Like Fluorophilic-Lipophilic-Hydrophilic Polymers for Nanocapsules as Ultrasound Contrast Agents

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1 Comb-like fluorophilic-lipophilic-hydrophilic  
2 polymers for nanocapsules as ultrasound contrast  
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16  
17 **Keywords**

18 Comb-like fluorinated polymers; fluorous interactions; nanocapsules; perfluorocarbons;  
19 ultrasound contrast agents

20

21 **Abstract**

22 Imaging the enhanced permeation and retention effect by ultrasound is hindered by the large  
23 size of commercial ultrasound contrast agents (UCAs). To obtain nanosized UCAs, triblock  
24 copolymers of poly(ethylene glycol)-polylactide-poly(1H,1H,2H,2H-heptadecafluorodecyl  
25 methacrylate) (PEG-PLA-PFMA) with distinct numbers of perfluorinated pendant chains (5,  
26 10 or 20) are synthesized by a combination of ring-opening polymerization and atom transfer  
27 radical polymerization. Nanocapsules (NCs) containing perfluorooctyl bromide (PFOB)  
28 intended as UCAs are obtained with a 2-fold increase in PFOB encapsulation efficiency in  
29 fluorinated NCs as compared to plain PEG-PLA NCs thanks to fluororous interactions. NC  
30 morphology is strongly influenced by the number of perfluorinated chains and the amount of  
31 polymer used for formulation, leading to peculiar capsules with several PFOB cores at high  
32 PEG-PLA-PFMA<sub>20</sub> amount and single-cored NCs with a thinner shell at low fluorinated  
33 polymer amount, as confirmed by small angle neutron scattering. Finally, fluorinated NCs  
34 yield higher in vitro ultrasound signal compared to PEG-PLA NCs and no in vitro  
35 cytotoxicity is induced by fluorinated polymers and their degradation products. Our results  
36 highlight the benefit of adding comb-like fluorinated blocks in PEG-PLA polymers to modify  
37 the nanostructure and enhance the echogenicity of nanocapsules intended as UCAs.

38

39

## 40 1. Introduction

41 Ultrasound is one of the most widely used clinical imaging modality owing to its low cost,  
42 safety, portability, and possible combination with therapy.<sup>1</sup> Nevertheless, this technique has  
43 been limited by the lack of effective ultrasound contrast agents (UCAs) to allow tissue  
44 differentiation.<sup>2,3</sup> Commercially available UCAs consist of 1 – 6  $\mu\text{m}$  gas microbubbles  
45 stabilized by a layer of lipids or proteins.<sup>4,5</sup> Their large size and high compressibility make  
46 them ideal ultrasound reflectors, currently used to enhance the blood pool signal and assess  
47 tissue blood flow at the microvascular level.<sup>6</sup> However, the fast diffusion of the encapsulated  
48 gas results in short circulation time in the bloodstream (a few minutes),<sup>7</sup> and their large  
49 micrometer size prevents their accumulation in solid tumors. For tumor imaging, more stable  
50 nano-sized UCAs able to extravasate into tumors by the enhanced permeation and retention  
51 (EPR) effect<sup>8</sup> are required. Since gas nanobubbles are difficult to stabilize,<sup>9</sup> research has  
52 focused on the encapsulation of liquid perfluorocarbons.<sup>10</sup> To further improve UCAs stability,  
53 several groups have reported the use of polymeric shells which are more stable than lipid or  
54 protein layers.<sup>11–14</sup>

55 In this context, polymeric UCAs have been developed recently in our group. These polymeric  
56 nanocapsules (NCs) of poly(lactide-co-glycolide) (PLGA) encapsulating liquid perfluorooctyl  
57 bromide (PFOB) showed long-lasting *in vitro* echogenicity and allowed *in vivo* blood pool  
58 imaging.<sup>15</sup> However, quick elimination by the mononuclear phagocyte system hampered their  
59 accumulation in the tumor. PLGA was then replaced by poly(lactide-co-glycolide)-  
60 poly(ethylene glycol) (PLGA-PEG) copolymer to formulate long-circulating NCs which  
61 accumulated in the tumor by the EPR effect, and yielded contrast enhancement by <sup>19</sup>F  
62 Magnetic Resonance Imaging.<sup>16</sup> Nevertheless, the acoustic response of these capsules  
63 remained too weak at clinical frequencies, owing mainly to the important thickness of their  
64 shells. The strategy of decreasing the polymer amount in the formulation, which previously

65 allowed reducing the thickness of plain PLGA NCs,<sup>17</sup> failed using PLGA-PEG due to local  
66 dewetting.<sup>18</sup>

67 Our aim is therefore to improve the nanocapsule echogenicity by reducing their shell  
68 thickness, while keeping their long-circulating properties with PEG chains. Recently, we  
69 reported that functionalizing PLA with a short linear perfluorinated end group induced an  
70 increase of PFOB encapsulation efficiency into PEGylated NCs made of a mixture of PLA-  
71 PEG and fluorinated-PLA, for perfluorinated chain length up to C<sub>8</sub>F<sub>17</sub>.<sup>19</sup> However, the shell  
72 thickness was not reduced, and although a higher *in vitro* echogenicity was obtained due to  
73 fluorination, there is still room for improvement since the ultrasound signal was much lower  
74 than commercial Sonovue microbubbles.

75 Plenty of reports on different triblock copolymers with hydrophilic, hydrophobic, and  
76 fluorinated blocks exist and nanoparticles formed using these polymers exhibit different  
77 morphologies.<sup>20-24</sup> Here, the strategy consists in synthesizing triblock hydrophilic-  
78 hydrophobic-fluorophilic copolymers of poly(ethylene glycol)-polylactide-  
79 poly(1H,1H,2H,2H-heptadecafluorodecyl methacrylate) (PEG-PLA-PFMA), containing a  
80 higher proportion of C<sub>8</sub>F<sub>17</sub> chains, to formulate nanocapsules. We intend to favor fluorous  
81 interactions between the fluorinated chains and PFOB to improve the wetting of the polymer  
82 around the PFOB core and yield capsules with thin shells as we decrease the amount of  
83 polymer in the formulation. We present here the polymer synthesis and the characterization of  
84 nanocapsules in terms of size distribution, morphology, PFOB encapsulation efficiency, shell  
85 thickness as measured by Small Angle Neutron Scattering, cytotoxicity and *in vitro*  
86 ultrasound scattering.

87

## 88 2. Materials and methods

### 89 2.1. Materials

90 DL-lactide was purchased from Biovalley, Polysciences Inc. (USA). Poly(ethylene glycol)  
91 methyl ether (OH-PEG-OCH<sub>3</sub>, average  $M_n = 5\ 000$  g/mol), stannous 2-ethyl hexanoate  
92 (stannous octoate, Sn(Oct)<sub>2</sub>), N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA),  
93 triethylamine (Et<sub>3</sub>N), dried toluene, cyclohexanone, magnesium sulfate (MgSO<sub>4</sub>), sodium  
94 cholate, D<sub>2</sub>O and trifluoroacetic acid (TFA) were provided by Sigma-Aldrich (France).  
95 1H,1H,2H,2H-heptadecafluorodecyl methacrylate (FMA) and perfluorooctyl bromide (PFOB)  
96 were purchased from Fluorochem (UK). 2-Bromo-2-methylpropionyl bromide and copper(I)  
97 bromide (CuBr) were provided by ACROS Organics (Belgium). Deuterated chloroform  
98 (CDCl<sub>3</sub>) was obtained from Euriso-top (France). All solvents were purchased from Carlo Erba  
99 (France). Cell culture reagents such as DMEM (Dulbecco's modified Eagle's medium), RPMI  
100 1640 (Roswell Park Memorial Institute medium), FBS (Fetal Bovine Serum), trypsin-EDTA  
101 solution and PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free phosphate buffer) were purchased from Sigma Aldrich  
102 (France). Water was purified using a RIOS/Synergy system from Millipore (France). NMR  
103 sample tubes and coaxial inserts were obtained from CortecNet (France).

### 104 2.2. Polymer synthesis

105 The synthesis route involved 3 steps as shown in Figure 1.

#### 106 2.2.1. Synthesis of PEG-PLA

107 PEG-PLA was synthesized by ring-opening polymerization (ROP) of DL-lactide initiated by  
108 OH-PEG-OCH<sub>3</sub> in the presence of stannous octoate catalyst.<sup>25</sup> All glassware was dried by  
109 heating under vacuum and handled under argon atmosphere. To a 250 mL round-bottom flask  
110 equipped with a magnetic stir-bar were added DL-lactide (156.14 mmol, 22.50 g) and OH-  
111 PEG-OCH<sub>3</sub> (1.13 mmol, 5.66 g). The flask was sealed with a rubber cap and a stannous

112 octoate solution (0.75 mmol, 306 mg) dissolved in 30 mL of dried toluene was added through  
113 the septum. The tube was purged with argon for 15 minutes and then placed into a 130°C oil  
114 bath. Polymerization was carried out for 1h under magnetic stirring, and then quenched by  
115 immersing the tube in a cold water bath. After evaporation of toluene, the reaction product  
116 was dissolved in chloroform and precipitated twice into excess cold diethyl ether. The  
117 polymer was finally dried under vacuum and 25.13 g of a white powder was obtained. Lactide  
118 conversion = 94% (<sup>1</sup>H NMR). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, δ, ppm): 5.10-5.28 (CHCH<sub>3</sub>COO  
119 of PLA), 3.64 (OCH<sub>2</sub>CH<sub>2</sub> of PEG), 3.38 (OCH<sub>3</sub> of PEG), 2.70 (end HOCHCH<sub>3</sub>COO of PLA),  
120 1.45-1.67 (CHCH<sub>3</sub>COO of PLA).  $M_n^{\text{NMR}} = 23,500$  g/mol;  $M_n^{\text{SEC}} = 24,300$  g/mol.

#### 121 2.2.2. Synthesis of PEG-PLA-Br macroinitiator

122 PEG-PLA-Br was synthesized by esterification of PEG-PLA with excess 2-Bromo-2-  
123 methylpropionyl bromide using a known procedure.<sup>26,27</sup> In a 250 mL round-bottom flask  
124 equipped with a magnetic stir-bar, PEG-PLA (1.06 mmol, 25 g) was dissolved in 160 mL of  
125 CH<sub>2</sub>Cl<sub>2</sub>. Excess of triethylamine (37.23 mmol, 5.2 mL) was added to the solution, and the  
126 mixture was stirred under argon and cooled to 0°C with an ice bath. 2-Bromo-2-  
127 methylpropionyl bromide (37.23 mmol, 4.6 mL) was added dropwise for 15 min. The mixture  
128 was allowed to stir at room temperature overnight, and was subsequently washed twice with  
129 saturated brine and once with water. The organic phase was dried over anhydrous MgSO<sub>4</sub>  
130 before being concentrated under reduced pressure and precipitated twice in excess cold  
131 diethyl ether. The product was finally dried under vacuum to provide 16.55 g of an off-white  
132 solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, δ, ppm): 5.10-5.28 (CHCH<sub>3</sub>COO of PLA), 3.64  
133 (OCH<sub>2</sub>CH<sub>2</sub> of PEG), 3.38 (OCH<sub>3</sub> of PEG), 1.95 and 1.98 (C(Br)(CH<sub>3</sub>)<sub>2</sub>), 1.45-1.67  
134 (CHCH<sub>3</sub>COO of PLA).  $M_n^{\text{NMR}} = 23,500$  g/mol;  $M_n^{\text{SEC}} = 25,200$  g/mol.

135 2.2.3. Synthesis of PEG-PLA-PFMA<sub>x</sub> polymers

136 PEG-PLA-PFMA<sub>x</sub> polymers were synthesized by ATRP of 1H,1H,2H,2H-  
137 heptadecafluorodecyl methacrylate (FMA) initiated by PEG-PLA-Br with CuBr/PMDETA as  
138 the catalyst system.<sup>28</sup> All glassware was dried by heating under vacuum and handled under  
139 argon atmosphere. To a 100 mL round-bottom flask equipped with a magnetic stir-bar were  
140 added PEG-PLA-Br (0.21 mmol, 5 g) and a solution of PMDETA (0.21 mmol, 37 mg) and  
141 FMA (1.06 mmol for x = 5, 2.13 mmol for x = 10, 4.26 mmol for x = 20) in cyclohexanone  
142 (15 mL). After dissolution of the macroinitiator, CuBr (0.21 mmol, 31 mg) was added and the  
143 mixture was subjected to two freeze-pump-thaw cycles. The reaction proceeded at 100 °C for  
144 42h. The mixture was cooled and diluted with THF, and the copper catalyst was removed with  
145 a neutral alumina column. After concentration under reduced pressure, the product was  
146 precipitated twice in excess cold diethyl ether and dried under vacuum. FMA conversion (<sup>1</sup>H  
147 NMR) = 84% (x = 20), 80% (x = 10), 67% (x = 5). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, δ, ppm):  
148 5.10-5.28 (CHCH<sub>3</sub>COO of PLA), 4.28 (OCH<sub>2</sub>CH<sub>2</sub>CF<sub>2</sub> of PFMA), 3.64 (OCH<sub>2</sub>CH<sub>2</sub>O of PEG),  
149 3.38 (end OCH<sub>3</sub> of PEG), 2.47 (OCH<sub>2</sub>CH<sub>2</sub>CF<sub>2</sub> of PFMA), 1.70-2.00 (CH<sub>2</sub>C(CH<sub>3</sub>)COO of  
150 PFMA), 1.45-1.67 (CHCH<sub>3</sub>COO of PLA), 0.80-1.45 (CH<sub>2</sub>C(CH<sub>3</sub>)COO of PFMA). Molar  
151 masses are displayed in Table 1.

152 2.3. Polymer characterization

153 Size exclusion chromatography (SEC) was performed at 30 °C with two columns from  
154 Polymer Laboratories (PL-gel MIXED-D; 300 × 7.5 mm) and a differential refractive index  
155 detector (Spectrasystem RI-150, Thermo Electron Corp.), using chloroform as an eluent, a  
156 Waters 515 pump at a flow rate of 1 mL/min, and toluene as a flow-rate marker. The  
157 polymers were dissolved at 5 mg/mL in the eluent and filtered on 0.2 μm PTFE syringe filters  
158 prior to injection. The calibration curve was based on poly(methyl methacrylate) (PMMA)  
159 standards from Polymer Laboratories. <sup>1</sup>H NMR and <sup>19</sup>F NMR spectroscopies were performed



160 in 5 mm diameter tubes in CDCl<sub>3</sub> on a Bruker Avance-400 (400 MHz) spectrometer. The DP  
161 and  $M_n$  of the commercial OH-PEG-OCH<sub>3</sub> were confirmed by <sup>1</sup>H NMR in CDCl<sub>3</sub> using the  
162 ratio of the methyl signal at 3.38 ppm versus the main chain signal at 3.64 ppm: DP = 118,  $M_n$   
163 = 5.2x10<sup>3</sup> g/mol. Differential scanning calorimetry (DSC) was performed using a DSC Q1000  
164 (TA Instruments). The polymers (2-5 mg) were sealed in aluminum pans and heated from -20  
165 to 100°C at a heating rate of 20°C/min and cooled to -20 °C before a second heating scan  
166 from -20 to 100°C at 20°C/min to determine the glass transition temperature ( $T_g$ ). A nitrogen  
167 flow was maintained throughout the test (20 mL/min).

#### 168 2.4. Interfacial tension measurements

169 Interfacial tension measurements were carried out using the pendant drop method, employing  
170 a Tracker tensiometer (Teclis, France). Drops of PFOB were formed using a syringe and a  
171 G22 stainless steel needle into a methylene chloride solution containing the polymers at 25  
172 mg/mL placed in an optical glass cuvette. The interfacial tension was determined from the  
173 drop profile using the Laplace equation and the forces balance between capillarity and  
174 gravity. The measurements were performed on at least three independent drops and the  
175 experiment was repeated on different days to ensure reproducibility.

#### 176 2.5. Nanocapsules preparation

177 Nanocapsules (NCs) of PFOB were prepared by an emulsion-evaporation process as  
178 previously described.<sup>16,19</sup> The polymer (50, 30 or 20 mg) was dissolved into 2 mL of  
179 methylene chloride along with 30 µL of PFOB. The organic phase was emulsified into 10 mL  
180 of 1.5 % sodium cholate (w/v) aqueous solution using a vortex for 1 min and then a vibrating  
181 metallic tip (Digital Sonifier, Branson Ultrasons, France) at 30% of maximal power, for 1 min  
182 over ice. Solvent was allowed to evaporate by magnetic stirring at 300 rpm at room  
183 temperature for 3 h. Suspensions of NCs were filtered through 0.45 µm PVDF filters, and if

184 necessary, were purified to remove sodium cholate by ultracentrifugation for 1h, at 4 °C and  
185 at 27 440 g (Optima LE-80K Ultracentrifuge Beckman Coulter). The pellet was finally  
186 resuspended in water to the desired concentration.

## 187 2.6. Size distribution and Zeta potential

188 The hydrodynamic diameter ( $d_H$ ) and polydispersity index (PDI) of the nanocapsules were  
189 measured by quasi-elastic light scattering, using a Zetasizer Nano ZS instrument (Malvern,  
190 France). Suspensions were diluted in water to a concentration of 1 mg/mL. Measurements  
191 were performed at 20 °C, at an angle of 173° to avoid multiple scattering. Zeta potential  
192 measurements were carried out with the same instrument, at 25 °C, in 1 mM NaCl.  
193 Measurements were performed in triplicate.

## 194 2.7. PFOB encapsulation efficiency by $^{19}\text{F}$ NMR spectroscopy

195 PFOB encapsulation efficiency was determined by  $^{19}\text{F}$  NMR on a Bruker Avance-400 (400  
196 MHz) spectrometer with a 5 mm dual probe  $^{19}\text{F}/^1\text{H}$ . The NMR quantitative method using stem  
197 coaxial inserts was originally proposed by Henderson for  $^{31}\text{P}$  compounds<sup>29</sup> and adapted  
198 previously for  $^{19}\text{F}$  NMR.<sup>30,19</sup> 1 mL of unpurified nanocapsules were freeze-dried for 24h  
199 using an Alpha-1-2 LD apparatus (Christ, France) and lyophilisates were dissolved into 1 mL  
200 of chloroform. Sodium cholate was removed by centrifugation for 5 min at 4696 g. The  
201 organic solution was collected and introduced into a usual 5mm-NMR sample tube loaded  
202 with a stem coaxial insert containing TFA in  $\text{D}_2\text{O}$  as an external standard ( $[\text{TFA}] = 9.4$   
203  $\mu\text{mol}\cdot\text{mL}^{-1}$ ). The total amount of PFOB in the suspension,  $n_{\text{PFOB}}$ , was determined after  
204 integration of the peak at -64.7 ppm corresponding to the  $\text{CF}_2\text{Br}$  group and normalization by  
205 the area of the TFA peak at -76.5 ppm. Absolute encapsulation efficiency,  $\eta_{\text{encaps}}$ , was  
206 calculated as follows:

207 
$$\eta_{encaps} = \frac{n_{PFOB}}{n_{PFOB}^{max}} \text{ with } n_{PFOB}^{max} = \frac{m_{PFOB}^{feed}}{M_{PFOB}} \frac{m_{NC}}{m_{PFOB}^{feed} + m_{polymer}^{feed} + m_{SC}^{feed}}$$

208 where  $m_{PFOB}^{feed}$ ,  $m_{polymer}^{feed}$  and  $m_{SC}^{feed}$  are the initial masses of the components introduced during  
 209 NCs preparation,  $m_{NC}$  corresponds to the mass of NCs recovered after freeze-drying and  
 210  $M_{PFOB}$  is the molar mass of PFOB (498.96 g/mol).

## 211 2.8. Transmission electron microscopy (TEM)

212 TEM was performed at I2BC (Gif-sur-Yvette, France) using a JEOL JEM-1400 operating at  
 213 80 kV. 5  $\mu$ L of purified suspensions of nanocapsules (0.5 mg/mL) were deposited for 1 min  
 214 on glow-discharged copper grids covered with formvar-carbon film. Samples were then  
 215 stained using 2% phosphotungstic acid (pH = 7) for 30 s. The excess solution was blotted off  
 216 using a filter paper. Images were acquired using an Orius camera (Gatan Inc, USA).

## 217 2.9. Cryo-transmission electron microscopy (cryoTEM)

218 CryoTEM was performed at I2BC (Gif-sur-Yvette, France) using a JEOL JEM-1400  
 219 operating at 120 kV. 5  $\mu$ L of purified suspensions of nanocapsules (25 mg/mL) were  
 220 deposited on glow-discharged Lacey copper grids covered with carbon film containing holes.  
 221 The excess solution was blotted off for 5 s using filter paper and the grids were subsequently  
 222 frozen in liquid ethane using a Leica EM GP automatic system (Leica, Austria) under a 90%  
 223 humidity atmosphere. Images were recorded on a US1000XP camera (Gatan Inc, USA) with a  
 224 -2  $\mu$ m defocus.

## 225 2.10. Small angle neutrons scattering (SANS)

### 226 2.10.1. SANS data collection

227 Small Angle Neutron Scattering (SANS) measurements were performed on the PACE  
 228 spectrometer of the Laboratoire Léon Brillouin (LLB, CEA-Saclay, France). Nanocapsules

229 were filtered, purified and resuspended at a final volume fraction of  $\phi_v = 1\%$  in a 40/60 (v/v)  
 230 H<sub>2</sub>O/D<sub>2</sub>O mixture to match the scattering length density of the PFOB core ( $\rho_{PFOB} = \rho_{core} = 3.6$   
 231  $\times 10^{10}$  cm<sup>-2</sup>). This mixture has a weak contrast with PFMA block polymer ( $\rho_{PFMA} = 3.0 \times 10^{10}$   
 232 cm<sup>-2</sup>). So, using contrast matching of the PFOB core, we focused on the polymeric shell of  
 233 PEG-PLA blocks. Two configurations were used: the first one with a sample-to-detector  
 234 distance of  $D = 4.7$  m and a neutron wavelength of  $\lambda = 13$  Å and the second one with  $D = 2.9$   
 235 m and  $\lambda = 4.6$  Å to cover a broad  $q$  range of  $3.2 \times 10^{-3} - 1.5 \times 10^{-1}$  Å<sup>-1</sup>. SANS measurements  
 236 were performed in 1 mm thick quartz cuvettes to minimize the incoherent scattering.  
 237 Scattered intensity curves were corrected from the scattering from the empty quartz cuvette  
 238 and the electronic background, and normalized by the incoherent signal of 1 mm water sample  
 239 to account for non-uniform efficiency of detector, using the LLB PASINET software.<sup>31</sup>

#### 240 2.10.2. SANS data modelling

241 The scattered intensity curves obtained in PFOB matching condition were fitted using the  
 242 vesicle model with Sasview software.<sup>32</sup> This model provides the form factor,  $P(q)$ , for a  
 243 unilamellar vesicle of inner radius  $R_{core}$  and thickness  $T$ , according to the following equation:

$$P(q) = \frac{scale}{V_2} \left[ \frac{3V_1(\rho_1 - \rho_2) J_1(qR_1)}{qR_1} + \frac{3V_2(\rho_2 - \rho_{solv}) J_1(qR_2)}{qR_2} \right]^2$$

244 Where  $scale$  is a scale factor,  $V_1$  is the volume of the core,  $V_2$  is the total volume of the  
 245 vesicle,  $R_1 = R_{core}$  is the radius of the core,  $R_2$  is the outer radius of the vesicle ( $R_2 = R_{core} +$   
 246  $T$ ), and  $J_1(x) = \frac{\sin x - x \cos x}{x^2}$ . For the vesicle,  $\rho_1 = \rho_{core} = \rho_{solv}$  is the scattering length density  
 247 of the core and the solvent,  $\rho_2 = \rho_{shell}$  is the scattering length density of the shell. The fit gives  
 248 geometrical parameters of the nanocapsules: the mean core radius  $R_{core}$ , the polydispersity of  
 249 the core radius  $\sigma_{core}$ , the thickness of the shell  $T$  and the polydispersity of the thickness  $\sigma_T$ . A  
 250 lognormal distribution was assumed for both radius and thickness.

251        2.11.        Hydrolytic degradation of polymers

252        50 mg of each polymer (except PEG-PLA-PFMA<sub>20</sub>) were suspended into 1 mL of NaOH 1M  
253        and vigorously stirred for 24h at 37°C in an incubator. After adjustment of the pH to 7 with  
254        HCl 1M, the cloudy solution was dialyzed against deionized water for 96h to remove salt and  
255        lactic acid (dialysis membrane molecular weight cut off: 100–500 Da).

256        2.12.        Cell culture

257        Human endothelial umbilical vein cells (HUVEC) obtained from ATCC (USA) were cultured  
258        in DMEM supplemented with 50 U.mL<sup>-1</sup> penicillin, 50 U.mL<sup>-1</sup> streptomycin, and 10% heat  
259        inactivated FBS. The J774.A1 murine macrophage monocyte cell line (ATCC, USA) was  
260        cultured in RPMI 1640 medium supplemented with 50 U.mL<sup>-1</sup> penicillin, 50 U.mL<sup>-1</sup>  
261        streptomycin, and 10% heat inactivated FBS. Cells were split twice a week, by trypsinisation  
262        for HUVEC and by scraping for J774.A1 cells. All cell lines were maintained at 37 °C and  
263        5% CO<sub>2</sub> under a humidified atmosphere.

264        2.13.        Cytotoxicity evaluation of NCs and polymer degradation products

265        To evaluate their *in vitro* cytotoxicity, NCs and polymer degradation products were diluted in  
266        cell culture medium before being added onto the cells. The cell viability was evaluated using  
267        the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells  
268        were seeded in 100 µL of culture medium (12 x 10<sup>3</sup> cells/well or 3 x 10<sup>3</sup> cells/well for 24 and  
269        72h incubation respectively) in 96 well plates (TPP, Switzerland) and pre-incubated for 24 h.  
270        100 µL of a serial dilution of NCs (0.01 to 10 mg/mL) or of polymer degradation products  
271        (10<sup>-5</sup> to 0.01 mg/mL of initial polymer) was then added to the medium. After 24 or 72h of  
272        incubation, the incubation medium was replaced by fresh medium containing 0.5 mg/mL of  
273        MTT (Sigma, France). After 1h incubation, the culture medium was gently aspirated and  
274        replaced by 200 µL dimethylsulfoxide (ACS grade, VWR, France) to dissolve the formazan

275 crystals. The absorbance of the solubilized dye, which correlates with the number of living  
276 cells, was measured with a microplate reader (LAB Systems Original Multiscan MS, Finland)  
277 at 570 nm. The percentage of viable cells in each well was calculated as the absorbance ratio  
278 between treated and untreated control cells.

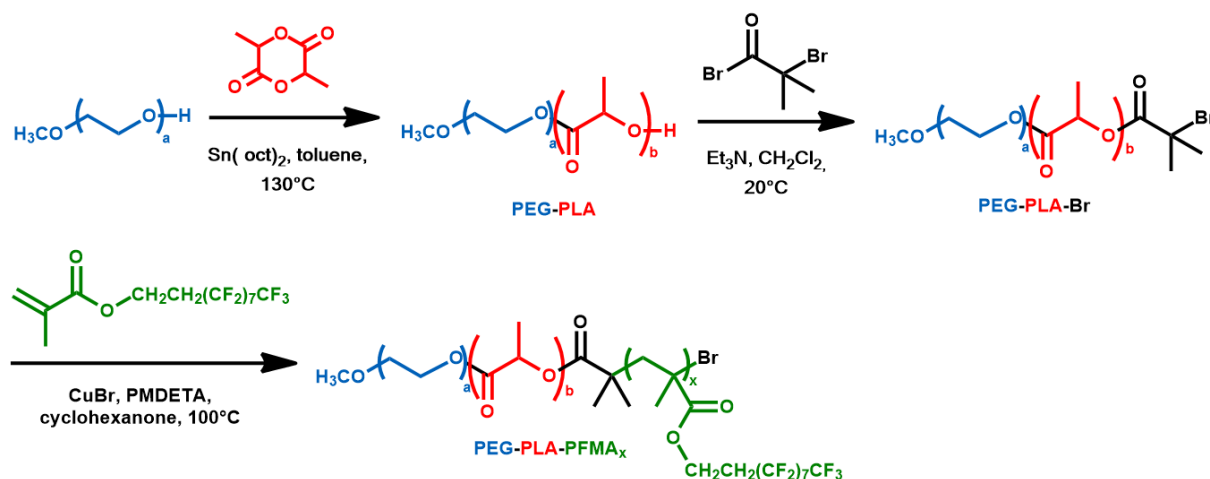
#### 279 2.14. *In vitro* ultrasound measurements

280 Suspensions of nanocapsules (1 mL, 5 mg/mL) were placed in a 10 mm x 10 mm x 45 mm  
281 polystyrene cuvette in which 5-mm diameter holes were cut out and covered by acoustically  
282 transparent mylar membrane, as shown in Figure S1. The cuvette was placed in a water bath  
283 at 37°C, and a small magnetic bar allowed agitating gently the suspension without disturbing  
284 the signal. Measurements were performed after 4 minutes to allow temperature equilibration  
285 and removal of potential bubbles. A Handyscope HS5 (TiePie engineering, Netherlands) was  
286 used as both an arbitrary wave generator and a USB oscilloscope connected to a computer  
287 using Matlab (Mathworks, USA). Samples were insonified by a transducer (focal length 51  
288 mm, model I3-0506-R-SU-F2.00IN, Olympus, France), which emitted 10 bursts of 500 cycles  
289 at 5 MHz. The scattered ultrasound signal was received by a second transducer (focal length  
290 51 mm, model I3-1506-R-SU-F2.00IN, Olympus, France) placed at a 90° angle compared to  
291 the transducer-transmitter (Figure S1). This scattered signal was preamplified before being  
292 measured by the oscilloscope connected to Matlab for signal processing. The signal was then  
293 windowed, Fourier-transformed and the scattered intensity within the bandwidth of each  
294 harmonics was summed. The resulting ultrasound scattered intensities were subtracted from  
295 the intensity obtained with the cuvette filled with Milli-Q water. At least 9 measurements  
296 were performed with each sample. Commercial SonoVue<sup>®</sup> microbubbles (diluted to 1/1000)  
297 were used as a reference positive control for the ultrasound set-up and parameters chosen for  
298 our study.

## 299 3. Results and discussion

### 300 3.1. Polymers synthesis and characterization

301 Triblock copolymers carrying perfluoroalkyl pendant chains were synthesized by a  
302 combination of ROP and ATRP as shown in Figure 1.



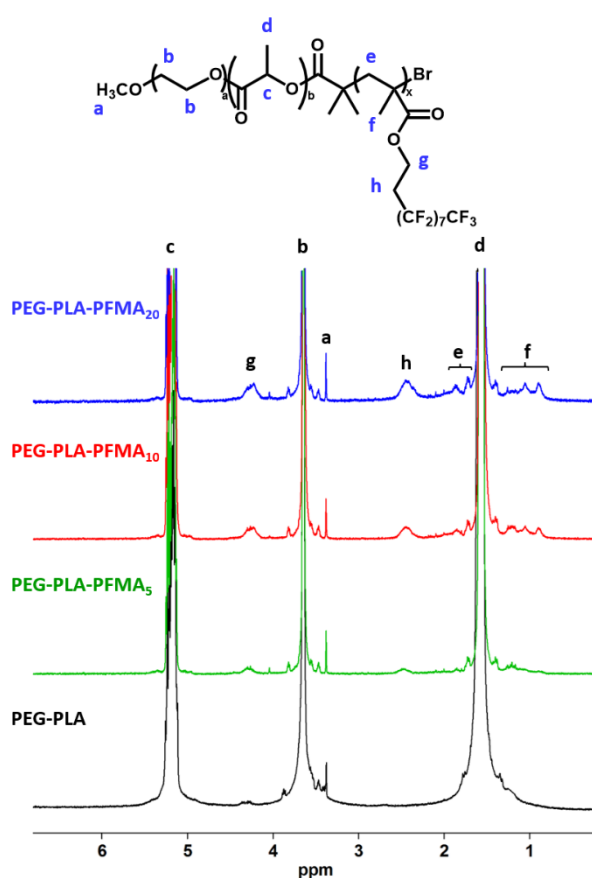
303

304 **Figure 1.** Synthesis of PEG-PLA-PFMA<sub>x</sub> triblock copolymers.

305 ROP of D,L-lactide with polyethylene glycol methyl ether ( $M_n = 5,000$  g/mol) as initiator and  
306 Sn(oct)<sub>2</sub> as catalyst first led to PEG-PLA diblock copolymer with molar mass close to the  
307 targeted  $M_n$  of 25,000 g/mol ( $M_n^{\text{NMR}} = 23,500$  g/mol,  $M_n^{\text{SEC}} = 25,500$  g/mol). PEG-PLA was  
308 subsequently converted to PEG-PLA-Br macroinitiator through esterification with excess 2-  
309 bromo-2-methylpropionyl bromide in the presence of triethylamine. Quantitative conversion  
310 was confirmed by <sup>1</sup>H NMR with disappearance of the end hydroxyl broad signal of PLA at  
311 2.70 ppm and appearance of signals at 1.95 and 1.98 ppm corresponding to the methyl protons  
312 from 2-bromo-2-methylpropionyl end group (supplementary material Figure S2).

313 The final PEG-PLA-PFMA<sub>x</sub> copolymers were prepared by ATRP of FMA in cyclohexanone  
314 with PEG-PLA-Br as macroinitiator and CuBr/PMDETA complex as catalyst. The nature and  
315 amount of solvent were initially varied to achieve final polymerization with satisfactory  
316 conversion (>65%). Three different monomer/initiator feed molar ratios ( $x = 5, 10, 20$ ) were

317 used to design polymers with various fluorinated block lengths. Successful polymerization  
 318 was confirmed by  $^1\text{H}$  NMR with appearance of the signals at 2.47 and 4.28 ppm  
 319 corresponding respectively to  $\text{CH}_2\text{CF}_2$  and  $\text{CH}_2\text{CH}_2\text{CF}_2$  protons of the PFMA block (signals h  
 320 and g, Figure 2), in addition to those of the methacrylate backbone 1.70 – 2.00 and 0.80 –  
 321 1.45 (signals e and f, Figure 2). The 4.28 ppm peak area was used to calculate the copolymer  
 322 molar composition and molar mass by normalization with the 3.64 ppm peak corresponding to  
 323 the  $\text{OCH}_2\text{CH}_2$  of PEG (Table 1).

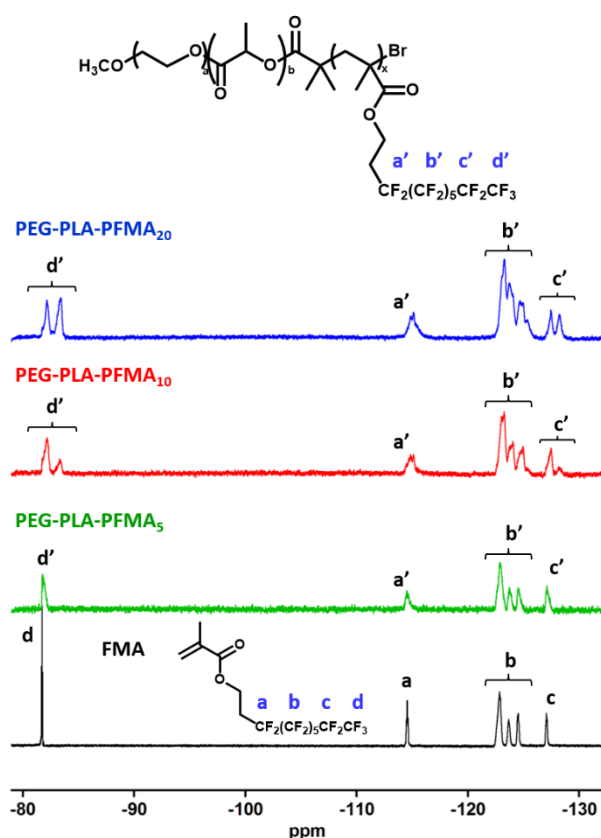


324  
 325 **Figure 2.**  $^1\text{H}$  NMR spectra of PEG-PLA and triblock copolymers PEG-PLA-PFMA<sub>x</sub> in  
 326  $\text{CDCl}_3$  (normalized with the PEG peak at 3.64 ppm).

327  $^{19}\text{F}$  NMR spectra of all polymers further support the successful polymerization of FMA  
 328 (Figure 3). A broadening of the fluorine resonances is observed in PEG-PLA-PFMA<sub>5</sub>  
 329 spectrum as compared to the monomer. This effect is more pronounced as the number of



330 perfluoroalkyl pendants increases, and leads to the overlapping of resonances of CF<sub>2</sub> groups  
 331 b' in PEG-PLA-PFMA<sub>10</sub> and PEG-PLA-PFMA<sub>20</sub> spectra, in agreement with previous  
 332 reports.<sup>33,34</sup> Moreover, a splitting of the peaks c' and d', corresponding to the CF<sub>2</sub> and CF<sub>3</sub>  
 333 groups at the extremity of the fluorinated pendants, is observed. This suggests the existence of  
 334 unaveraged conformations and therefore indicates some degree of self-association in  
 335 chloroform.



336  
 337 **Figure 3.** <sup>19</sup>F NMR spectra of FMA (black line) and triblock copolymers PEG-PLA-PFMA<sub>x</sub>  
 338 in CDCl<sub>3</sub>.

339 The molar masses were also determined by conventional SEC in chloroform (Table 1). All  
 340 polymers present a single peak (supplementary material Figure S3), confirming the successful  
 341 formation of copolymers rather than homopolymer blends. Although  $M_n^{\text{SEC}}$  values are close to  
 342  $M_n^{\text{NMR}}$  values,  $M_n^{\text{SEC}}$  does not increase with the polymerization degree of the PFMA block, as  
 343 shown by the superposition of chromatograms and the values in the 24,000 – 26,500 g/mol

344 range. Because of fluorophilicity of the perfluorinated chains, full swelling of the triblock  
345 polymers in chloroform is limited by a probable folding or formation of micellar domains.  
346 Indeed, the triblock polymers appeared more difficult and longer to solubilize than non-  
347 fluorinated PEG-PLA in common organic solvents such as chloroform, methylene chloride  
348 and acetone. To confirm this different behavior, DLS analysis of fluorinated polymers at 25  
349 mg/mL in methylene chloride and chloroform was performed. It revealed an important  
350 increase of scattered intensity compared to plain PEG-PLA (around 15000 kcps for the  
351 triblocks compared to 200 kcps for PEG-PLA, see supplementary material Figure S4). This  
352 indicates presence of aggregates such as micelles which confirms that the fluorinated block  
353 strongly impacts the solubility and conformation of the polymers chains.

354

355 **Table 1.** Degree of polymerization (DP), number-average molar mass ( $M_n$ ), dispersity ( $\mathcal{D}$ ) and  
 356 glass transition temperature ( $T_g$ ) of PEG-PLA-PFMA<sub>x</sub> polymers and their precursors.

Polymer	$M_n^{\text{theoretical}}$ (x 10 <sup>3</sup> g/mol)	DP PLA:PFMA <sup>1</sup>	$M_n^{\text{NMR}}$ (x 10 <sup>3</sup> g/mol)	$M_n^{\text{SEC}}$ (x 10 <sup>3</sup> g/mol)	$\mathcal{D}$	$T_g$ (°C)
PEG-PLA	25.0	257	23.5	24.3	1.29	13 ± 1
PEG-PLA-Br	25.0	260	23.7	25.2	1.28	ND
PEG-PLA-PFMA <sub>5</sub>	27.7	272:4	26.7	25.0	1.38	16 ± 1
PEG-PLA-PFMA <sub>10</sub>	30.3	291:7	29.7	24.9	1.39	20 ± 2
PEG-PLA-PFMA <sub>20</sub>	35.6	277:15	32.9	26.2	1.29	18 ± 1

357 <sup>1</sup>The DP and  $M_n^{\text{NMR}}$  were determined from the areas of the peak at 3.64 ppm (OCH<sub>2</sub>CH<sub>2</sub> of  
 358 PEG) versus 5.19 ppm (CH of PLA) for PLA, and versus 4.28 ppm (CH<sub>2</sub>CH<sub>2</sub>CF<sub>2</sub> of PFMA)  
 359 for PFMA.

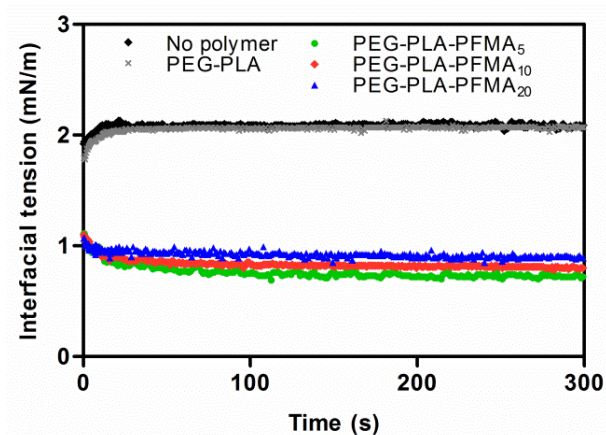
360 DSC thermograms of all polymers exhibited a single glass transition temperature ( $T_g$ ) in the  
 361 16-20°C range for triblock copolymers, slightly higher than plain PEG-PLA ( $T_g$  = 13°C)  
 362 (Table 1). The  $T_g$  of the PEG block is too low to be detected, and the  $T_g$  of both PLA and  
 363 PFMA blocks are probably very close to each other to be detected separately. Indeed, Li et al.  
 364 reported the synthesis of PEG-PFMA block copolymers and found a  $T_g$  of -24.3 or -41.7 °C  
 365 for the PEG block, and 19.5 °C for the PFMA block,<sup>28</sup> in agreement with our observations.  
 366 Moreover, the glass transition of PEG-PLA-PFMA<sub>20</sub> appears less sharp than the others  
 367 (supplementary material Figure S5), suggesting a stronger entanglement of polymer chains  
 368 induced by a higher proportion of perfluoroalkyl units. This is consistent with the study of Li  
 369 et al. where the glass transition of PFMA was observed only with the shorter PFMA block (28  
 370 fluorinated units) and not with increasing number of fluorinated units (36 and 38).<sup>28</sup>

371 A series of triblock copolymers containing a fluorinated block with a comb-like structure was  
 372 therefore successfully synthesized using a combination of ROP and ATRP. This will allow  
 373 studying the influence of the number of perfluoroalkyl pendant chains on polymers interfacial  
 374 properties and on nanocapsules characteristics.

### 375 3.2. Polymers interfacial properties

376 The importance of polymer interfacial behavior during the formulation process of  
377 perfluorocarbon micro- and nanocapsules was highlighted in previous reports.<sup>18,35,36</sup> In  
378 particular, reducing the amount of PEG-PLGA in the formulation of PFOB nanocapsules led  
379 to local dewetting and expulsion of some PFOB droplets stabilized by sodium cholate.<sup>18</sup> The  
380 interfacial tension at the PFOB/organic phase interface was therefore measured with each  
381 triblock polymer (Figure 4). A reduction from 2.0 to 0.9-1.0 mN/m is observed upon addition  
382 of each PEG-PLA-PFMA<sub>x</sub> polymer at 25 mg/mL in methylene chloride, regardless of the  
383 number of fluorinated units, whereas no decrease is induced by addition of plain PEG-PLA ( $\gamma$   
384 = 2.0 ± 0.3 mN/m). The profile as a function of time after the drop formation is also different  
385 between fluorinated polymers and the non-fluorinated one, showing a decrease of interfacial  
386 tension during the first few seconds with fluorinated polymers only (Figure 4). These results  
387 confirm that perfluoroalkyl pendant chains interact with PFOB and adsorb at the interface.  
388 Such finding is consistent with the reported fluorophilic character of perfluorinated chains,  
389 which usually form fluorous domains.<sup>37-39</sup> In a previous study, no reduction of interfacial  
390 tension could be observed with PLAs terminated by a linear fluorinated chain of length  
391 ranging from C<sub>3</sub>F<sub>7</sub> to C<sub>13</sub>F<sub>27</sub>.<sup>19</sup> Adsorption of PEG-PLA-PFMA<sub>x</sub> polymers could potentially  
392 be favored by their higher fluorine content as compared to PLA-C<sub>x</sub>F<sub>2x+1</sub> polymers. However,  
393 the difference between PLA-C<sub>13</sub>F<sub>27</sub> and PEG-PLA-PFMA<sub>5</sub> is not so important as compared to  
394 differences between all triblock polymers when taking into account the real degrees of  
395 polymerization: 25 mg of polymer contains ~45 μmol of fluorine for PLA-C<sub>13</sub>F<sub>27</sub> and ~64  
396 μmol for PEG-PLA-PFMA<sub>5</sub>. This quantity then reaches ~100 μmol for PEG-PLA-PFMA<sub>10</sub>  
397 and ~194 μmol PEG-PLA-PFMA<sub>20</sub>. Adsorption of triblock polymers is therefore probably  
398 favored by their comb-like architecture. In linear fluorinated PLAs, the fluorinated chain  
399 might be hidden by the PLA chain, whereas with the methacrylate backbone, the fluorinated

400 part is more rigid and more voluminous, making it more exposed and able to adsorb at the  
401 PFOB-solvent interface.



402

Polymer	Interfacial tension (mN/m)
No polymer	2.0 ± 0.3
PEG-PLA	2.0 ± 0.3
PEG-PLA-PFMA <sub>5</sub>	0.9 ± 0.1
PEG-PLA-PFMA <sub>10</sub>	0.9 ± 0.1
PEG-PLA-PFMA <sub>20</sub>	1.0 ± 0.1

403 **Figure 4.** Interfacial tension measurements at the interface between PFOB and methylene  
404 chloride solutions of each polymer at 25 mg/mL with the pendant drop method: typical  
405 profiles as a function of time after the drop formation (top) and interfacial tension values at  
406 200 s presented as mean ± SD (n > 6) (bottom).

### 407 3.3. PFOB nanocapsules: physical characterization

408 PFOB nanocapsules (NCs) were formulated using an emulsion evaporation process.<sup>16,17</sup> Since  
409 the objective was to reduce shell thickness, the initial amount of polymer in the organic phase  
410 was decreased (50, 30 and 20 mg), while keeping the amount of PFOB constant.

#### 411 *Size distribution and zeta potential*

412 For a fixed polymer quantity, NCs mean hydrodynamic diameters are slightly larger with all  
413 PEG-PLA-PFMA<sub>x</sub> (117 – 147 nm) compared to PEG-PLA (113 – 131 nm) (Table 2). As  
414 observed with interfacial tension results, the number of fluorinated pendants does not seem to

415 have an influence on size and zeta potential. The polymer mass in the formulation is a more  
 416 important parameter. Indeed, NCs formulated from 20 mg of polymer exhibit larger sizes than  
 417 NCs from 50 and 30 mg, especially with the fluorinated polymers (136 – 147 nm at 20 mg  
 418 compared to 117 – 119 nm at higher polymer amounts). For all polymers, the polydispersity  
 419 (PDI) increases as the polymer quantity decreases ( $< 0.19$  at 50 mg,  $> 0.28$  at 20 mg),  
 420 suggesting a wider size distribution at low polymer amount. This might arise from the  
 421 presence of a second population of objects, such as non-encapsulated PFOB droplets, despite  
 422 the addition of perfluorinated chains in the polymer, as previously observed with PLGA-  
 423 PEG.<sup>18</sup> For all polymers, the zeta potential is negative and no significant difference is induced  
 424 by the number of perfluoroalkyl chains (Table 2).

425 **Table 2.** Characterization of nanocapsules prepared from 50, 30 or 20 mg of each polymer.

426 Data are presented as mean  $\pm$  SD ( $n > 3$ ) for  $d_H$  and  $\zeta$  values.

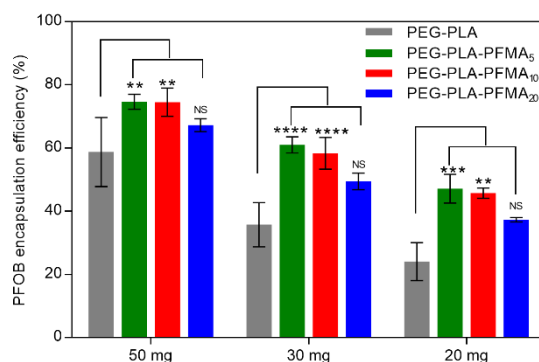
Polymer	50 mg			30 mg			20 mg		
	$d_H$ (nm)	PDI	$\zeta$ (mV)	$d_H$ (nm)	PDI	$\zeta$ (mV)	$d_H$ (nm)	PDI	$\zeta$ (mV)
PEG-PLA	113 $\pm$ 3	0.19	-17 $\pm$ 10	113 $\pm$ 3	0.22	-23 $\pm$ 9	131 $\pm$ 8	0.28	-29 $\pm$ 7
PEG-PLA-PFMA <sub>5</sub>	117 $\pm$ 2	0.17	-15 $\pm$ 8	120 $\pm$ 5	0.24	-20 $\pm$ 4	140 $\pm$ 9	0.34	-30 $\pm$ 15
PEG-PLA-PFMA <sub>10</sub>	116 $\pm$ 5	0.17	-15 $\pm$ 7	120 $\pm$ 6	0.26	-20 $\pm$ 5	136 $\pm$ 12	0.35	-21 $\pm$ 6
PEG-PLA-PFMA <sub>20</sub>	119 $\pm$ 3	0.16	-15 $\pm$ 9	123 $\pm$ 2	0.26	-21 $\pm$ 5	147 $\pm$ 3	0.44	-23 $\pm$ 10

427

#### 428 *PFOB encapsulation efficiency*

429 PFOB encapsulation efficiency in NCs was measured by <sup>19</sup>F NMR after freeze-drying to  
 430 remove any non-encapsulated PFOB droplets remaining. As shown in Figure 5, more PFOB  
 431 is encapsulated in PEG-PLA-PFMA<sub>x</sub> NCs compared to plain PEG-PLA NCs. When  
 432 comparing to the PLA-PEG control, the encapsulation efficacy of PFOB is significantly  
 433 higher for PEG-PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub> for all initial masses of polymer but  
 434 not for PEG-PLA-PFMA<sub>20</sub>. The difference appears more important at low polymer amounts,

435 with 49-61% PFOB encapsulated in PEG-PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub> NCs  
 436 compared to 35% in PEG-PLA NCs at 30 mg, and 37-47% compared to 24% at 20 mg. Such  
 437 results are consistent with interfacial tension measurements and demonstrate that fluorophilic  
 438 interactions between PFOB and the perfluorinated units of the polymers allow entrapping  
 439 more PFOB into the NCs. One can also observe that PFOB encapsulation efficiency is slightly  
 440 lower with PEG-PLA-PFMA<sub>20</sub> compared to PEG-PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub>  
 441 (about 9 – 12% of difference). This could be due to a stronger self-association of the polymer  
 442 with the largest fluorinated block of 20 pendants, reducing their ability to interact with PFOB  
 443 as compared with shorter blocks of 5 – 10 fluorinated units.

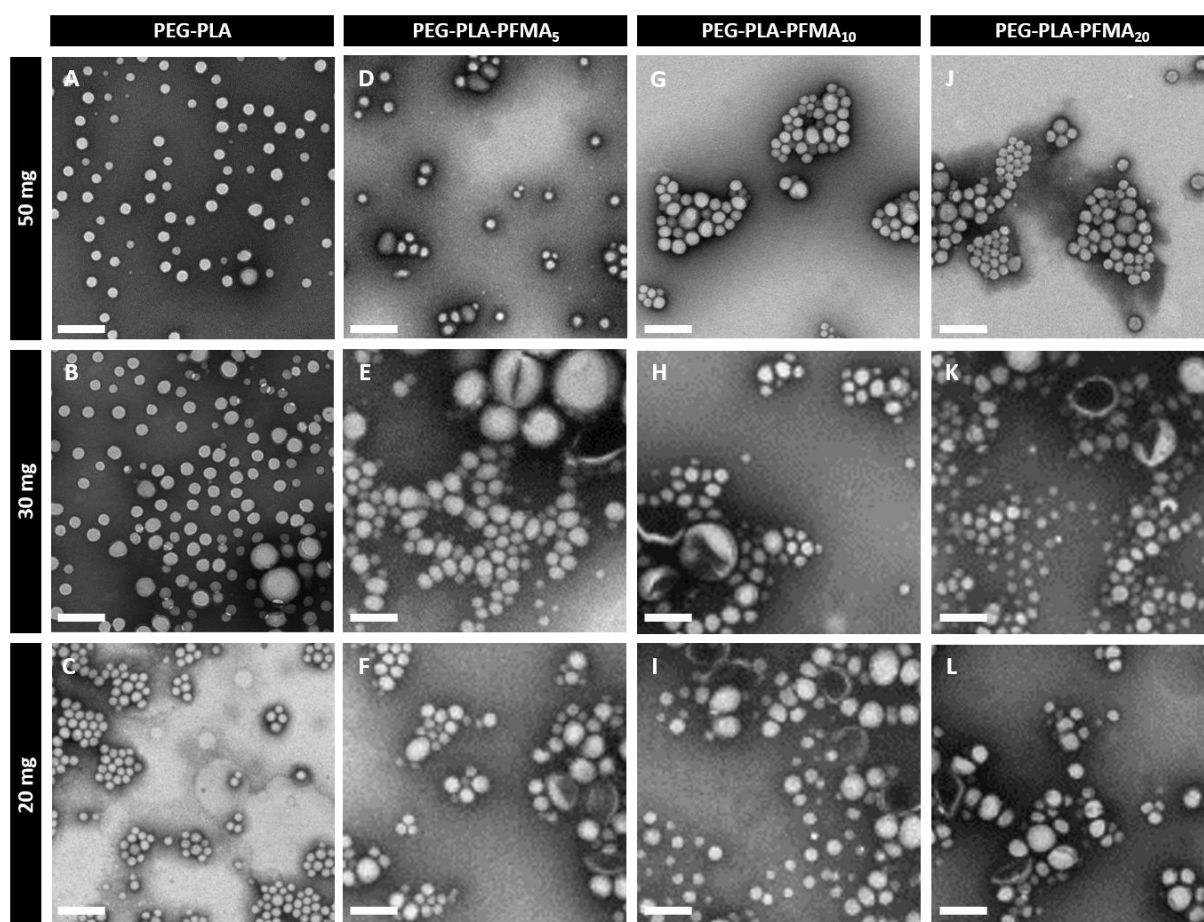


444  
 445 **Figure 5.** PFOB encapsulation efficiency in freeze-dried NCs. Results are presented as mean  
 446  $\pm$  SD (n = 4). Statistical significance was analyzed using an ANOVA test: \*\*\*\* p<0.0001,  
 447 \*\*\* p<0.001, \*\* p<0.01.

448  
 449 *Nanocapsules morphology*

450 Nanocapsules were then imaged by TEM with negative staining and cryo-TEM. In TEM  
 451 images, one can observe mostly spherical objects with PEG-PLA (Figure 6A,B,C) whereas  
 452 PEG-PLA-PFMA<sub>x</sub> NCs exhibit more heterogeneous morphologies (Figure 6 and  
 453 supplementary material S6 to S9). Indeed, capsules appear less spherical and a lot of broken  
 454 capsules can be observed at low polymer amounts (Figure 6E,F,H,I,K,L). These broken

455 capsules, which were neither observed in plain PEG-PLA samples nor for NCs made from 50  
456 mg of PEG-PLA-PFMA<sub>x</sub>, are sometimes very large (up to 1 μm diameter, see supplementary  
457 material). Such finding is surprising since NCs are filtered on 0.45 μm after the emulsion  
458 evaporation process. These objects might be very deformable to pass through the filter, and  
459 might be broken because of the filtration or because they are under vacuum during TEM  
460 observation. These large capsules can therefore explain the high PDI values observed at low  
461 fluorinated polymer amounts (Table 2).



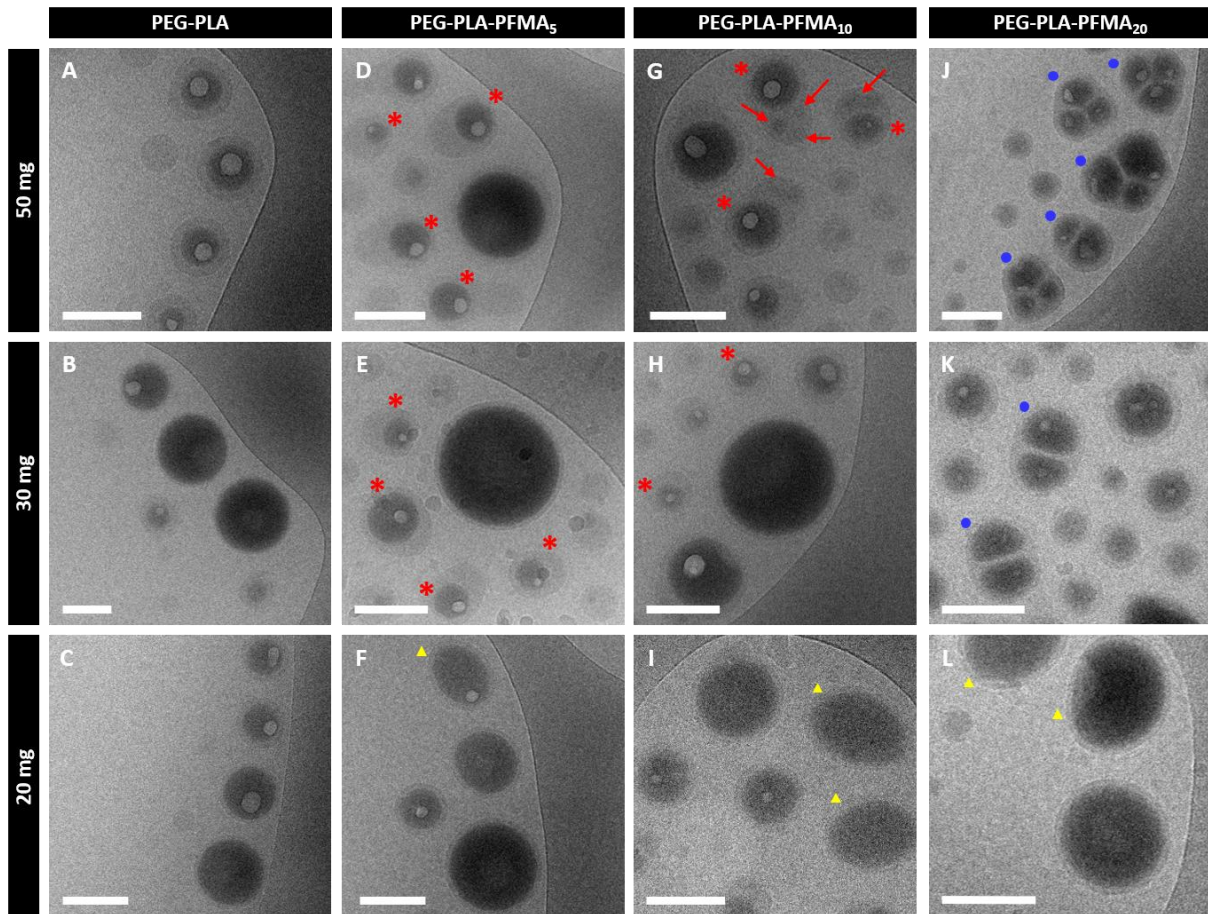
462  
463 **Figure 6.** TEM images of formulated NCs with negative staining (scale bars = 200 nm).

464 Additional images are shown in supplementary material.

465 Cryo-TEM images allow distinguishing the dark PFOB core, with high electronic density,  
466 from the lighter polymeric shell (Figure 7 and supplementary material S6 to S9). NCs also  
467 present a white meniscus in the core, due to rapid freezing, which causes solidification and



468 shrinking of PFOB. PFOB therefore does not fill entirely the shell cavity anymore as observed  
469 previously.<sup>18,19,40</sup> One can observe that NCs morphology is strongly influenced by the number  
470 of fluorinated chains and the amount of polymer used for formulation. Indeed, all PEG-PLA  
471 NCs possess a spherical core-shell structure (Figure 7A,B,C), whereas the presence of five  
472 perfluoroalkyl chains induces the formation of some elongated NCs with a non-centered  
473 PFOB core looking like sunny-side up eggs (shown by red stars in Figure 7D,E). Such  
474 elongated morphology had already been observed with COOH-PEG-PLA NCs. This was  
475 attributed to an interfacial instability due to electrostatic repulsions between deprotonated  
476 carboxy functions leading to an increase of the total surface area.<sup>30</sup> Comparatively, with PEG-  
477 PLA-PFMA<sub>5</sub>, we can imagine that the incompatibility between the various blocks may force  
478 the chains to adopt a particular conformation leading to an increase of the total surface area.  
479 The same type of morphologies (shown by red stars) is observed with 50 and 30 mg of PEG-  
480 PLA-PFMA<sub>10</sub>, but with additional dark domains (shown by red arrows) in the same object  
481 (Figure 7G,H), which correspond either to other PFOB cores, or to fluorinated rich domains  
482 formed by aggregation of the perfluoroalkyl chains of the polymer.<sup>41</sup> PEG-PLA-PFMA<sub>20</sub>  
483 leads to NCs with 2 or 3 distinct PFOB cores at high polymer amounts, as shown by blue  
484 circles in Figure 7J,K. Such multi-core structures are probably favored by strong interactions  
485 between PFOB and perfluoroalkyl pendants. However, at 20 mg of each fluorinated polymer,  
486 although morphologies are still a bit heterogeneous within a same sample, one can observe a  
487 majority of capsules with a single centered PFOB core and a thin shell (Figure 7F,I,L).  
488 Interestingly, some are not perfectly spherical and seem ellipsoidal/elongated (shown by  
489 yellow triangles), which may indicate different mechanical properties. In this type of capsules,  
490 the PFOB core is not spherical and the shell thickness is homogeneous within the whole  
491 capsule, which is different from the other elongated NCs observed at higher polymer amounts.



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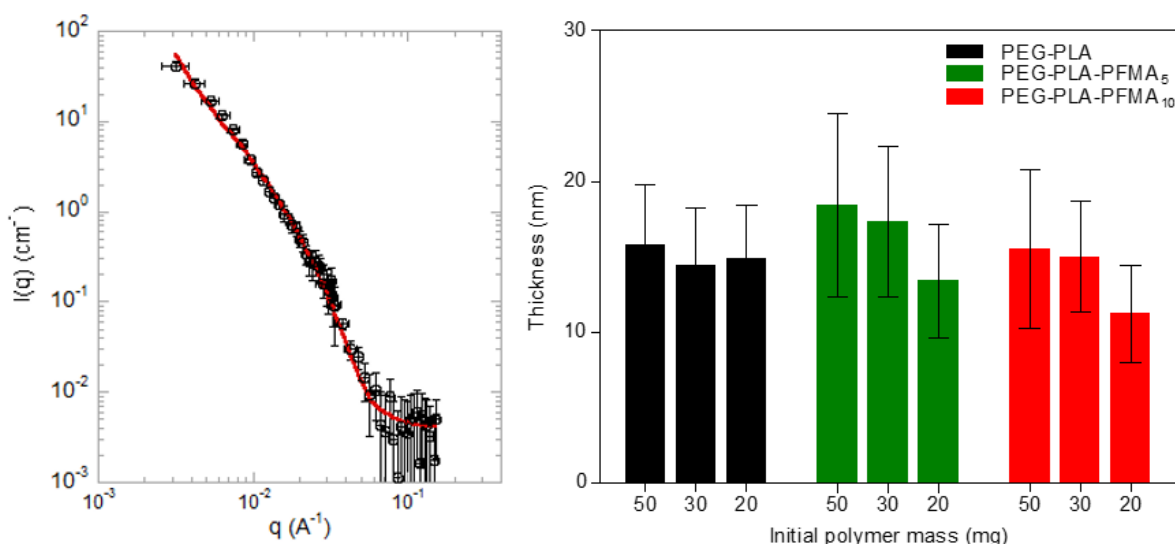
504

**Figure 7.** Cryo-TEM images of formulated NCs (scale bars = 100 nm). Additional images are given in supplementary material. Red stars show elongated NCs with a non-centered spherical PFOB core at high amounts of PEG-PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub>. In the case of PEG-PLA-PFMA<sub>10</sub>, this type of elongated NCs also possesses additional darker domains shown by red arrows. Blue circles show NCs with 2 or 3 distinct PFOB cores. Yellow triangles show ellipsoidal NCs with a single non-spherical PFOB core and a shell thickness homogeneous within the same capsule.

#### *Shell thickness*

Small Angle Neutron Scattering (SANS) was used to statistically determine shell thickness. We focused on PEG-PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub> which showed better PFOB encapsulation efficiency and higher solubility in methylene chloride than PEG-PLA-PFMA<sub>20</sub>. NCs were resuspended in the appropriate mixture of H<sub>2</sub>O and D<sub>2</sub>O to match the scattering

505 length density of the PFOB core and allow focusing on the polymeric shell. Scattered  
 506 intensity curves were fitted with the vesicle model, assuming a log-normal distribution for  
 507 both shell thickness and core radius. One example of a satisfying fit and the best fit  
 508 parameters obtained for shell thickness are represented in Figure 8. Fitted curves and  
 509 numerical values of shell thickness and core radius are given in supplementary material  
 510 (Figure S10 and Table S1). Mean PFOB core radii are all in the same range (53 – 56 nm) with  
 511 a high polydispersity, in agreement with DLS measurements. The mean thickness of PEG-  
 512 PLA NCs is not impacted by the amount of polymer, with values between 16 and 14.5 nm.  
 513 However, with fluorinated triblock polymers, the mean thickness is decreasing down to 13.5  
 514 nm and 11 nm for 20 mg of PEG-PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub>, respectively, as  
 515 compared with 50 and 30mg polymer where no difference can be observed. This decrease is  
 516 meaningful as the precision of SANS fitting is on the 1-2 nm order. A high polydispersity is  
 517 nevertheless observed for all samples, especially for NCs made from higher fluorinated  
 518 polymer amounts. This can be attributed to PFOB cores not being always well centered as  
 519 seen on cryo-TEM images.

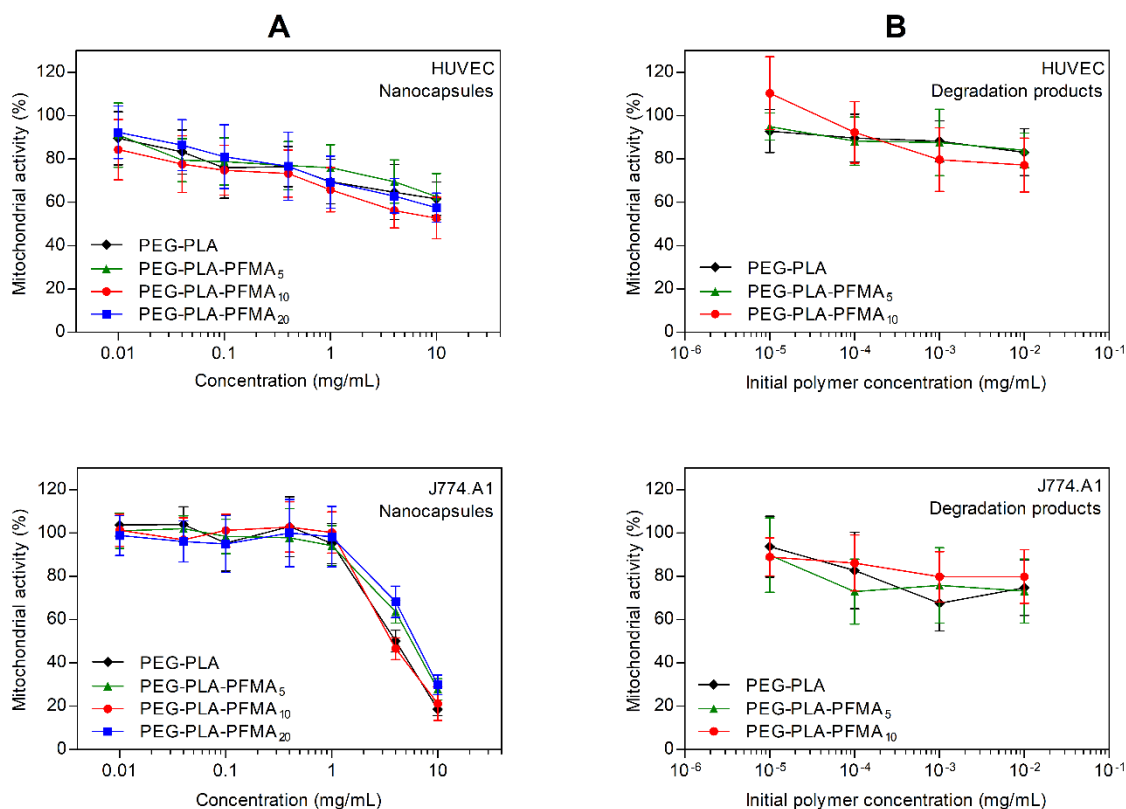


520  
 521 **Figure 8.** Experimental scattered intensity curve (black circles) of NCs made from 20 mg  
 522 PEG-PLA-PFMA<sub>10</sub> in PFOB matching condition fitted with the vesicle model (red line) (left)

523 and shell thickness values of NCs determined by fitting with the vesicle model (mean  $\pm$  SD)  
524 (right).

### 525 3.4. Cytotoxicity studies

526 The biological inertness of perfluorocarbons is well documented.<sup>42</sup> Nevertheless,  
527 perfluoroalkyl moieties are often associated to a certain toxicity due to their low excretion  
528 profiles and prolonged retention in the organism, especially linear perfluorinated chains  
529 longer than eight carbons.<sup>43–45</sup> Therefore the potential cytotoxicity of PEG-PLA-PFMA<sub>x</sub> NCs  
530 was investigated using an MTT assay on two representative cell types. Human umbilical vein  
531 endothelial cells (HUVEC) were chosen for their high sensitivity and rapid response to  
532 external stimuli which make them a widely used *in vitro* model for polymer cytotoxicity  
533 evaluation.<sup>46</sup> J774.A1 cells play a key role in phagocytosis and were chosen to highlight the  
534 possible toxicity of NCs after being engulfed by macrophages. At both 24 and 72h incubation  
535 times, cell viability of HUVEC slightly decreases as NCs concentration increases, but remains  
536 above 70% until 1 mg/mL, and above 50% at 10 mg/mL, with no difference between the  
537 polymers (Figure 9A and Figure S11A). Regarding J774.A1, cellular viability remains above  
538 80% until 1 mg/mL at both incubation times. A strong viability decrease down to 45% after  
539 24h and 20% after 72h is observed at 10 mg/mL, but this high concentration is unlikely to be  
540 reached *in vivo* (Figure 9A and Figure S11A). For both cell types and both incubation times,  
541 the perfluoroalkyl chains do not induce any specific toxicity in comparison to plain PEG-  
542 PLA, with no influence of the number of perfluoroalkyl chains. These results concur with  
543 reports from Koda et al. on the low toxicity of an amphiphilic fluororous random copolymer  
544 containing PEG and C<sub>8</sub>F<sub>17</sub> pendants.<sup>34</sup>



545  
 546 **Figure 91.** Viability assays on HUVEC (top) and J774.A1 (bottom) cell lines after 72h  
 547 incubation with nanocapsules (A) or polymers degradation products (B). Results are presented  
 548 as mean  $\pm$  SD (n = 3).

549 PEG-PLA diblock copolymer is well known to be degradable into non-toxic products (lactic  
 550 acid and polyethylene glycol). However, toxicity of the remaining short PFMA block after  
 551 degradation of the triblock copolymers must be investigated. Accelerated hydrolytic  
 552 degradations of PEG-PLA-PFMA<sub>5</sub>, PEG-PLA-PFMA<sub>10</sub>, and PEG-PLA as control, were  
 553 carried out in basic conditions. The *in vitro* cytotoxicity of degradation products was then  
 554 evaluated using an MTT assay on the same cell lines as for NCs. Due to insolubility issues,  
 555 only low concentrations could be tested (maximum 0.01 mg/mL of initial polymer). In this  
 556 range of concentrations, high HUVEC cellular viabilities (> 70%) were observed at both  
 557 incubation times (Figure 9B and Figure S11B). J774.A1 cells show a slightly higher  
 558 sensitivity depending on the incubation time, with cell viabilities above 87% after 24h and

559 above 67% after 72h. Within experimental error, no significant differences between polymers  
560 can be observed for both cell types, indicating an absence of obvious toxicity arising from  
561 fluorinated degradation products (Figure 9B and Figure S11B). These results are encouraging  
562 and additional *in vivo* studies will be required to evaluate the potential toxicity arising from a  
563 prolonged exposure to the degradation products.

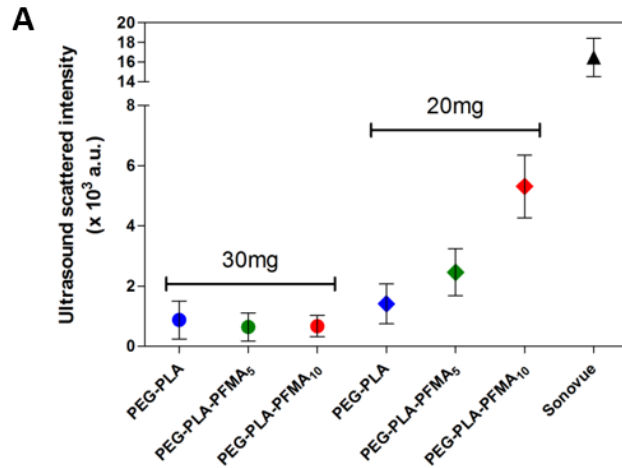
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### 565 3.5. *In vitro* ultrasound measurements

566 Finally, the acoustic response of NCs made from 30 and 20 mg polymer was evaluated *in*  
567 *vitro* at 5 MHz. Figure 10A presents the ultrasound scattered intensities measured at  
568 fundamental frequency and subtracted by the background level produced by Milli-Q water.  
569 No non-linear response could indeed be detected with our capsules, in agreement with  
570 previous studies showing the absence of nonlinear scattering with nano-sized agents.<sup>47,48</sup> At  
571 30 mg polymer, all samples did not yield an ultrasound signal much higher than the  
572 background (intensity < 0.9 x 10<sup>3</sup> arbitrary units, a.u.). However, at 20 mg polymer, the  
573 ultrasound scattered intensity increases with the number of fluorinated pendant chains, from  
574 1.4 x 10<sup>3</sup> a.u. with plain PEG-PLA to 2.5 x 10<sup>3</sup> a.u. with PEG-PLA-PFMA<sub>5</sub> and 5.3 x 10<sup>3</sup> a.u.  
575 with PEG-PLA-PFMA<sub>10</sub>. Such enhancement probably arises from several contributions which  
576 are summarized in Table 3. As shown by De Jong et al., the scattering cross section of a  
577 particle is defined as  $SCS = \frac{4\pi}{9}k^4R^6 \left[ \left( \frac{\kappa_d - \kappa}{\kappa} \right)^2 + \frac{3(\rho_d - \rho)^2}{4(\rho_d + \rho)^2} \right]$  where  $k$  is the wavenumber,  $R$  is  
578 the radius of the particle,  $\kappa_d$  and  $\kappa$  the compressibilities of respectively the particle and the  
579 medium,  $\rho_d$  and  $\rho$  the densities of respectively the particle and the medium.<sup>49</sup> A contrast agent  
580 will therefore backscatter ultrasound waves more effectively if its radius is larger and if its  
581 physical properties (compressibility and density) differ a lot from the ones of the surrounding  
582 medium. Here, the slight diameter difference (up to 27 nm) could hardly be considered

583 responsible for the higher efficacy in scattering ultrasound. One of the main contribution is  
584 probably the reduction of shell thickness (down to 13.5 nm for PEG-PLA-PFMA<sub>5</sub> and 11 nm  
585 for PEG-PLA-PFMA<sub>10</sub>) which may increase the capsules compressibility and echogenicity as  
586 previously observed.<sup>15</sup> Indeed, AFM indentation experiments performed on PFOB  
587 microcapsules have shown that a thinner shell leads to overall softer capsules.<sup>50</sup> Moreover,  
588 higher encapsulated PFOB contents and higher amounts of fluorinated chains in the polymers  
589 both increase capsule density and strengthen the difference of acoustic impedance with the  
590 surrounding medium, as previously observed with linear fluorinated PLAs.<sup>19</sup> Although the  
591 quantity of PFOB and fluorinated chains is also high in samples made from 30 mg of PEG-  
592 PLA-PFMA<sub>5</sub> and PEG-PLA-PFMA<sub>10</sub>, these two samples possess a larger shell thickness (>  
593 15 nm) and a different morphology with a non-centered PFOB core (Figure 10B). The  
594 observed differences in capsules morphology are probably related to some differences in their  
595 mechanical properties which impact their response to ultrasound waves. AFM indentation  
596 experiments should be performed in the future to relate mechanical properties and ultrasound  
597 signal.

598 Nanocapsules made from 20 mg of fluorinated triblock polymers therefore appear as  
599 promising UCAs. Although the measured ultrasound scattered intensities were still lower than  
600 with SonoVue<sup>®</sup> microbubbles ( $16.5 \times 10^3$  a.u.) due to their large radius (20-fold larger than  
601 NCs) and gaseous core, these NCs have greater potential to accumulate into the tumors by the  
602 EPR effect and allow contrast ultrasound imaging of tumors.



**B**

Initial polymer mass	30 mg			20 mg		
Polymer	PEG-PLA	PEG-PLA-PFMA <sub>5</sub>	PEG-PLA-PFMA <sub>10</sub>	PEG-PLA	PEG-PLA-PFMA <sub>5</sub>	PEG-PLA-PFMA <sub>10</sub>
Main morphology						
mean $d_H$ (nm)	113	120	120	131	140	136
mean T (nm)	14.5	17.5	15	15	13.5	11
PFOB ( $\mu\text{mol}$ )	6.9	11.8	11.3	7.0	13.7	13.3
C <sub>8</sub> F <sub>17</sub> chains ( $\mu\text{mol}$ )	0.0	0.7	1.2	0.0	0.7	1.2

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**Figure 10.** (A) Ultrasound scattered intensity by NCs resuspended at 5 mg/mL made from 30 mg (circles) and 20 mg (diamonds) of each polymer and by SonoVue microbubbles (dark triangle) as positive control. Results are presented as mean  $\pm$  SEM ( $n > 9$ ). (B) Table summarizing NCs characteristics which could account for differences of ultrasound scattering: simplified representation of their morphology, mean hydrodynamic diameter ( $d_H$ ), mean shell thickness (T), PFOB quantity and C<sub>8</sub>F<sub>17</sub> chains quantity contained in 1 mL of formulation resuspended at a final polymer concentration of 5 mg/mL for ultrasound measurements.



## 612 4. Conclusion

613 Triblock PEG-PLA-PFMA polymers with distinct lengths of PFMA block (5, 10 or 20  
614 fluorinated pendant chains) were successfully synthesized and were shown to adsorb at the  
615 PFOB/methylene chloride interface. These favorable fluororous interactions led to an increase  
616 of the PFOB encapsulation efficiency into nanocapsules made of fluorinated polymers  
617 compared to plain PEG-PLA. The morphology of the nanocapsules was strongly influenced  
618 by the number of perfluorinated chains and the amount of polymer used for formulation:  
619 capsules with several PFOB cores or fluorinated-rich domains are obtained at high polymer  
620 amount, while a single PFOB core and a thinner shell are observed at low polymer amount.  
621 SANS measurements confirmed the observed reduction of mean shell thickness down to 11  
622 nm with PEG-PLA-PFMA<sub>10</sub>, which led to a 3.7-fold higher *in vitro* ultrasound response at 5  
623 MHz compared to plain PEG-PLA nanocapsules. Finally, no *in vitro* cytotoxicity was induced  
624 by both the fluorinated polymers and their degradation products. Results are encouraging, and  
625 future work will consist in performing *in vivo* studies to confirm the potential of these  
626 PEGylated/fluorinated nanocapsules to be used as ultrasound contrast agents for tumor  
627 imaging.

## 628 5. Supporting information

629 Supporting information is available: scheme of the experimental set up for *in vitro* ultrasound  
630 measurements, <sup>1</sup>H NMR spectra of PEG-PLA and PEG-PLA-Br, SEC chromatograms, DLS  
631 of polymers in methylene chloride and chloroform, DSC thermograms, additional TEM and  
632 cryo-TEM images, SANS curves fitting, cytotoxicity results after 24h incubation, DLS curves  
633 of NCs (correlation and distribution functions).

634

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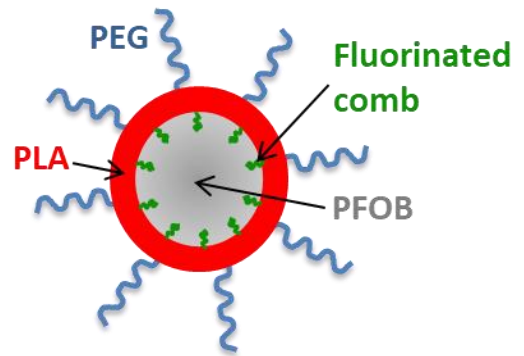
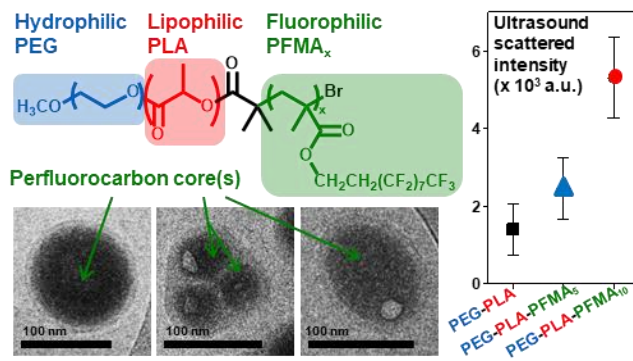
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790 **Table of Contents graphic**



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