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

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Abstract

Poly(lactide) (PLA) polymers containing five distinct lengths of fluorinated (from C_3F_7 to $C_{13}F_{27}$) and non-fluorinated (C_6H_{13}) end-groups were successfully synthesized by ring-opening polymerization of D,L-lactide. Fluorination was expected to increase the encapsulation efficiency of perfluorohexane (PFH). 150 nm nanocapsules were obtained and ^{19}F nuclear magnetic resonance revealed that nanocapsules formulated with fluorinated polymers increased by 2-fold the encapsulation efficiency of PFH compared with non-fluorinated derivatives, without any effect of fluorine chain length. Fluorination of the polymers did not induce any specific *in vitro* cytotoxicity of nanocapsules towards HUVEC and J774.A1 cell lines. The echogenicity of fluorinated-shelled nanocapsules was increased by 3-fold to 40-fold compared to non-fluorinated nanocapsules or nanoparticles devoid of a perfluorohexane core for both fundamental and harmonic ultrasound imaging modalities. In particular, an enhanced echogenicity and harmonic response was observed as the fluorinated chain-length increased, probably due to an increase of density and promotion of bubble nucleation. When submitted to focused ultrasound, both intact and exploded nanocapsules could be observed, also with end-group dependency, indicating that PFH was partly vaporized. These results pave the way to the design of theranostic perfluorohexane nanocapsules co-encapsulating a drug for precision delivery using focused ultrasound.

Keywords: Nanocapsules, perfluorohexane, fluorinated polymers, ultrasound imaging, focused ultrasound, fluorine interactions; cell viability.

1. Introduction

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Materials

D,L-lactide was purchased from Polysciences (Germany) and perfluorohexane (purity>98%) was acquired from Alfa Aesar (Germany). 1-hexanol from Acros Organics (Belgium), 2,2,3,3,4,4-heptafluoro-1-butanol, 1H,1H perfluoro-1-heptanol, 1H,1H perfluoro-1-nonanol, 1H,1H perfluoro-1-dodecanol and 1H,1H perfluoro-1-tetradecanol were acquired from Fluorochem (United Kingdom). Acetone, tetrahydrofuran (THF) and dichloromethane were purchased from Carlo Erba Reactifs (France), chloroform and diethyl ether from VWR (France). Stannous octoate, dry toluene, D₂O, sodium cholate, trifluoroacetic acid (TFA) and polyvinyl alcohol (PVA, 30-70kDa, 87-90% hydrolyzed) were provided by Sigma-Aldrich (France). Deuterated solvents (CDCl₃ and acetone-d) were purchased from Eurisotop (France), Cell culture reagents such as DMEM (Dulbecco's modified Eagle's medium), RPMI 1640 (Roswell Park Memorial Institute medium), FBS (Fetal Bovine Serum), trypsin-EDTA solution and PBS (Ca²⁺ and Mg²⁺ free phosphate buffer) were purchased from Sigma Aldrich (France). The ultrapure water was produced by a Millipore Synergy 185 apparatus coupled with a RiOs5TM (Millipore, France) with a resistivity of 18.2 MΩ.cm. The NMR sample tubes and coaxial inserts were obtained from CortecNet (France).

2.2 Polymer Synthesis

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

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

2.3 Characterization

All polymers were characterized by size exclusion chromatography (SEC) in 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 $300 \times 7.5 \text{ mm}$) calibrated against a curve based on poly(methyl methacrylate) standards (PMMA, Polymer Laboratories, Varian Inc.). The system was coupled to a refractive index detector (Spectrasystem RI-150, Thermo Electro Corp.). ^1H and $^{19}\text{F-NMR}$ spectra were obtained on a Bruker Advance 400 MHz in CDCl_3 at room temperature.

2.4 Differential Scanning Calorimetry (DSC)

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

2.5 Nanocapsules Formulation

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

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

2.6 Size and ζ -potential

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

2.7 Electron Microscopy

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

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

2.8 PFH encapsulation efficiency

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

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

2.9 Cell viability

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

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

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 $p<0.05$.

3. Results and discussion

3.1 Synthesis and characterization of fluorinated polymers

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

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

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

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

Insert Figure 2 here

Insert Table 1 here

3.2 PFH Nanocapsules

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

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

Insert Table 2 here

Insert Figure 3 here

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

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

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

Insert Figure 4 here

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

Insert Figure 5 here

3.3 *In vitro* cytotoxicity of NCs

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

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

Insert Figure 6 here

3.4 *In vitro* Ultrasound imaging

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

Insert Figure 7 here

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

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

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

Insert Figure 8 here

3.5 PFH vaporization by focused ultrasound

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

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

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

Insert Figure 9 here

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

4. Conclusion

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

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612 Tables and Figures captions

613 Table 1. Number-average molar masses (M_n) obtained by SEC/ ^1H -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. ^1H -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

between fluorinated samples in conventional mode ($p < 0.001$). In contrast specific imaging Again, significant differences were observed between fluorinated samples except for PLA- $C_{11}F_{23}$ and PLA- $C_{13}F_{27}$ which are not statistically different.

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

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