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Influence of polymer end-chemistry on the morphology of perfluorohexane polymeric microcapsules intended as ultrasound contrast agents

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ABSTRACT

Ultrasound contrast agents (UCAs) composed of a liquid perfluorocarbon (PFC) core surrounded by a polymer shell have shown promising echogenicity as well as stability. In a strategy to optimize the ultrasound properties of these systems, encapsulating a liquid PFC with a low boiling point such as perfluorohexane (PFH) was suggested. The ultimate aim of these systems would be to induce phase-transition of the liquid PFH into gas by acoustic droplet vaporization (ADV) to further increase the UCA acoustic response. Microcapsules with a perfluorohexane core have been developed by an emulsion–evaporation process, using three biodegradable polymers: PLGA and PLA with acid (PLA-COOH) and ester (PLA-COOR) terminations. Despite their similar properties, these polymers were found to strongly influence the final microcapsule morphology. While PLGA was able to form nice core–shell microcapsules, the use of PLA-COOH led to decentered microcapsules and big "eye" morphologies, and PLA-COOR induced the formation of "acorn" morphologies. To shed light on morphologies disparities, polymer interfacial behavior was studied at the dichloromethane–water and the PFH–dichloromethane interfaces. One can conclude that the core–shell structure is the result of a significant adsorption of the polymer at the dichloromethane–water interface together with a good stability of the PFH droplet within the emulsion globule. Previous work has shown that the capsule's thickness-to-radius (T/R) ratio can be controlled easily by varying the polymer to perfluorocarbon proportions. This versatility was confirmed for PFH capsules with PLA-COOH and PLGA shells. Finally, the encapsulation efficiency of PFH was assessed by relating the T/R ratio to the volume fraction of PFH and by ^{19}F NMR spectroscopy.

1. Introduction

Ultrasound is a popular imaging modality which is safe, non-invasive and cost-effective. However, due to a very weak difference of backscattering between tissues, this modality requires the use of ultrasound contrast agents (UCAs) (Correas et al., 2001). For this purpose, the echogenic properties of perfluorocarbons (PFCs) are widely known as well as the other interesting characteristics like their low-toxicity and their low solubility in blood (Correas and Quay, 1996; Schutt et al., 2003). The first commercial formulations of PFCs such as Definity® or Optison®, consists of microbubbles of gaseous PFC, stabilized by phospholipids or albumin, respectively (Diaz-Lopez et al., 2010; Kang and Yeh, 2012; Mullin et al., 2011; Quaia, 2007). Nevertheless, these agents still lack stability specially when exposed to ultrasound and consequently their blood half-life time remains short (<10 min). To promote UCA stability, research turns toward liquid perfluorocarbons, well described for their safety and biocompatibility as oxygen carriers (Lowe, 2003). These compounds were formulated in different systems such as emulsions stabilized by lipids (Kornmann et al., 2008) or surfactants (Grayburn, 1997) and polymer capsules (Diou et al., 2012; Pisani et al., 2008a, 2006; Valette et al., 2012). Among these objects, UCAs composed of a polymer shell encapsulating liquid perfluorocarbon droplets such as perfluorooctyl bromide exhibited a longer-lasting signal-to-noise ratio (SNR) (Pisani et al., 2008b) as well as a better resistance to

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pressure and mechanical fluctuations. Despite their echogenic properties, the signal arising from these capsules remains low, and there is room for improvement.

In a strategy to benefit from polymer capsule stability while enhancing echogenicity, encapsulating a liquid PFC of lower boiling point was proposed. The aim being afterwards to vaporize liquid PFC by an ultrasound process called acoustic droplet vaporization (ADV) (Kripfgans et al., 2000). Above a certain threshold value of acoustic energy, the PFC droplet would become gaseous, therefore increasing their echogenicity. These in situ-created microbubbles are highly echogenic and can trigger a drug release locally (Fabiilli et al., 2010), and also revealed to be of interest for occlusion therapy (Kripfgans et al., 2002, 2005), thermal therapy (Zhang et al., 2010) and targeted tissue penetration via nanomachines (Kagan et al., 2012).

To confer ADV properties to polymer capsules, perfluorooctyl bromide was replaced by perfluorohexane (PFH) as the latter possesses a lower boiling point (56 °C at 1 atm). Microcapsules with a PFH core have been formulated by an emulsion–evaporation process using polylactide (PLA) or poly(lactide-co-glycolide) (PLGA) biodegradable and biocompatible polymers (Shive and Anderson, 1997) as described previously (Pisani et al., 2006). The influence of polymer hydrophobicity (PLA versus PLGA) or end-chemistry (COOH versus COOR) on the final morphology has been investigated in detail and correlated to interfacial tensions of the three phases in the presence of aqueous solution of surfactant, methylene chloride and PFH. Finally, the ability to modulate the capsule's thickness-to-radius (T/R) ratio was investigated for PLA-COOR and PLGA. The encapsulation efficiency of PFH of all types of capsules was also assessed by confocal microscopy imaging and ^{19}F NMR spectroscopy (Diou et al., 2012).

2. Experimental

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA) Resomer RG502 (intrinsic viscosity 0.16–0.24 dL/g; Mn = 7000–17,000 g/mol; ester terminated), poly(D,L-lactide) (PLA-COOH) Resomer R203H (intrinsic viscosity 0.25–0.35 dL/g; Mn = 18,000–28,000 g/mol; acid terminated) and poly(D,L-lactide) (PLA–COOR) Resomer R202S (intrinsic viscosity 0.16–0.24 dL/g; Mn = 10,000–18,000 g/mol; ester terminated) were provided by Evonik Röhm Pharma GmbH (Germany). Sodium cholate (SC), poly(vinyl alcohol) (PVA) (M_w 30,000–70,000, 89% hydrolyzed), and nile red were purchased from Sigma–Aldrich (France). Perfluorohexane (PFH) and Perfluoro-15-crown-ether (PFCE) were obtained from Fluorochem (United Kingdom). Methylene chloride RPE-ACS 99.5% was provided from Carlo-Erba Reactifs (France). Water was purified using a RIOS/Milli-Q system (Millipore, France).

2.2. Microcapsules preparation

The desired amount of polymer was dissolved into 4 mL of methylene chloride, along with 60 μL of PFH. To obtain microcapsules, this organic solution was then emulsified into 20 mL of 1.5% sodium cholate (w/v) aqueous solution using an Ultra-Turrax T25 (IKA) operating with a SN25-10G dispersing tool, at a velocity of 8000 rpm. Emulsification was carried out in a 50 mL glass beaker placed over ice for 1 min. Methylene chloride was then evaporated under magnetic stirring at 300 rpm for about 3 h in a thermostated bath at 20 °C. For fluorescent and confocal microscopy, nile red was added to the organic solution before emulsification. For storage, the fresh capsules were frozen in liquid nitrogen to avoid sedimentation and freeze-dried for 24 h using Alpha 1-2LD Plus apparatus (Christ).

2.3. Bright field and fluorescence microscopy

Suspensions in water were placed between glass slides and observed with a Leitz Diaplan microscope fitted with a Coolsnap ES camera (Roper Scientific). Fluorescent samples dyed with nile red were excited at 543 nm and observed at 560 nm (long pass filter).

2.4. Confocal microscopy

The samples mixed with glycerol to avoid capsules motion were observed with a Zeiss LSM-510 confocal scanning microscope equipped with a 1 mW helium neon laser, and using a Plan Achromat 63× objective (NA 1.40, oil immersion). The pinhole diameter was set at 71 nm. Stacks of images were gathered every 0.42 μm along the z-axis. The microcapsules' sizes and thicknesses were measured directly on the confocal images using the Zeiss LSM Image Browser software, considering that the size of a pixel was 70 nm. The measurements were performed in the equatorial plane of each capsule to minimize the error due to the position of the slice on about hundred capsules per sample.

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using a LEO 1530 (LEO Electron Microscopy, Inc., Thornwood, NY) operating at 1 kV with a filament current of about 0.5 mA. The microcapsules were washed twice by centrifugation to remove the excess sodium cholate before imaging. The liquid samples were deposited on carbon conductive double-sided tape (Euromedex, France) and dried at room temperature. They were then coated with a palladium–platinium layer of about 3 nm using a Cressington sputter-coater 208HR with a rotary-planetary-tilt stage, fitted with an MTM-20 thickness controller.

2.6. Granulometry

The size measurements on microcapsules were performed using a Malvern Mastersizer 2000 granulometer based on laser diffraction, and equipped with a wet dispersion unit (Hydro 2000SM). Small amounts of capsules suspension were added to MQ water in the measurement cuvette until getting a laser obscuration between 10 and 20%. The measurements were carried out in triplicate. The size distribution was evaluated using either the Fraunhoffer or the Mie model, according to the size estimated with microscopy.

2.7. Interfacial tension measurements

The interfacial tension measurements were carried out using the pendant drop method, employing a Tracker tensiometer (Teclis, France). Drops of PFH, methylene chloride with or without polymers or a mixture of both solutions were formed using a syringe and a G18 stainless steel needle into an aqueous solution containing or not sodium cholate placed in an optical glass cuvette. The temperature was maintained at 20 °C. The interfacial tension was determined from the drop profile using the Laplace equation and the forces balance between capillarity and gravity. The measurements were performed on at least ten independent drops. To ensure reproducibility, the measurements were divided in two sessions, between which the syringe was cleaned and refilled.

2.8. Encapsulation efficiency determination by ^{19}F NMR spectroscopy

The microcapsules lyophilisates were dissolved into a deutered chloroform solution containing Perfluoro-15-crown-ether (PFCE) as an internal standard at 0.74 mmol/L. To ensure accuracy and