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Counterbalancing the use of ultrasound contrast agents by a cavitation-regulated system

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ABSTRACT

The stochastic behavior of cavitation can lead to major problems of initiation and maintenance of cavitation during sonication, responsible of poor reproducibility of US-induced bioeffects in the context of sonoporation for instance. To overcome these disadvantages, the injection of ultrasound contrast agents as cavitation nuclei ensures fast initiation and lower acoustic intensities required for cavitation activity. More recently, regulated-cavitation devices based on the real-time modulation of the applied acoustic intensity have shown their potential to maintain a stable cavitation state during an ultrasonic shot, in continuous or pulsed wave conditions. In this paper is investigated the interest, in terms of cavitation activity, of using such regulated-cavitation device or injecting ultrasound contrast agents in the sonicated medium. When using fixed applied acoustic intensity, results showed that introducing ultrasound contrast agents increases reproducibility of cavitation activity (coefficient of variation 62% and 22% without and with UCA, respectively).

Moreover, the use of the regulated-cavitation device ensures a given cavitation activity (coefficient of variation less 0.4% in presence of UCAs or not). This highlights the interest of controlling cavitation over time to free cavitation-based application from the use of UCAs. Interestingly, during a one minute sonication, while ultrasound contrast agents progressively disappear, the regulated-cavitation device counterbalance their destruction to sustain a stable inertial cavitation activity.

1. Introduction

Even if the presence of gas bubbles is usually highly undesirable *in vivo* (in bloodstream for instance), a growing number of biomedical applications are based on the benefit of using microsized bubbles. For such applications, ultrasound contrast agents (UCAs) can be injected intravenously. UCAs consist in small (diameter < 10 μm) gas-filled and encapsulated bubbles. Since the first generation of this kind of microbubbles (Echovist, Levovist, or Albunex), adapting the gas content and shell materials led to new generations of UCAs with an improved stability and echogenicity, such as SonoVue (Bracco International Imaging, Milan, Italy) or Definity (Lantheus Medical Imaging, North Billerica (MA), USA). They have been widely used for ultrasound imaging to enhance the acoustic contrast between blood and surrounding tissues [1], and are extensively studied for therapeutic applications such as sonothrombolysis [2] and sonoporation [3,4].

In the context of ultrasound-mediated sonoporation, the first advantage of the presence of UCAs is to provide cavitation nuclei and hence, to reduce the pressure required to produce cavitation bubbles and cavitational effects [5]. Particularly, inertial bubble collapse, which is commonly admitted to be one of the main mechanism underlying cell sonoporation [6], is enhanced. While the collapse of a free bubble is governed by the inertia of the surrounding fluid, the linear and nonlinear acoustic response of UCAs is dominated by the stiffness and viscosity of its shell [7]. The acoustic behavior of UCAs in the nonlinear regime has been recently investigated both theoretically [8–10] and experimentally by acoustic and optical measurements [11–15] to describe their destruction thresholds in terms of fragmentation, cavitation, rupture and collapse. The second advantage of using UCAs is the ability to release them at a target site, providing an excellent delivery vehicle for localized cavitation activity [16].

Even if it is recognized that shelled microbubbles have potential for US-induced cavitation activity and resultant bioeffects, both *in vitro* [17–19] and *in vivo* [20], the use of UCAs presents several drawbacks: they are not available worldwide [21] and destructive

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effects of UCAs have been reported, such as hemorrhaging [22] and lysis [23].

In consequence there is an interest in developing alternative technologies based on *in situ* induction of cavitation bubbles without using ultrasound contrast agents. Most studies on ultrasound cavitation are performed at a fixed acoustic intensity, making results difficult to analyze due to the stochastic behavior of cavitation [24]. Recent studies deal with the design of cavitation-based monitoring and control, in continuous or pulsed-wave conditions [25,26]. These technologies rely on the real-time modulation of the applied acoustic intensity allowing to maintain a target cavitation level. Such regulated-cavitation devices have shown their efficiency in terms of cavitation initiation [25], accurate control of cavitation over the sonication time [26], as well as resulting bioeffects [6]. Considering these technological developments, the question arises of the real interest of using ultrasound contrast agents for applications such as transfection, particularly *in vitro*.

Thus this paper aims to investigate the interest of using *in vitro* a regulated-cavitation device in comparison to the introduction of UCAs in the sonicated medium. This comparison is performed by studying the cavitation activity reproducibility and the acoustic intensity supplied to the medium. The combined use of a regulation device and UCAs is also tested. Finally sonicated UCAs behavior is characterized and discussed.

2. Material and method

A schematic diagram of the experimental apparatus is shown in Fig. 1. Two plane piezoelectric transducers (Ferroperm PZ 26, frequency 426.5 kHz, dimensions 18×3 mm) face each other on the two opposite sides of a chambered coverglass (Lab-Tek, Thermo Scientific Nunc, NY, USA) of dimensions 20×20 mm. The signal supplied to the transducers is synthesized within a FPGA system (Field-Programmable Gate Array, NI PXIe-7965R card, Austin, TX) and is provided by the Digital-to-Analog Converter of the FPGA module (16-bit, 100 MHz sampling frequency, NI-5781R module), subsequently amplified by a power amplifier (24 V, 4.8 A, 50 MHz, Kalmus). The transducers generate 426.5 kHz sinusoidal pulsed ultrasonic waves with a period of 250 ms and a duty cycle of 0.2 (200 ms time off). The acoustic intensities in the culture well may vary from 0 to 7 W/cm^2. A flush-mounted needle hydrophone (Onda HNR-0500) located on another side of the well passively listens the inertial cavitation activity in the sonicated medium. The hydrophone signal is amplified (Müller preamplifier, 16 dB) and acquired by the Analog-to-Digital Converter device of the FPGA module (14-bit resolution, 100 MHz sampling frequency, NI-5781R module). The inertial cavitation activity is quantified using a cavitation index $\xi(t)$, calculated by estimating the broadband noise enhancement during the sonication of the medium [26]. The broadband noise is evaluated as the mean arithmetic value of the overall frequency dB magnitude of the hydrophone signal spectra. It is worth noting that, by applying a logarithmic scale before calculating the mean value, the weight of the harmonics, sub-harmonics and ultra-harmonics due to non-inertial cavitation is minimized, and their contribution within the $\xi(t)$ values is negligible. The designed cavitation device is consequently based on the acquisition of the hydrophone signal which is performed in every feedback loop (i.e. every 300 μs). Also based on this work, two sonication strategies are considered in this study: the open loop case (OL) for which the electrical voltage supplied to the transducer is fixed, and the closed loop case (CL) for which the electrical voltage supplied to the transducer is modulated in real-time to maintain a constant level of inertial cavitation in the sonicated medium and to reach a target cavitation index ξ^f. For both strategies, electrical voltages $u(t)$ supplied to the transducers, cavitation index $\xi(t)$ evolutions, and acoustic spectra are saved during the experiments.

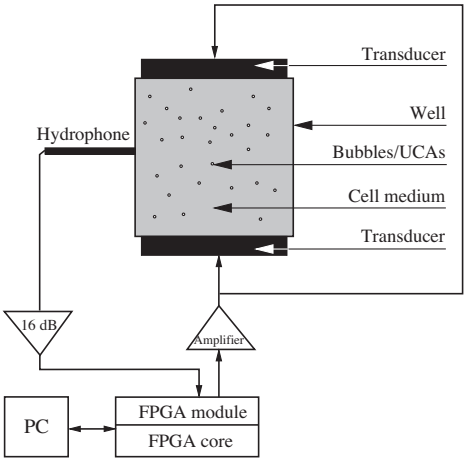

Fig. 1. Schematic diagram of the experimental apparatus.

The sonicated medium is composed of 2 mL of RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 200 UI/mL of penicillin and 200 μg/mL of streptomycin. All reagents were purchased from Invitrogen (Carlsbad, CA, USA). This medium is stored in a refrigerator for a few days after being made. The initial O_2 concentration in the medium is 7.88 ± 0.2 mg/L. The UCAs used for the experiments are SonoVue (Bracco International Imaging, Milan, Italy), i.e. sulfur hexafluoride microbubbles surrounded by a phospholipid shell with a mean size of 2.5 μm [27]. SonoVue are dissolved into 5 mL NaCl 0.9% solution, and 10 μL of freshly made solution, corresponding to a bubble concentration of 2.5 10^6 bubbles/mL, are added into each well just before sonication. All experiments are made at the same conditions of atmospheric pressure (1020 hPa) and temperature (23 °C).

The experimental protocol consists in comparing the cavitation index $\xi(t)$ measured by the hydrophone into the medium with or without UCAs for various target cavitation indexes ξ^f or various acoustic fixed intensities I_a, with three replicates on a day for each condition, and repeated on two different days. To avoid accumulation of UCAs in the culture well, the cell medium and UCAs are removed between each measurement, and the wells are rinsed five times with ultrapure water. Data are analyzed with the non-parametric Kruskal–Wallis test. For all statistical tests, the significance level (alpha) is set to 0.05 and calculations are performed using Statistica 8.0 software (Stat soft, Inc.).

3. Results

To evaluate the reproducibility of the measurements, Fig. 2(a–d) present the evolution of the mean cavitation index <ξ> over the 60 s sonication for each of the two days of measurements, and for all combinations of conditions used in this study: with or without UCAs in the cell medium, and with (CL) or without (OL) regulation of the inertial cavitation activity.

Without regulation and no UCAs [Fig. 2(a)], there are significant differences on <ξ> values between the two days of measurements ($p < 0.05$ for all the acoustic intensities). Without regulation and with UCAs [Fig. 2(c)], statistical differences are observed for three acoustic intensities: $p = 0.049$ for $I_a = 0.7, 1.0,$ and 5.5 W/cm^2. Fig. 2(a) shows that, without regulation of the cavitation activity (OL), the standard deviation varies from 3% to 62% (day 1) and from 1% to 28% (day 2) of the <ξ> value when there are no UCAs in the cell medium. When UCAs are present in the sonicated medium [Fig. 2(c)], the standard deviation varies from 1% to 22% and from