Ultrasound-Responsive Microdroplets for Multi-Drug Delivery Applications

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***Abstract*—The controlled release of multiple drugs represents an exciting avenue that can be exploited for targeted therapies and for promoting tissue regeneration. In this context, perfluorocarbon droplets are promising ultrasound-responsive carriers, which can release their cargo upon acoustic droplet vaporization. In this study, we investigate the release behavior of two different droplet types, in response to low-frequency (38 kHz) and low-pressure (50-100 kPa) ultrasound. These two droplet formulations, consisting of the same perfluoropentane core but different shells (phospholipid, surfactant-based), are designed to respond to different US pressures. Therefore, they can be triggered separately by modulating the acoustic pressure, potentially enabling the ultrasound-controlled, temporally independent delivery of two drugs.**

Keywords—perfluorocarbon droplets, drug delivery, low-frequency ultrasound, acoustic droplet vaporization

I. Introduction

Tissue regeneration is a complex process involving multiple stages, biological pathways and a constantly changing environment. Therefore, multifunctional therapies are significantly more effective than treatments providing single cues and acting on single aspects of the regenerative processes. Furthermore, proper timing is crucial for providing the right cues at the most appropriate stages of the regeneration process, maximizing drug efficacy and reducing harmful side effects [1]. Similar considerations apply for certain targeted therapies, in which the alternation of different drugs and the timing of their delivery is crucial (e.g, in chronotherapies). For this reason, delivering multiple drugs and having external control over their release can significantly increase the therapeutic outcomes. In this context, smart and multifunctional systems that can perform multiple actions in response to an external triggering biophysical stimulus, are extremely promising platforms for tissue regeneration [2]. Among biophysical stimuli, ultrasound (US) is particularly promising, as it represents a non-invasive, safe and easily tunable trigger, capable of penetrating deep into tissues and already approved for clinical use in multiple applications [3]. Among ultrasound-responsive carriers, perfluorocarbon (PFC) droplets offer significant advantages, as they exhibit higher stability than microbubbles, good tunability and a sharp on-off response to US [4].

PFC droplets consist of a liquid core, based on PFCs, and a stabilizing shell, which can keep the core stable in the liquid state even at temperatures above its nominal boiling point [4]. PFC droplets have been widely investigated over the last two decades, mainly as contrast agents in US imaging, but also for therapeutic purposes, such as in histotripsy, embolotherapy, drug delivery and theranostics [5]. One of their most interesting features is their responsiveness to US: indeed, when subjected to sufficient negative pressure, the PFC core undergoes a liquid-to-vapor phase change, a phenomenon called acoustic droplet vaporization (ADV), which is accompanied by a rapid volume expansion [6]. This can result in the release of the loaded cargo, making PFC droplets efficient US-triggerable drug carriers.

In the literature, a wide range of droplet formulations has been investigated, but there is no clear consensus over the best US parameters for ADV activation across different formulations. Moreover, the fact that US stimulation is often performed in non-standardized and non-controlled configurations hinders the possibility of comparing different studies and results. Furthermore, the vast majority of studies use US in the MHz range, employing very high pressures (several MPa), which may pose safety concerns in view of a preclinical or clinical translation [7-8]. In the last years, however, some studies have explored lower frequencies, below 1 MHz, unveiling an interesting potential in reducing the activation pressure of PFC droplets and lowering the mechanical index employed [9-10]. In a recent study, we demonstrated that it is possible to trigger efficient release from a stable droplet formulation, consisting of a perfluoropentane (PFP) core and a surfactant shell, using low-intensity pulsed US (LIPUS) at 38 kHz and 100 kPa [11]. The use of such a low frequency, which has never been investigated for this application but is already approved for clinical use in physiotherapy settings, allowed to lower the activation pressure of this stable PFC droplet formulation of one order of magnitude compared to the pressures commonly used in literature.

Here, we investigated a new formulation of microdroplets, with the aim of activating them with low-frequency and low-pressure US, using a different set of parameters with respect to our first formulation. In particular, we modified the previously developed formulation (PFP+P188) [11], changing the composition of the shell to a phospholipid-based one (PFP+L), which is known in literature for being less rigid and robust with respect to surfactant-based shells [12]. The production of this new droplet formulation (PFP+L) was optimized and characterized. Furthermore, we explored its response to 38 kHz US stimulation and compared it with the response of the PFP+P188 formulation, with the final aim of creating a

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potential dual system for US-controlled sequential and independent release of two drugs.

II. Materials and Methods

A. Droplet Synthesis and Characterization

Both droplet types have a PFP core, encapsulating fluorescein sodium salt as a drug model, but different shell composition: phospholipid-based (PFP+L) and surfactant-based (PFP+P188), respectively.

The PFP+P188 formulation was synthesized as described in our previous work [11]. The PFP+L formulation consisted of the same core, but a different shell solution. Briefly, the core consisted of a mixture of liquid PFP and a triblock fluorosurfactant, emulsified with an aqueous solution of fluorescein sodium salt (FSS). The lipidic shell solution was synthesized starting from a protocol from literature, optimized for the purpose [13]. 5 mg of DSPC (CAS 816-94-4, Sigma Aldrich) were added to 0.150 mL of DSPE-PEG2000 (CAS 474922-77-5, Sigma Aldrich) dissolved in chloroform (10 mg/mL). The chloroform was evaporated under nitrogen, after which 5 mL of deionized water were added, and the resulting solution was sonicated for 20 minutes in an ultrasonic bath. Subsequently, 4.7 mL of deionized water, 0.2 mL of glycerol (CAS 56-81-5, Sigma Aldrich), 0.1 mL of propylene glycol (CAS 57-55-6, Sigma Aldrich) and 1 mL of a 100 mg/mL Kolliphor P188 solution were added, and this solution was sonicated for 10 minutes. The droplets were synthesized through the same microfluidic setup used for the PFP+P188 formulation, employing a commercial microfluidic chip (Cat#: 3200146, quartz, junction 14 x 17 µm, hydrophilic coating, Dolomite) and pumping the core emulsion and shell solution in its inlets at a flow rate of 2 and 10 µL/min, respectively.

High-resolution optical microscopy (HRX-01 Digital Microscope, Hirox) was employed to assess the morphology of the PFP+L droplets in suspension. Data on size and polydispersity were extracted from the images using MATLAB. The encapsulation efficiency of FSS was quantitatively evaluated using a multiwell plate reader (VICTOR Nivo Multimode Plate Reader, PerkinElmer) to assess FSS concentration in the supernatant.

B. Stability tests

The stability of the PFP+L droplets suspension in storage conditions (4°C) was evaluated over three days. At each timepoint (day 0, 1 and 2), the passive leakage of FSS was evaluated through fluorescence analysis using the multiwell plate reader, and the concentration and size of the droplets were monitored through high-resolution optical microscopy.

C. Ultrasound-triggered drug release tests

All droplets were stimulated using low-intensity pulsed US with the following parameters: frequency 38 kHz; pressure ranging from 25 to 100 kPa (rms), with a 25 kPa step; duty cycle (DC) 20%; pulse repetition frequency (PRF) 100 Hz; time 5 min. Stimulation was carried out using a patented system designed to minimize perturbations to the ultrasound wave propagation and thus have optimal control over the ultrasound dose reaching the target [14]. The system consisted of a tank filled with degassed water at 37 $°C$, a housing for three transducers and a housing for the positioning of a sealed and US-transparent sample-retaining disk, covered by an acoustic absorber. Piezoceramic transducers centered at 38 kHz (SIRIO, BAC Technology), driven by a commercial single-channel signal generator (SIRIO, BAC Technology), were previously characterized using a calibrated hydrophone, both in terms of pressure field mapping and the relationship between driving voltage and resulting US pressure.

After stimulation, Hirox microscopy was used to assess morphological changes in the samples. Drug release was evaluated by performing fluorescence analysis on filtered samples, in order to separate the remaining droplets from the supernatant, which was analyzed through a multiwell plate reader. The concentration of FSS in the supernatant of the stimulated samples was compared to the total FSS embedded, determined from a positive control, for calculating the percentage of FSS release induced by US. For all the conditions tested, a negative control underwent the exact same treatment than the stimulated samples.

D. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8. No outliers were identified in any of the analyses performed, and all data were normally distributed according to Shapiro-Wilk test. Comparisons were performed using the one-way ANOVA test with multiple comparisons (Tukey's post-hoc test). A p-value lower than 0.05 was considered as statistically significant. P-values were categorized as *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, and ****: $p < 0.0001$.

III. Results

A. Droplet Synthesis and Characterization

The PFP+L droplets had an average diameter of 7.8 µm, with a low polydispersity index of 0.012 (Fig. 1a). Encapsulation efficiency was found to be 86 ± 0.4%, which means that less than 15% of the total FSS loaded remained non-encapsulated within the droplets.

B. Stability tests

The concentration of PFP+L droplets in suspension showed a decreasing trend over time, with a marked drop at day 2 (Fig. 1b). This suggests that some of the droplets may undergo spontaneous vaporization, thereby disappearing from the sample. Conversely, the drug content in the supernatant increased in time, reaching values of passive leakage above 50% at day 2 (Fig. 1c). These results are consistent with the concentration data, since it is reasonable to think that the spontaneous vaporization of a droplet leads to the release of its content.

C. Ultrasound-triggered drug release tests

PFP+L droplet samples were stimulated at 38 kHz, the same frequency that induced the most efficient drug release from PFP+P188 droplets, within the same pressure range (25-100 kPa). As shown in Fig. 2a, which reports the quantitative drug release from PFP+L droplets in the whole pressure range, a pressure as low as 25 kPa did not induce any effect, displaying no significant difference with respect

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to the negative control. However, at 50 kPa, samples stimulated with US showed a significantly higher release, around 28%. This value remained mostly unvaried also for pressures higher than 50 kPa, such as 75 and 100 kPa. The

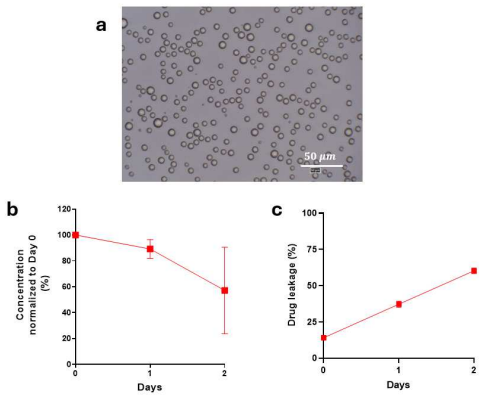


Fig. 1. Representative optical microscopy image of PFP+L droplets suspension after production (a); stability of the PFP+L droplets in suspension at 4°C over 3 days, in terms of droplet concentration (b) and passive drug leakage (c).

quantitative release data are also supported by the optical microscopy images of the samples, reported in Fig. 3: while the sample stimulated at 25 kPa presented a very similar aspect with respect to the unstimulated sample (Fig 3a-b), the samples stimulated at 50 kPa pressure or higher all showed a significant reduction in droplet number and a marked fragmentation of the remaining droplets (Fig 3c-e).

Considering these results, the PFP+L formulation clearly showed a threshold behavior in its response to low-frequency US stimulation, reflecting a typical ADV behavior, exactly as the PFP+P188 formulation did. However, for PFP+L droplets the activation threshold appeared to be between 25 and 50 kPa, whereas for PFP+P188 droplets the threshold was found to be between 75 and 100 kPa (Fig. 2b) [17].

IV. Discussion

In this work, we started from a previously developed droplet formulation (PFP+P188), which was based on a perfluoropentane core and a surfactant shell, and characterized by high stability and a US activation threshold around 100 kPa at 38 kHz [11]. It is known from literature that using cores based on PFCs with different boiling points has a strong influence on the modulation of the US activation threshold [15]. However, this is true at high frequencies (several MHz), which implies the use of

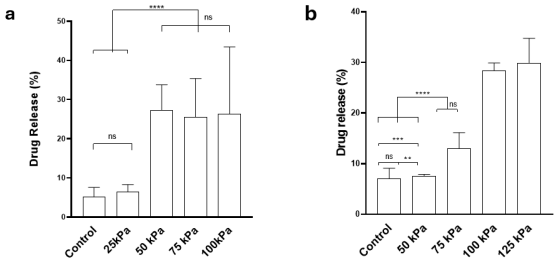


Fig. 2. Drug release from PFP+L droplets (a) and PFP+P188 droplets (b) under US stimulation at 38 kHz at different pressures (25-125 kPa).

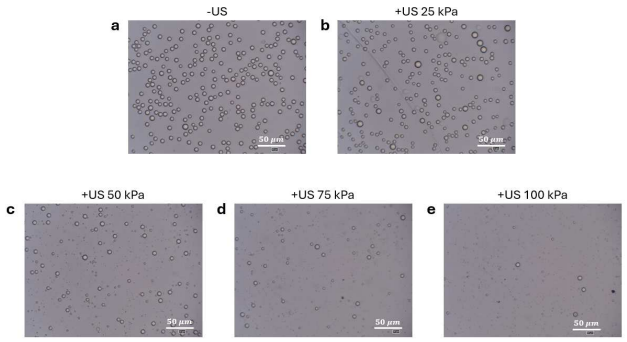


Fig. 3. Optical microscopy images of the stimulated PFP+L samples.

very high pressures. Conversely, it has been found that at lower frequencies (i.e., 300 kHz), the effect of the core boiling point on the activation threshold is almost negligible [9]. This is why we have decided to play with the shell composition in order to change the activation threshold. Indeed, we chose a phospholipid shell, with is less robust with respect to surfactant-based shells, which could result in a lower ADV threshold [4,12].

For droplets production, we used a previously optimized microfluidic technique, which has the advantage of providing repeatable results and highly homogeneous droplet populations, with respect to other techniques [6]. Here, microfluidic production generated satisfactory results in terms of monodispersity and efficient encapsulation. The flow rates reported in this study (2 µL/min for the core and 10 µL/min for the shell) were optimized starting from a wider range. Other two droplet families have been investigated, produced with twice and half these flow rates, keeping constant the core-to-shell flow rate ratio. However, the family produced with 1 and 5 µL/min rates resulted to be too unstable, passively releasing more than 80% of its content over 3 days. This might be due to a poor arrangement of the phospholipid chains in the microfluidic chip junction, where the droplets are formed. Instead, the family produced with 4 and 20 µL/min rates appeared to have a higher US activation threshold, showing no release when stimulated at 50 kPa. This might be due to their significantly smaller size, which might correlate with a higher ADV threshold [15].

Compared to PFP+P188 droplets, which remained unaltered in number and passively released less than 10% of their content over 7 days at 4°C, the PFP+L formulation exhibited lower stability. This was expected, since a phospholipid shell is more porous than a compact surfactant layer, providing a less efficient barrier against diffusion. Furthermore, a phospholipid shell, being less rigid, also tends to exert a lower Laplace pressure on the core, having a poorer stabilizing performance and making droplets more susceptible to spontaneous vaporization. It is difficult to make a direct comparison with the literature on these data, since there are no studies assessing stability at 4°C of similar droplet formulations in terms of droplets concentration and passive drug release. However, we believe that it might be possible to improve the stability of this formulation to some degree, by optimizing the shell composition.

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The frequency selected for the US-triggered drug release experiments, 38 kHz, is unconventional in the field of drug delivery. However, it has previously been shown to efficiently induce ADV-driven drug release from a similar PFC droplet formulation (PFP+P188), at very low and safe pressures (100 kPa) [11]. In this study we exploited the same US frequency, exploring the response of the PFP+L formulation within the same pressure range (25-100 kPa). We demonstrated that the shell composition indeed influences the ADV threshold: US at 50 kPa induced rupture and fragmentation of the lipid-shelled droplets, resulting in a 28% release, while it had no effect on surfactant-shelled droplets. To trigger an efficient release (33%) from the latter, a higher pressure of 100 kPa was required. Therefore, PFP+L and PFP+P188 represent two droplet formulations that can be activated with two different US stimulation protocols, at the same frequency but at different pressure levels, while keeping all the other parameters fixed (DC, PRF, time), which is advantageous in a clinical setting.

Therefore, sequential delivery of two drugs could be achieved with this approach. For example, it would be possible to first activate only the phospholipid-shelled droplets, through US at 50 kPa, inducing the release of a first drug; in a second moment, a second drug could be delivered from the surfactant-shelled droplets in response to 100 kPa stimulation. The negligible response to 50 kPa stimulation and the high stability of PFP+P188 droplets would ensure a good retention of the second drug until activation, guaranteeing an efficient release at the needed moment. In future work, dual drug delivery tests will be performed to demonstrate this potential and to further investigate the interactions between these two droplet formulations when used in combination.

V. Conclusions

In this study, we developed a microdroplet formulation based on a fluorescein-loaded PFP core and a phospholipid shell. We optimized the production of this PFP+L formulation and characterized it in terms of stability and response to low-frequency US. As expected, PFP+L droplets were found to be less stable than the previously developed PFP+P188 droplets, and also responsive to lower US pressures. Indeed, efficient release was obtained from PFP+L droplets at 38 kHz and 50 kPa, while 100 kPa were necessary to obtain a comparable release from PFP+P188 ones. It is worth remarking that both formulations can be triggered with very low and safe US doses, which could significantly facilitate clinical translation. The different ADV thresholds of these formulations make them promising for multi-drug delivery applications, where the local and temporized delivery of two different drugs is beneficial, such as in many tissue regeneration applications (e.g., wound healing, myocardial repair). With this approach, externally controlled dual delivery could be achieved with a single type of stimulus (i.e., US), by just modulating the acoustic pressure, while ensuring safety for the surrounding tissues. A potential application scenario could involve a patch, in which the two droplet families are embedded in a soft matrix, serving the dual purpose of protecting the surrounding tissues from the mechanical effects of ADV and limiting the passive drug release from PFP+L droplets, thereby maximizing the therapeutic efficacy of the US-induced release.

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