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Acoustic characterization of a new trisacryl contrast agent. Part I: In vitro study

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Abstract

The objective of the study was to acoustically characterize trisacryl polymeric microparticles (TMP), which are derived from biocompatible embolic agents.

With significant acoustic properties, these polymeric particles could be potentially used as targeted ultrasound contrast agents, directed towards a specific site, with ligands conjugation on the polymeric network surface. In the in vitro study, a pulser/receiver (PRF of 1 kHz), associated to different transducers (5, 10 and 15 MHz), was used to measure the acoustic properties of the TMP inserted in a Couette flow device. Acoustic characterization according to TMP concentration (0.12–15.63 mg/ml), frequency (4.5–17 MHz, defined by each transducer bandwidth), ultrasound pressure (137–378 kPa) and exposure time (0–30 min) was conducted. Particle attenuation was also evaluated according to TMP concentration and emission frequency. Backscattering increased non linearly with concentration and maximum enhancement was of 16.4 dB $\pm$ 0.89 dB above 7.8 mg/ml. This parameter was found non-linear with increasing applied pressure and no harmonic oscillation could be noticed. Attenuation reached approximately 1.4 dB/cm at 15 MHz and for the 15.6 mg/ml suspension.

The TMP have revealed in vitro ultrasound properties comparable to those observed with known contrast agents, studied in similar in vitro systems. However, such set-ups combined with a rather aqueous suspending medium, have some limitations and further investigations need now to be conducted to approach in vivo conditions in terms of flow and blood environment.

Keywords: Ultrasound; Contrast agent; Polymeric microparticle; Trisacryl; In vitro; Backscattering enhancement

1. Introduction

Neoangiogenesis, defined as the formation of new vessels from existing vessels, is a key stage in the development of malignant tumors. Within tumors above 2-mm-diameter, the existing vascular network is not sufficient anymore and angiogenic substances are secreted to induce the creation of new vessels. The invasive potential of a tumor is thus strongly linked to its vascularization [1]. Noninvasive imaging techniques assessing tumor angiogenesis are currently being developed to diagnose primary and metastatic disease and to assess prognosis. New methods for evaluating angiogenesis, such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) or positron emission tomography (PET), mostly rely on the detection of tumoral perfusion, microvascular blood volume, or vascular permeability [2]. Among these noninvasive modalities, Doppler ultrasonography allows both morphological studies and accurate analysis of tumor vascularity. Moreover, the use of contrast agents for fifteen years, has strongly improved the sensitivity of ultrasound (US) imaging by increasing intratumoral vessels detectability. Indeed, these agents induce larger ultrasound backscattering than red cells and

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are thus able to optimize visualization through better detection of blood flow in micro-vessels [3] and perfusion while also allowing tumor blood flow and blood volume measurement [4]. The enhanced sensitivity of US imaging relies on the physical properties of these contrast agents. Usually, they consist of micron-sized gas-filled stabilized bubbles compatible with blood thus presenting an interface between two materials with different acoustic impedances. The first step in the development of an ultrasound contrast agent involves the investigation of its physical and acoustic properties. These include ultrasound attenuation, backscattering enhancement of different concentrated suspensions as well as potential non-linear properties. In this development process, it is also essential to work first on an in vitro set-up in order to perform characterization studies under controlled and stable conditions.

Some of recently developed agents have been approved for clinical applications [5], for instance Sonovue® and Levovist® in Europe, and have shown particular promise for generating high echogenicity. These agents have actually been widely used in echocardiography for noninvasive assessment of myocardial perfusion [6], or in tumor perfusion imaging to evaluate early treatment efficiency [7]. However, none of the contrast agents available today are tissue-specific and they usually flow passively within the blood circulation once they are intravenously administered. A number of particle suspensions are currently under development either as non-specific or as targeted ultrasound contrast agents [8]. By targeting, for instance, the vascular endothelium in tumors, some US contrast agents have been shown to be useful by specifically improving the angiogenesis detection sensitivity: indeed, targeted agents are able to access to events occurring within the vascular compartment such as diameter variation, vascular density and up-regulation of some receptor molecules [9]. As an application, they have proved of great interest in enhancing the distinction between normal and pathologic or inflammated tissue [10] and in the selective delivery of drugs to an area of interest [11].

Among the various investigated systemic contrast agents, polymeric particles are often chosen for their known biocompatibility. Moreover, this class of chemical formulation allows an easy surface link with antibodies or ligands that can specifically bind to endothelial cell adhesion molecules. Trisacryl particles are hydrophilic and microporous beads made of a cross-linked acrylic copolymer. Initially developed in France by Biosphere Medical (Roissy-en-France, France), they have been approved in 2001 by the Food and Drug Administration for embolization treatment of hypervascular tumors [12]. These embole particles are inert spheres that have demonstrated biocompatibility, further confirmed by the use of different cell lines such as BHK [13], HeLa Ohio, HeLa Oxford and HTC [14]. In vitro and in vivo studies in rats and rabbits [13,15] have also shown that these emboles are rather deformable, tend not to aggregate and do not produce toxic tissue reaction [16]. Moreover, preliminary clinical studies have proved the particles' safety in the embolization of highly vascularized tumors and vascular malformations [16] with non-circulating particles.

Since cyanoacrylate particles (SHU 563 A) had already demonstrated significant ultrasound properties [17], we explored the inherent US imaging properties of the trisacryl material and first studied the backscattering enhancement of trisacryl particles in the range of 200– 1000 μm. Preliminary measurements revealed high acoustic properties up to 30 dB, involving useful applications in the ultrasound guidance of tumor embolizations [18]. The main objectives of our study were then (1) to decrease the particle mean diameter and size dispersion in the order of few micrometers and (2) to provide an in vitro quantitative assessment of the acoustic characteristics of these trisacryl microparticles (TMP) for a potential ultrasound contrast agent application. For this purpose, we used a validated experimental technique [19] allowing, under different and stable conditions, accurate measurements of TMP attenuation and backscattering enhancement.

2. Methods

2.1. TMP fabrication

The TMP fabrication process was achieved by Biosphere Medical (Roissy-en-France, Ile de France, France). To prepare particles of a few micrometers in diameter, the chemical protocol differed in terms of stirring rate and duration from the protocol initially conducted to prepare embolization spheres of 200–1000 μm.

The lipophilic phase, considered as continuous, consisted of heptane (1 liter) and Span 80 (10 ml) as emulsifier. This solution was then stirred at 45 °C. For the trisacryl aqueous phased (63 g), methylenebisacrylamide (MBA-15 g) and water (125 ml) were mixed at 45 °C and filtered. The initiator V-50 (1.26 g) was dissolved in water (15 ml) at room temperature and added to the water phase at 45 °C under magnetic stirring. The water phase was continuously added to the continuous phase and the emulsion was obtained by stirring at 22,000 revolutions per minute (rpm) during 30 min with an Ultra-Turrax (IKA® – Werke Gmbh & Co. KG, Staufen, Germany). The temperature of the water-jacketed reactor was then increased to 70 °C and maintained at this temperature during 19 h under stirring at 300 rpm. After cooling to room temperature, particles were isolated by centrifugation at 5000 rpm for 10 min (Heraeus® Labofuge® 200, DJB Labcare, Buckinghamshire, England), washed several times with hexane and acetone and then lyophilized to remove residual solvents and water (Martin Christ, Osterode, Germany). Particles were stored in a dessicator (Nalgene, Rochester, USA) at room temperature. Their size repartition was analyzed under a microscope (BH2, Olympus, Rungis, France) equipped with a granulometry analyzer software (Ellix, Microvision, Evry, France). For this purpose, particles were

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re-suspended in a mixture of serum and tensioactive agents and dispersed with ultrasound.

Chemicals, solvents and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). The initiator V-50 was purchased from Wako (Wako Pure Chemical Industries, Ltd, Neuss, Germany).

For the in vitro experiments, dried TMP were weighted and dispersed in a defined medium suspension volume in order to prepare different suspension concentrations.

All ultrasound field parameters related to the in vitro study are reported, according to the "Guidelines for *Journal of Ultrasound in Medicine* Authors and Reviewers on Measurement and Reporting of Acoustic Output and Exposure" [20].

2.2. Experimental Couette device and emission/acquisition system

The experimental device consisted of an hydrodynamic Couette device coupled to an unfocussed and immersed mono-element transducer A, B or C (Panametrics, Waltham, MA, USA), excited by a pulser/receiver 5072PR® (Panametrics, Waltham, MA, USA), working at variable pulse energy levels (13, 26, 52 and 104 μJ). The three transducers worked at a theoretical nominal frequency of 5, 10 or 15 MHz, respectively (Accuscan-S serie: A310S-SU, A312S-SU and A313S-SU).

The Couette device was composed of two coaxial cylinders: one cylinder was fixed while the other was rotating at a rate electronically controlled [19]. The 3 mm-gap between the two cylinders allowed to work with a particle suspension of 40 ml. For all experiments, a constant shear rate was generated at 200 s^{-1} within the gap by a step-by-step motor to avoid sedimentation phenomena. One of the transducers was inserted through the wall of the outside cylinder in order to be directly in contact with the suspensions. The transducer was activated by the pulser–receiver at a pulse repetition frequency of 1 kHz (emitted pulse duration of 0.5 μs), also used to amplify by 45 dB the radiofrequency (RF) signals received after TMP backscattering. Signals originated from a 1 mm-window positioned in the gap at 0.6 cm from the transducer to eliminate likely stationary echoes. The output of the pulser–receiver was connected to the input of a WavePro® 950 digital scope (8 bits resolution) equipped with mathematical functions (Lecroy, Chestnut Ridge, NY, USA). The amplified signals were digitized at a sampling rate of 16 GHz. For each measurement, one thousand 2-μs sequences of backscattered signals were computed with a Fast Fourier Transform (FFT) using a Hamming window. An averaged spectrum of these spectra was then calculated.

2.3. B-transducer calibration: acoustic pressure determination

To measure the backscattering enhancement under different acoustic pressure conditions, the B-transducer (theoretical nominal frequency of 10 MHz given by manufacturer) was calibrated in an ultrasound test tank ($30 \times 20 \times 35$ cm, in length, width and height, respectively). The power spatial distribution of the transducer was determined within the ultrasound beam in order to map the acoustic pressure produced at 10 MHz. The water tank, whose inner walls were covered with an acoustic absorber 10-mm-thick (Aptflex-NPL® F28, Precision Acoustics Ltd., Dorchester, UK), was partly filled with 7.8 l of demineralized and degassed water, just before experiments. The transducer was partially immersed in the tank with its emitting surface facing an hydrophone HGL 0200 (PZT-Onda Corporation®, SunnyVale, CA, USA), connected to an amplifier (A101, Onda Corporation®, SunnyVale, CA, USA). The distance between the B-transducer and the hydrophone was fixed at 0.6 cm to reproduce the conditions of the in vitro Couette device (Fig. 1). The transducer could shift every millimeter in the ($x, y, z = 6$ mm) plan with a 3-axis millimetric displacement device (Microcontrol, Evry, France). For each spatial location, the 5072PR® pulser–receiver excited the transducer at each pulse energy level. The hydrophone's response was visualized on the digital scope and recorded. These signals were converted into pressure values according to the calibration curve of the hydrophone supplied by the manufacturer. The maximal negative pressure values were recorded and could be further used to calculate the mechanical index (MI) with $MI = \frac{P_{max}^{-}}{\sqrt{f}}$ with f corresponding to the nominal frequency of the transducer.

2.4. In vitro particle acoustic characterization

Using the B-transducer, TMP acoustic properties were studied in the Couette device according to (1) concentration ranged from 0.12 to 15.63 mg/ml and (2) acoustic pressure, at the four energy levels generated by the pulser/receiver. When evaluating TMP backscattering dependence on frequency, the two other transducers A and C were also used and suspensions were studied from 2 to 17 MHz.

Each combination of acoustic pressure, frequency and concentration was repeated four times for in vitro

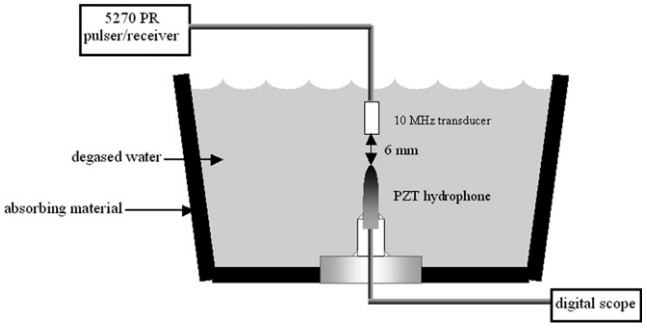

Fig. 1. Ultrasound set-up for the determination of the power spatial distribution of the B-transducer (theoretical nominal frequency of 10 MHz). This set-up was composed of a water tank filled with degassed water and a PZT hydrophone immerged in the tank and connected to a digital scope. This hydrophone was placed at 6 mm from the B-transducer excited by the pulser/receiver system at different energy values.

characterization reproducibility. In order to work with the three A, B and C transducers of 5, 10 and 15 MHz, respectively, each impulse response was established using a brass reflector through the bandwidth defined at −6 dB. Subsequently, when expressing a parameter according to emission frequency, only values within the used transducer's bandwidth were considered and measurements were normalized with each impulse response.

2.4.1. TMP suspensions

TMP were suspended in a 40 ml reference medium composed of glycerol and physiological serum (40:60, v/v), simulating blood viscosity (4 cP). Ten concentrations were considered from 0.12 to 15.63 mg/ml. To express concentration in terms of number of microparticles per milliliter, it was assumed a spherical shape and diameters equal to the mean diameter. Using these assumptions, we approximately evaluated at 2.33×10^7 particles per ml the particle concentration in the 0.12 mg/ml suspension. Aggregate formation was avoided by sonicating solutions prior to acoustic tests in an ultrasound bath (Bioblock Scientific, Illkirch, France) during 10 min at 35 kHz, producing no particle degradation. Samples were then stored at room temperature and stirred a few minutes for homogenization just before measurements in the Couette device.

2.4.2. In vitro characterization protocol in the Couette device

Whatever concentration, acoustic pressure or frequency applied, acoustic measurements started first with the acquisition of weak but non negligible signals backscattered by the reference medium. Once these reference measurements, the medium was removed and replaced by a 40 ml TMP suspension. Acquisitions started 30 s later in order to allow homogenization and avoid any transitory phenomenon. To prevent cross-contamination between samples of different concentrations, the stator was removed and washed prior to each new set. For each measurement, 1000 FFT spectra of RF signals were collected and averaged. Each backscattering averaged spectrum was then normalized with the backscattering spectrum generated by the reference medium. Finally, the backscattering enhancement P_{us} considered the maximal value of the normalized averaged spectrum measured at the central frequency of the transducer.

To investigate P_{us} stability over time, one hundred FFT were recorded every minute for the first 6 min and afterward every 2 min over 30 min using the B-transducer (10 MHz), at 275 kPa. In order to average the hundred FFT at short intervals within the first seconds after particles insertion, appropriate amounts of TMP were suspended in 5 ml of reference medium. This volume was added through a syringe into the 35 ml of reference medium already placed inside the Couette device in order to achieve the desired concentration ranged from 0.98 to 7.80 mg/ml. This protocol prevented thereby from measuring a transitory phenomenon with air bubbles creation within the first seconds after TMP inclusion.

2.4.3. Acoustic attenuation measurements

On a brass reflector, two sets of experiments were performed at 26 μJ. In a first set, attenuation was measured according to frequency using the three transducers A, B and C at two intermediate TMP concentrations. In a second set, attenuation was studied using the C transducer with concentrations ranging from 0.25 mg/ml to 15.63 mg/ml. Each condition was repeated 4 times and for each measurement, 50 power spectra were acquired and averaged during the post-treatment process. Given the distance $d = 2.6$ cm from the transducer to the brass, the attenuation parameter could be calculated according to the relation: $\alpha = \frac{1}{2d}(P_{\text{us part}} - P_{\text{us ref}})$ with $P_{\text{us part}}$ being the brass response when immerged into particles suspension and $P_{\text{us ref}}$ being its response without particle. The parameter α, measured in linear data, included the different responses at the nominal frequency of each transducer and was expressed in dB/cm.

2.5. TMP size distribution

Following the acoustic tests, the TMP size distribution was measured with a size analyzer LS230® (Beckman Coulter, Fullerton, CA, USA) using low angle laser light scattering. The calculation of particles size was based on the Mie's theory accounting for the optical properties of the polymer. Measurements showed homogenous dispersion of spherical microparticles with 99.9% of the population smaller than 4 μm and a mean diameter of 2.62 μm ± 1.4 μm (Fig. 2).

2.6. Statistical analyses

Linear regressions were performed to evaluate the relationship between P_{us} and the different physical parameters, using Pearson's correlation coefficients (R^2): p-values were considered significant when below 0.05.

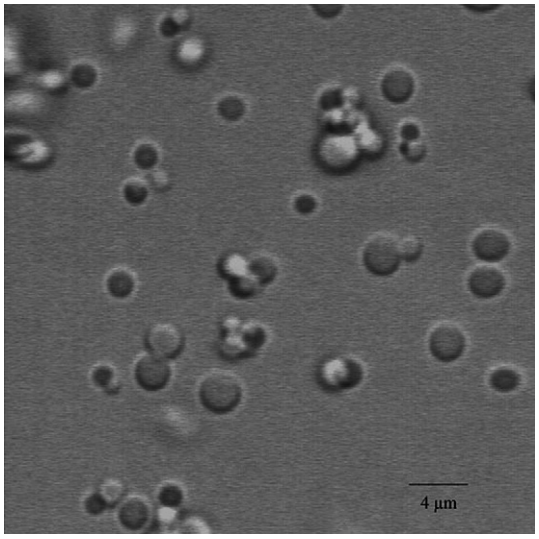

Fig. 2. Microscopic slide of trisacryl microparticles. (bar corresponds to 4 μm). The observed field shows the spherical shape of the TMP with a dispersed diameter between 1.2 and 4 μm.

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3. Particles characterization

For each A, B and C transducer, the −6 dB bandwidth was measured of 3.5–7.3, 8.3–11.1 and 10.9–17.1 MHz with a central frequency of 5.1, 9.3 and 14.5 MHz, respectively. All measurements described in the following sections include corrections with each transducer's impulse response.

3.1. Acoustic pressure produced by the B-transducer

At 9.3 MHz and at $z = 6$ mm, maximal acoustic pressure was evaluated along the transducer axis as 137, 191, 275 and 378 kPa for the 13, 26, 52 and 104 μJ pulser/receiver energy values, respectively. According to Fig. 3 showing the transducer pressure spatial repartition at 52 μJ, the effective surface (defined as the plan with values above 90% of the maximal value) was of 80 mm^2. That surface corresponded to 70% of the active surface of the B-transducer (6 mm activ diameter).

P_{us} was measured in this study as a function of TMP concentration, acoustic pressure, emission frequency and over time.

3.2. Backscattering enhancement as a function of particle concentration

P_{us} is represented on Fig. 4 as a function of concentration at 9.3 MHz. For an applied pressure of 275 kPa, the relative backscattering coefficient increases linearly ($R^2 = 0.985$) from the value of 1.59 to 43.65 times (2 to 16.4 dB) within the dose ranging from 0.12 to 7.8 mg/ml. Above that concentration, no clear increase in the relative backscattering intensity can be seen and the curve reaches a plateau from 7.8 mg/ml to 15.6 mg/ml, which may be caused by saturation due to multiple scattering between particles.

3.3. Backscattering enhancement as a function of frequency

The frequency-dependence was studied on the Couette device for the 3.9 mg/ml concentration at the four emitted energy values of 13, 26, 52 and 104 μJ.

To calculate the frequency-dependence of the backscattering coefficient, mean P_{us} and frequency were expressed in log values and represented on a linear scale on Fig. 5. On this figure, backscattering exhibited a linear frequency-dependence with a correlation coefficient of $R^2 = 0.65$ ($p = 0.02$). Error bars represent the standard

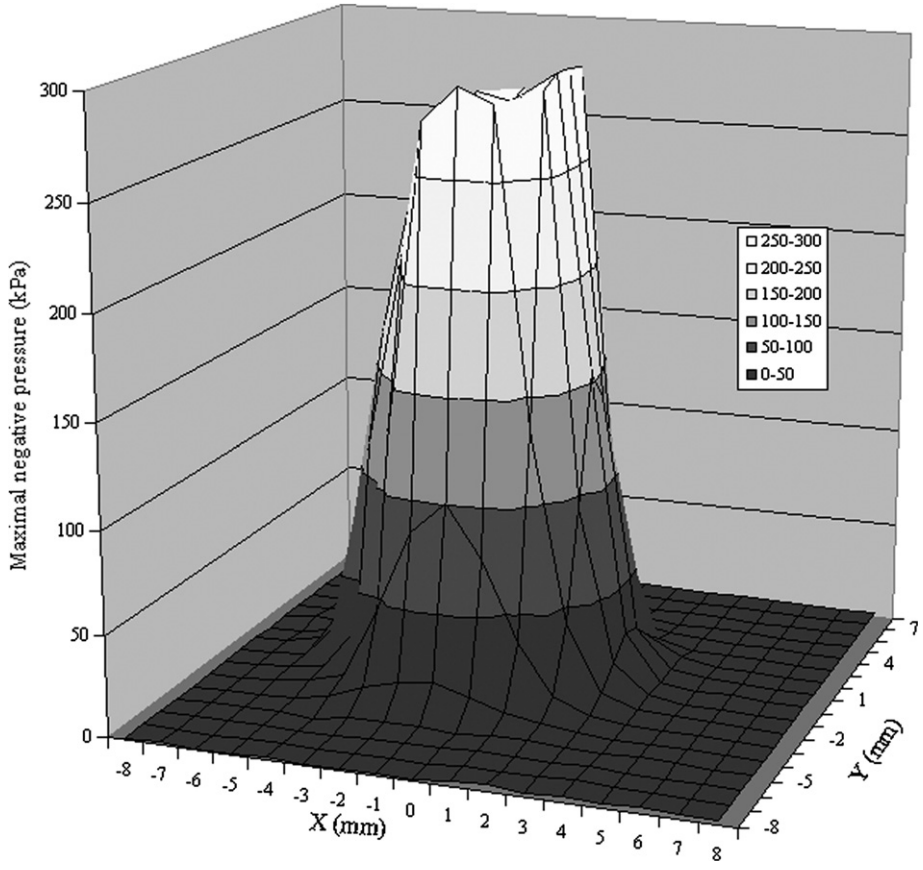

Fig. 3. Acoustic pressure spatial distribution of the ultrasound beam produced by the B-transducer placed at 0.6 cm from the hydrophone (52 μJ). In the ultrasound set-up, signals received by the hydrophone and transmitted to the digital scope were converted into pressure values. The maximal negative value was then recorded for each (X, Y) spatial position.

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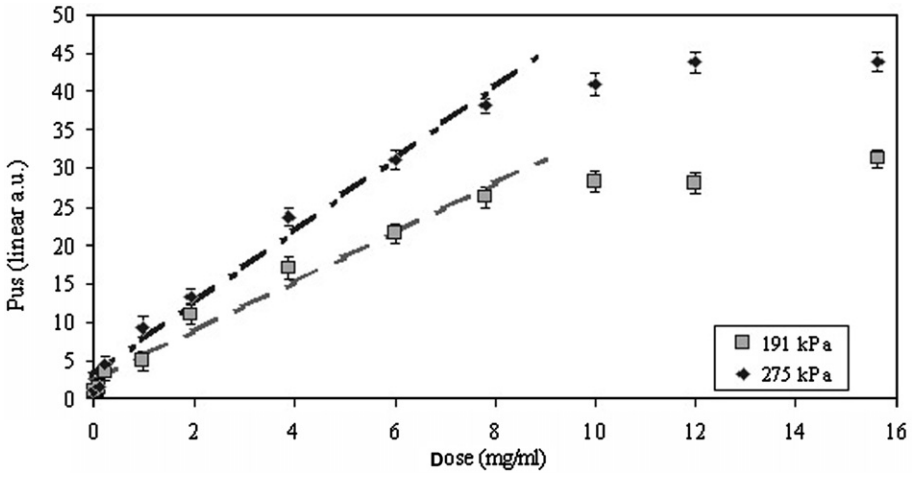

Fig. 4. P_{us} (linear arbitrary units) according to the dose measured with the 9.3 MHz transducer. P_{us} was measured on the Couette device and is represented under two acoustic pressure conditions corresponding to 191 and 275 kPa (errors bars indicating the average within the 4 experiments). Linear regressions in dash lines with $R^2 = 0.976$ and $R^2 = 0.985$ for 191 kPa and 275 kPa, respectively.

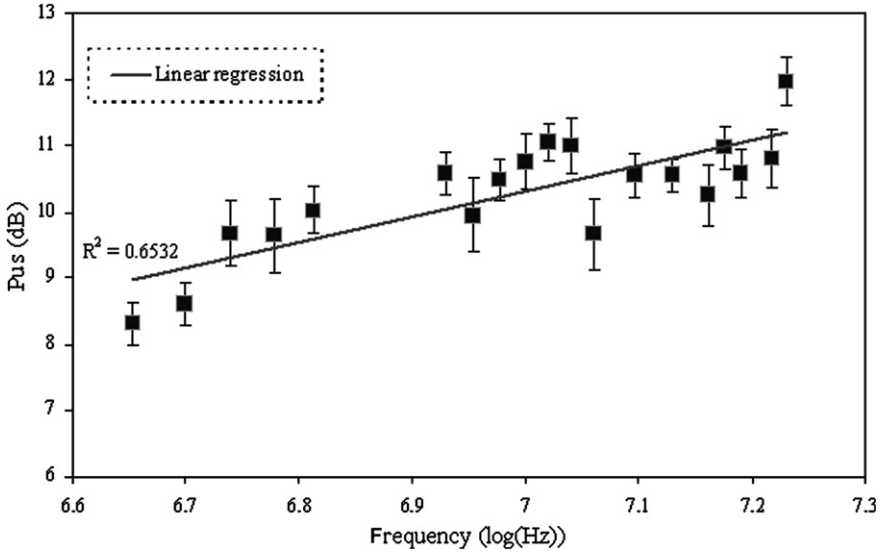

Fig. 5. P_{us} as a function of frequency for the 9.3 MHz transducer. Backscattering was measured in the Couette device at the 3.9 mg/ml concentration under an emitted energy of 52 μJ. Frequency and P_{us} are expressed in log values. Linear regression in solid lines with $R^2 = 0.6532$ and $p < 0.05$. Error bars represent the standard deviation of the measurements.

deviations of the four measurements: maximal deviation reaches 1.12 dB at 6 MHz (6.79 log(Hz)). Within the range 4.5–17 MHz, the regression slope of 3.83 corresponds to a dependence of P_{us} as the power of 3.83 of the frequency for an energy of 52 μJ. Regarding the other energy values of 13, 26 and 104 μJ, linear regressions had slopes in the same order of magnitude: 3.34 ($R^2 = 0.63$), 3.41 ($R^2 = 0.68$) and 3.57 ($R^2 = 0.62$) ($p < 0.05$), respectively.

3.4. Backscattering enhancement according to acoustic pressure (B-transducer)

In Fig. 6, non-linear effect of the applied emitted intensity on P_{us} could be observed within the range below 378 kPa for the 3 concentrations of 0.975, 1.95 and 3.9 mg/ml. This behaviour suggests a progressive TMP deformability inducing a lower backscattering from the second measurement point at 191 kPa. However, the TMP deformation was not associated to particle destruction and the backscattered signal increased continuously up to the maximal pressure value.

3.5. Stability of backscattering enhancement

Backscattered signals were measured with the 9.3 MHz transducer just after the TMPs insertion into the reference medium already rotating in the Couette device ($T = 0$). The

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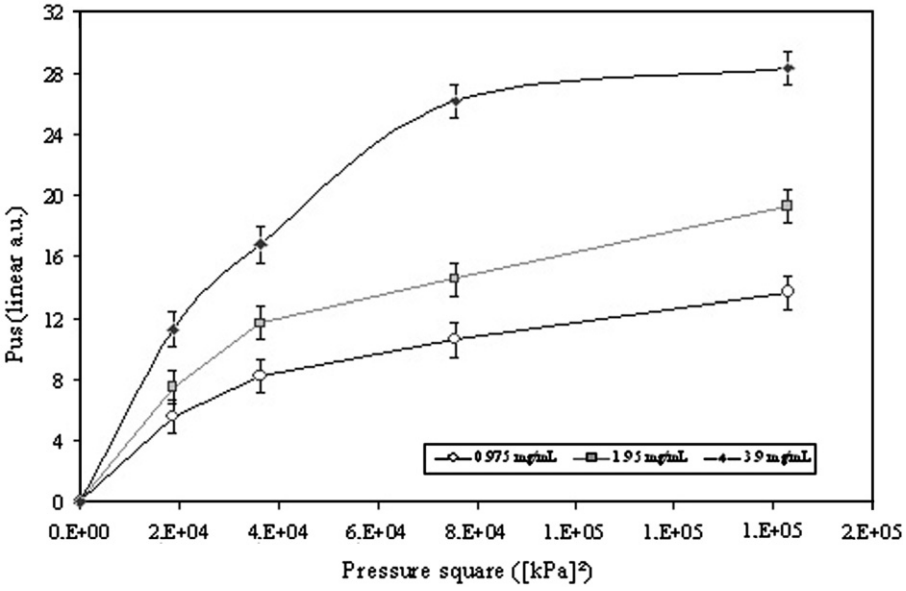

Fig. 6. Mean values of P_{us} with the corresponding SDs according to the square of applied pressure (ultrasound intensity) for the 9.3 MHz transducer. P_{us} is represented for three particle concentrations of 0.975, 1.95 and 3.9 mg/ml (error bars represent the standard deviation of the four measurements). This Figure shows a non linear but continuous increase in backscattered intensity with increasing incident pressure.

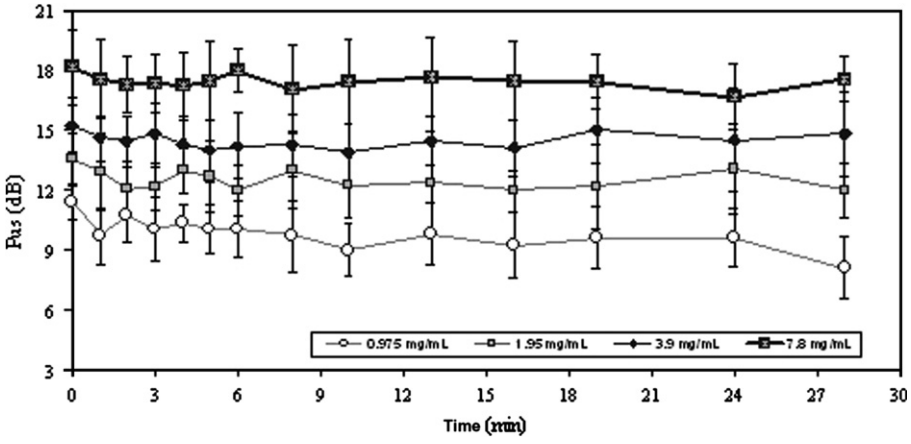

Fig. 7. Mean values of P_{us} with SD measured in the Couette device as a function of time. P_{us} was recorded over 30 min for three particle concentrations: 0.975, 1.95, 3.9 and 7.8 mg/ml (error bars represent the standard deviation of the four measurements). Measurements were performed at 9.3 MHz and 275 kPa.

acoustic enhancement as a function of time is depicted in Fig. 7 for the 0.975, 1.95, 3.9 and 7.8 mg/ml doses. All curves demonstrate marked enhancement over more than 20 min after injection, at 275 kPa (52 μJ). The 7.8 mg/ml dose produces the highest enhancement of 17.4 dB as it could be expected considering the results shown in Fig. 4 (16.4 dB at 7.8 mg/mL). Results presented in Fig. 7 indicate also that evaluating the mean P_{us} by averaging over 1000 consecutive FFT like we did is correct since no significant change in the P_{us} is observed over a time interval of about 3 min. Although enhancement stability is observed under controlled conditions with a constant shear rate ($200\ s^{-1}$), this result is quite important for the in vivo applications. Indeed, if the enhancement induced by these TMPs is stable over time in vivo, the trisacryl particles could be used as a drug vector. This system could be imaged by ultrasonography during the whole delivery process towards the targeted site.

3.6. Attenuation determination

The particle attenuation was investigated by measuring on the brass reflector/transducer system, the signal backscattered by suspensions of particles or by the reference medium. Attenuation was studied first as a function of frequency and then as a function of particle dose. With intermediate particle concentrations of 1.95 and 3.9 mg/ml, no attenuation through particle suspensions was observed according to frequency. A maximal measurable value of 0.4 dB/cm was found at 14.5 MHz. At this frequency value,

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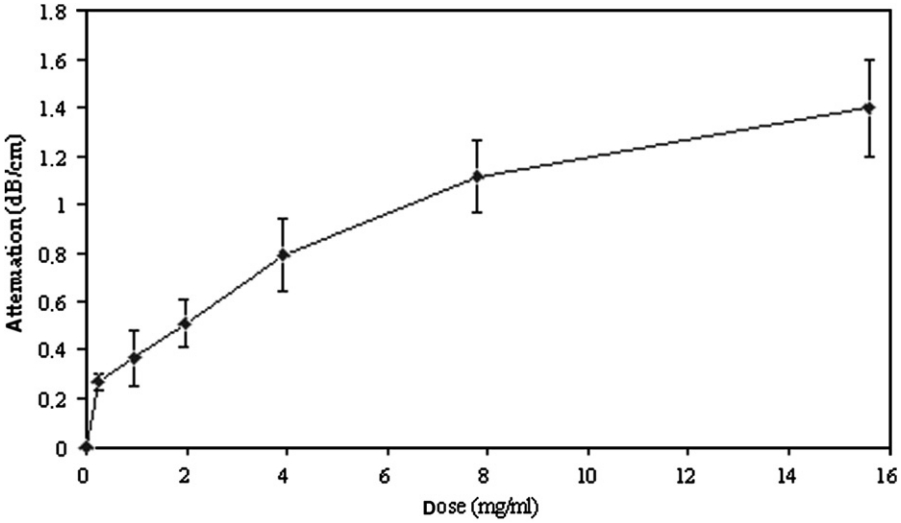

Fig. 8. Attenuation (dB/cm) as a function of the dose from 0 to 15.7 mg/ml (error bars represent the standard deviation of the four measurements). Attenuation was measured here in the brass reflector at 14.5 MHz and for an energy value of 26 μJ.

attenuation coefficient is depicted as a function of dose in Fig. 8. On this Figure, we can observe that attenuation is only increasing from 0.25 dB/cm for the lowest dose to 1.4 dB/cm for the maximal dose. This attenuation value of 1.4 dB/cm at the maximal dose induced an incident and scattered waves attenuation of less than 3% in the Couette device over the depth of the gated volume. The error introduced by neglecting attenuation when evaluating the P_{us} is therefore negligible.

4. Discussion and conclusion

In this study, TMP were acoustically characterized to establish their potential application as ultrasound contrast agents. The choice of polymeric particles for an ultrasound contrast agent application is based on their particular biocompatibility properties, which produce no toxic effect. Moreover, their polymeric network allows chemical reaction and anchor of ligands or antibodies towards angiogenesis markers. Among the polymeric formulation, the trisacryl particles have been already used in human embolization application when associated to gelatin. They have been shown to be biocompatible but also non-resorbable when used as vascular occlusive agents with diameters ranging from 500 to 1000 μm [15]. However, this feature could be less noticeable in further in vivo investigations with microparticles, since their much smaller diameter around 2 μm might lead to easier immune response with macrophages and lymphocytes [part II].

For the TMP in vitro characterization, backscattering enhancement and attenuation were measured under different conditions of particle concentration, acoustic pressure and emission frequency. As a first step in the process of developing and testing a new contrast agent, it is actually important to first collect data about the acoustic patterns under controlled and stable shear conditions. For this purpose, the Couette device described in this paper, has been validated in the establishment of new ultrasonic indexes for erythrocyte aggregation [19] or more recently, for the quantitative estimation of backscattering enhancement of PLGA microparticles [21].

The TMP backscattering enhancement was defined in this study as the maximal value of the averaged backscattered spectrum normalized with a reference medium. This parameter increased linearly with TMP concentration below a specific dose of 7.8 mg/ml. Such a behaviour demonstrated that the measurements were not affected by multiple scattering, whereas backscattering saturation was observed above the 7.8 mg/ml dose and P_{us} reached a maximal value of 16.4 dB corresponding to an increase of more than 40 times. This enhancement value was in the same order of magnitude than other values ranging from 8 to 27 dB measured in vitro with different contrast agents: ST68 (Span 60 and Tween 80 surfactants) with 3.5×10^5 particles/ml, PLGA particles by Forsberg and Wheatley [22,23] with 1×6.10^8 particles/ml or Quantison® by Frinking and de Jong [24] with 6.6×10^6 particles/ml. Indeed, by using similar equipments composed of a pulser/receiver, transducers with nominal frequencies from 2 to 15 MHz and a digital scope, Forsberg et al. [23] studied the ST68 in vitro properties (backscatter and attenuation) according to dose, emission frequency and time. After a first enhancement increase, they observed a plateau above a dose corresponding to 5.3×10^4 particles/ml (mean diameter of 3.8 μm) with a maximal value of 13 dB at 7.5 MHz and approximately 100 kPa. In the same way, El-Sherif et al. [22] found a mean backscattering power of 15 dB at a PLGA concentration of 0.6 mg/ml (10 MHz, 13 μJ) and Frinking et al. observed a Quantison® backscattering increase reaching 12 dB with an acoustic pressure of approximately 1 MPa. Even if the different particle compositions make it difficult comparisons to perform, the TMP

in vitro enhancement properties measured in the present study were found similar to those observed with microbubbles. One possible explanation for these observations is that a lyophilisation step is included at the end of the TMP preparation process during which, some air microbubbles could be trapped into the polymeric network. However, we still need to go thoroughly into that aspect to explain the acoustic properties of theses TMP (density seems to be around 1 g/cm^3): we need to explore the intrinsic characteristic of the trisacryl polymer in free or condensed form and repeat this experiment at each step of the preparation process.

Furthermore, the TMP demonstrated a non-linear but continuous increase in backscattered intensity with increasing acoustic pressure. This behaviour suggested that the MP could be distorted but were not flexible enough to break. Moreover, with a R^2 regression coefficient around 0.65, the P_{us} parameter exhibited a likely linear frequency-dependence with no resonance occurrence. The corresponding slopes ranged between 3.34 and 3.83 according to the emitted energy ($p < 0.05$). Slope coefficients were found in the same order of magnitude than the theoretical power of 4, described in the Rayleigh scattering. The difference however could be probably associated to the concentration of TMP used in the experiments. Indeed, 7.6×10^8 particles per ml were mixed in the 3.9 mg/mL TMP suspension. This particle quantity could induce a minor multi-scattering bias, leading to a scattering intensity dependence on frequency with a coefficient somewhat lower than the Rayleigh scattering coefficient, which reflects independent scatterers behaviour. The TMP characteristics dependent upon concentration, pressure and especially frequency, revealed that these small particles (2 μm in diameter compared to a minimal ultrasound wavelength of 700 μm) could rather conform to the hypothesis of Rayleigh scattering, with no resonance behaviour. Consequently, it could be inappropriate for the moment to use these TMP in harmonic ultrasound modes, based on the non-linear properties of some contrast agents. However, as the grade of cross-linkage correlates with the proportion of MBA tensio-activ in the matrix of the polymer, TMP will be able to acquire more flexible characteristics by decreasing this proportion. Some experiments with MBA percentage of 9, 6 and 1% instead of 23% are currently conducted: TMP backscattering is further studied on the Couette device according to acoustic pressure and sonication time.

The attenuation parameter was also measured in this study according to dose and frequency. In spite of negligible attenuation with increasing frequency, this parameter showed a maximal value of 1.4 dB/cm for the 7.8 mg/ml dose and at 14.5 MHz. This estimation is much smaller than those measured in vitro, for example by Forsberg et al. [23] with a maximal ST68 attenuation value of 12 dB/cm at 5 MHz (resonance phase) or by El-Sherif et al. [22] measuring 1.2 dB/cm at 14.5 MHz for PLGA (50:50) particles. In that study, the attenuation parameter exhibited a resonance peak of more than 4 dB/cm at 2 MHz. In fact, higher levels of attenuation are rather more often observed with particles containing microbubbles than with more rigid particles. Indeed, less flexible particles do not resonate in their actual form and thus absorb few ultrasound energy. Contrast microbubbles such as Albunex®, Quantison® or Sonovue® [24–26] have attenuation coefficients of 4 dB/cm or even higher depending on concentration, frequency and acoustic pressure conditions. In fact, both backscattering enhancement and attenuation parameters have to be involved in the acoustic characterization of contrast agents and particles acoustic effectiveness depends on the optimal ratio of these two parameters [27]. In this study, backscattering coefficient and attenuation were both only measured along the direction of the ultrasound beam. Nevertheless, the ratio of these two patterns seemed to be favourable, especially to avoid ultrasound shadowing when exploring organs or tumors of few centimeters in diameters in future in vivo applications.

The in vitro experiments in the Couette and brass reflector devices allowed us to study the ultrasound properties of TMP, namely attenuation and backscattering enhancement, under controlled conditions. We are aware of the limitations of studying such particles using an in vitro system with TMP suspension in a rather aqueous medium. Indeed, when injected intravenously into human circulatory system, contrast particles can be usually subject to several effects that modify their physical and acoustical properties. There is therefore a real need to investigate further these TMP first in a flow phantom to work closer to in vivo circulation conditions but also in a blood medium and, as a last step, in vivo with intravenously injected TMP.

5. Conclusion

The polymeric TMP have revealed in vitro ultrasound properties comparable to those observed with other agents in similar in vitro set-ups. These properties have now to be further investigated, in particular, in blood medium associated to a flow phantom but also in vivo, in order to measure the TMP acoustic characteristics and their potential interactions in the presence of moving blood cells.

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