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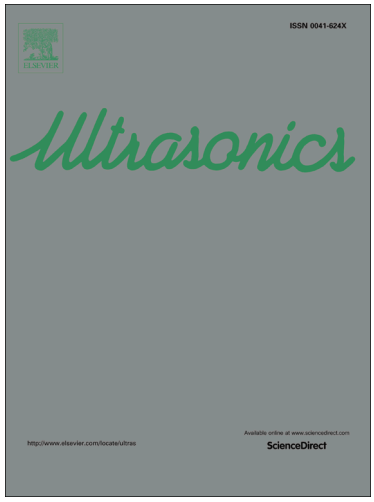

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Influence of temperature, needle gauge and injection rate on the size distribution, concentration and acoustic responses of ultrasound contrast agents at high frequency

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ABSTRACT

This paper investigated the influence of needle gauge (19G and 27G), injection rate (0.85ml·min^{-1}, 3ml·min^{-1}) and temperature (room temperature (RT) and body temperature (BT)) on the mean diameter, concentration, acoustic attenuation, contrast to tissue ratio (CTR) and normalised subharmonic intensity (NSI) of three ultrasound contrast agents (UCAs): Definity, SonoVue and MicroMarker (untargeted). A broadband substitution technique was used to acquire the acoustic properties over the frequency range 17–31 MHz with a preclinical ultrasound scanner Vevo770 (Visualsonics, Canada). Significant differences ($P<0.001$ - $P<0.05$) between typical *in vitro* setting (19G needle, 3ml·min^{-1} at RT) and typical *in vivo* setting (27G needle, 0.85ml·min^{-1} at BT) were found for SonoVue and MicroMarker. Moreover we found that the mean volume-based diameter and concentration of both SonoVue and Definity reduced significantly when changing from typical in *vitro* to *in vivo* experimental set-ups, while those for MicroMarker did not significantly change. From our limited measurements of Definity, we found no significant change in attenuation, CTR and NSI with needle gauge. For SonoVue, all the measured acoustic properties (attenuation, CTR and NSI) reduced significantly when changing from typical *in vitro* to *in vivo* experimental conditions, while for MicroMarker, only the NSI reduced, with attenuation and CTR increasing significantly. These differences suggest that changes in physical compression and temperature are likely to alter the shell structure of the UCAs resulting in measureable and significant changes in the physical and high frequency acoustical properties of the contrast agents under typical *in vitro* and preclinical *in vivo* experimental conditions.

KEY WORDS: High frequency ultrasound, microbubble, needle gauge, injection rate, temperature, acoustic characterisation

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1 INTRODUCTION

There is an increasing number of applications for the use of UCAs in the preclinical field [1], with an associated increase in the number of published studies utilising UCAs both *in vivo* and *in vitro* [2-5]. However, the differences between the administration of UCAs in an *in vivo* environment at body temperature and an *in vitro* experiment at room temperature need to be considered. The size of needle gauges used for small animal *in vivo* injections are often not cited in the literature but generally range from 24G for rat tail vein injection [6] , 27G for mouse tail vein injection [7] and in larger animals - 30G for pig cervical region of nerve [8] and dog lymphatic system [9]. Generally *in vitro* experiments of UCAs are performed at room temperature using 18G - 20G needle gauges – sizes which are widely used in clinical practice and specifically recommended by some contrast agent manufacturers [10]. When bolus injections are undertaken in small animals, although the bolus is considerably smaller (of the order of microliters), it is generally performed at an injection rate of between 0.5 - 3 ml/min, a rate that is commonly used for clinical bolus intravenous injections.

1.1 The influence of temperature

The three commercial UCAs: Definity (Lantheus Medical Imaging, USA), SonoVue (Bracco, Italy) and MicroMarker (untargeted) (Visualsonics, Canada) are lipid-coated microbubbles (MBs). The shells of the three UCAs are monolayers but each is formed from a varying combination of different lipids. As a result, it is difficult to determine a specific phase transition temperature for the lipid shells of each and hence the effect of temperature on the lipid-coated MBs is unknown and its resultant influence on microbubble characteristics is unclear. The fluid-gel phase transition temperature of

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lipids in bilayer states varies from -1°C to 75°C [11] and -1°C to 55°C [12] depending on the phospholipids acyl chain length. Lipid molecules are limited on the membrane plane in two phases – either fluid: liquid phase; or gel: solid phase, where the liquid phase allows a freer diffusion of molecules than in the solid phase [13]. For this reason, the viscosity, elasticity, free energy and diffusion coefficient of the MB lipid shell can be changed below, at, or above the transition temperature [12]. The lipid shell of Definity consists of three phospholipids (DPPA, DPPC and MPEG 5000 DPPE) [3]. The lipids incorporated into MicroMarker shell are polyethylene glycol, phospholipids (unspecified) and fatty acids (VisualSonics PN11691) [14]. SonoVue possesses an amphiphilic phospholipid shell (a hydrophilic surface outside and a hydrophobic surface inside) [15] and involves two lipids (DSPC and DPPG) [16].

The thermal response of SonoVue has previously been studied over the temperature range 37-43^{o}C and its lipid transition temperature was inferred to be 40^{o}C, from observation of changes in its maximal diameter and backscatter intensity (ultrasound frequency: 2.5- 8MHz, low MI: 0.0081- 0.113) [17]. For Definity and MicroMarker, the attenuation was measured and found to be higher at 37°C than at 25°C over a frequency range from 2 to 25MHz. Measurements were undertaken in bovine serum albumin and whole blood and no difference in attenuation was found between the two diluents [14]. Individual Definity and SonoVue MBs have previously been measured at room temperature (21^{o}C) and body temperature (37^{o}C) using a high speed optical camera under an insonation of 1.7MHz, 10-80kPa pulse [18]. Compared with the performance at room temperature, Definity and SonoVue showed an increase in radial expansion and a decrease in onset of acoustic oscillation at body temperature. Extensive temperature dependence studies of SonoVue were completed using 3.5

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MHz-ultrasound [16, 19]. Increasing the temperature close to the transition temperature resulted in bubbles exhibiting greater radial expansion. Expanding MBs and rapid diffusion was shown to increase the mean diameter, attenuation, backscatter and nonlinear components.

1.2 The effect of needle size and injection rate

Talu [20] measured the variation in concentration and mean diameter of one in-house, lipid-encapsulated UCA at 3 concentrations (1, 5, 10×10^8 MBs·ml^{-1}) using 3 needle gauges (23G, 27G and 30G) at 5 injection rates (0.01, 0.03, 0.1, 0.3 and 0.5 ml.sec^{-1}). With increasing needle gauge (decreasing inner needle diameter), the measured concentration of microbubbles was shown to decrease suggesting destruction of the MBs. In addition, a reduction in mean diameter was observed and attributed to an overall decrease in number of MBs in the population. It was suggested that this was due to diffusion by the forced compression and possible preferential destruction of large MBs. A similar study investigating the influence of administration variables (2 needle gauges: 18G and 25G; 2 syringes: 5ml and 10ml; 5 discrete flow rates from 2 to 3.3 ml·min^{-1}; 2 suspending fluids: distilled water and 95% volume-glycerol) was performed using another in-house lipid MB [21]. The results from this study showed that in addition to the hydrostatic pressure, shear stress due to the increasing pressure and velocity gradient played a key role in destroying MBs. However, this study found that an increasing volume flow rate (i.e., injection rate) reduced the destruction of MBs, which is not in agreement with the study of Talu [20]. The discrepancy was attributed to the difference in initial diameter, size distribution, concentration and composition of MBs. An *in vivo* experiment to improve plasmid transfection using SonoVue MBs-mediated gene transfection tested the effects of using 3 needle gauges

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(25G, 27G and 29G) and showed that increasing needle gauge increased MB destruction and reduced MB diameter [22].

From the above studies, temperature, needle gauge and injection rate have been shown to have significant effects on the size distribution and acoustic properties of UCAs. However, the implications of these results for preclinical studies remain unclear. This is due to several factors. Firstly, the previous studies are limited to the ultrasound frequencies relevant for clinical applications and little research has been performed at high frequencies applicable for preclinical applications. Secondly, the effect of temperature, needle gauge and injection rate have been discussed separately but the potential interactions between them remain indistinct. Thirdly, a wide range of in-house MBs have been studied while commercial UCAs (both clinical and preclinical) are the products that are most commonly used for preclinical studies and the influence of these parameters on these commercial agents has not been fully investigated at high frequencies.

The aim of this paper is to investigate the influence of needle gauge, injection rate and temperature on the mean diameter, concentration, acoustic attenuation, contrast to tissue ratio (CTR) and normalised subharmonic intensity (NSI) of solutions of Definity, SonoVue and MicroMarker (untargeted) over the frequency range of 17-31MHz. In particular, the changes observed in these measurements for SonoVue and MicroMarker, the most commonly used contrast agents for preclinical studies, under typical *in vivo* and *in vitro* experimental conditions will be discussed.

2 METHOD AND MATERIALS

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2.1 UCAs preparation

UCAs were reconstituted based on the manufacturers' guidelines and were ready for experimental use after standing at room temperature for 20 minutes. The information of the three UCAs is listed in Table 1. Based on the maximum concentration from the manufacturer's published literature, MBs were diluted in air saturated distilled water to reach a concentration of 0.8×10^{6} MBs·ml^{-1}. At this concentration, the occurrence of multiple scattering and shadowing of Definity MBs was previously shown to be avoided [23].

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Table 1: *The parameters and dilution of contrast agents, *Definity [10], SonoVue [24, 25], ‡MicroMarker [26]*

Name	**Gas**	**Shell**	**Number-based mean diameter** (μm)	**Maximum concentration after reconstitution** (mbs/ ml)	**Dilution in this study**	**Manufacturer**
* Definity®	C_3F_8	Phospholipids	1.1-3.3	1.2×10^{10}	1:15000	Lantheus Medical Imaging, USA
†SonoVue®	SF_6	Phospholipids	2-3	5×10^{8}	1:625	Bracco, Italy
‡MicroMarker™ untargeted contrast agent	C_4F_{10} / N_2	Polyethylene glycol, Phospholipids and fatty acid	2.3-2.9	2×10^{9}	1:2500	Visualsonics, Canada

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2.2 The control of needle gauge and injection rate

In this study, two needles (19G and 27G) (Becton, Dickinson and Company (BD), USA) are selected: a 19G (internal diameter (ID) = 0.686mm) needle is generally used in human intravenous injection and also in some *in vitro* experiments while a 27G (ID = 0.21mm) needle is commonly used for mouse-tail injections and mouse intra-cardiac injections. Two injection rates are studied: 0.85 $ml \cdot min^{-1}$ and 3$ml \cdot min^{-1}$, where 0.85 $ml \cdot min^{-1}$ is a typical injection rate used in bolus injections into a mouse tail vein during preclinical studies and 3 $ml \cdot min^{-1}$ a typical injection rate applied in *in vitro* experiments. The steady and reproducible injection rate was controlled using a syringe pump (Aladdin, World Precision Instruments Inc., USA) by connecting the test needle with 1ml-syringe (ID = 4.78 mm) to reach an injection rate of 0.85 $ml \cdot min^{-1}$ and 2ml-syringe (ID=8.66 mm) to reach a 3$ml \cdot min^{-1}$injection rate.

2.3 Temperature control and the measurement of dissolved oxygen level

The entire experiment was undertaken in air saturated distilled water (diluent) at both room temperature (RT: $20 \pm 2^{o}C$) and body temperature (BT: $37 \pm 2^{o}C$). Two-degree Celsius is the maximal range of variation in temperature. The water was determined to be air-saturated using the dissolved oxygen content in the water. This was monitored using a dissolved oxygen probe (Vernier Software & Technology, OR, USA) using the assumption that oxygen (O_2) and nitrogen (N_2) are the dominant gases in the water and that the dissolved O_2 is proportional to the N_2 at atmospheric pressure under Henry's law [27]. The air-saturated water at RT was prepared by leaving the distilled water over 24 hours. The air-saturated water at BT was obtained by heating the water to $42^{o}C$ then degassing using a vacuum pump. The resultant air saturated water at BT

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was sealed in a bottle and placed on a hotplate, to maintain the temperature of the water throughout the experiment.

For the process of withdrawing Definity and MicroMarker, a 19G needle was inserted into the vial of activated contrast agent, a second 19G was inserted for venting the gas as per the manufacturer's recommendations. One of the 19G needles was connected to a syringe and the Definity or MicroMarker was withdrawn at a steady slow rate of approximately 1.5ml·min^{-1}. For SonoVue, once it was formulated it was withdrawn from its vial using a needleless syringe provided by the manufacturer at a speed similar to that used to withdraw Definity and MicroMarker. The choice of syringe selected for the experiments depended on the injection rate selected (1ml-syringe for 0.85 ml·min^{-1} and 2ml-syringe for 3ml·min^{-1}). The syringe was then placed horizontally on a syringe pump and connected with one of the needles under test. At the injection rate setting, UCAs were collected from the needle tip into an Eppendorf PCR tube and were then diluted ready for sizing and acoustical measurement. The experiments undertaken at BT were performed in an identical manner except that the diluent was the prepared air-saturated water at BT. Because the MBs suspension was placed in an open tank and stirred continuously throughout the acoustic measurements, the water tank was placed on a hotplate to maintain the temperature at BT.

Details of the different combinations of needle gauge, injection rate and temperature for each experiment are shown in Table 2. In this study, the choice of a 19G needle, an injection rate of 3ml·min^{-1} in a diluent at RT is defined as '***in vitro***' representing a typical *in vitro* situation. The choice of a 27G needle, an injection rate of 0.85ml·min^{-1} in a diluent at BT is defined as an ***'in vivo'*** experimental setting. For analysis of the

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data, the '*in vitro*' setting was defined as the control group and all data was compared to this. Consequently for each agent, comparison between the 19G and 27G experimental data at the same injection rate ($3ml \cdot min^{-1}$) and temperature (RT) reveals impact of the needle gauge. Comparison between the $3ml \cdot min^{-1}$ and $0.85ml \cdot min^{-1}$ at the same needle gauge (19G) and temperature (RT) enables the impact of the injection rate to be determined, and between the RT and BT at the same needle (19G) and injection rate ($3ml \cdot min^{-1}$) the impact of temperature can be determined for MicroMarker and SonoVue. Finally between the *in vitro* and *in vivo* settings the combined impact of temperature, injection rate and needle gauge size can be determined. For Definity and SonoVue, the mean diameter was acquired for all experimental settings, however for MicroMarker only the data from the *in vitro* and *in vivo* were acquired. The acoustical properties of Definity at 37^0C are not included in this study.

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Table 2. *The specific experimental settings for the five groups*

Group name	*In vitro*	Needle Gauge	Injection Rate	Temperature	*In vivo*
Needle gauge	19G	27G	19G	19G	27G
Injection rate ($ml \cdot min^{-1}$)	3	3	0.85	3	0.85
Temperature	RT	RT	RT	BT	BT

2.4 Characterisation of diameter and concentration

The mean diameter of the three UCAs was measured by a laser diffraction particle analyser Mastersizer 2000 Hydro MU (Malvern Instruments Ltd**,** Malvern, UK). Three measurements were taken from each sample and the mean values displayed. The variation in concentration was measured using haemocytometers (FastRead 102 Counting Slides, Immune Systems Ltd, UK) and an inverted microscope (Zeiss Axiovert 25) under a 40× magnification. MBs were manually counted in a 4×4-counting grid for each experimental set-up. Each experimental setting was repeated three times for each agent yielding a mean value of 9 counts for each agent at each experimental setting.

2.5 Acoustic characterisation

A Vevo 770 preclinical ultrasound scanner (VisualSonics Inc., Canada) was used to measure the acoustic properties of the contrast agents. Details of the measurement technique can be found in [28, 29] and only a brief summary will be described here. The transducer was placed perpendicular to a polymethylpentene (TPX) reflector

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(Boedeker Plastics, Shiner, TX, USA) in a water tank (length: width: height = 4cm: 3cm: 5cm) on a magnetic stirrer (RCT basic, IKA, US). A magnetic bar (3mm-OD, 1cm-length) was placed in the tank to ensure a homogeneous MB suspension. One transducer 707B with nominal centre frequency of 30MHz, a focal length of 12.7mm, a 3dB bandwidth ranging from 17-31MHz, a 6dB bandwidth of 13-35MHz at 3% transmitting power (the peak negative pressure (PNP) was measured to be -0.56 MPa) performed as both a transmitter and receiver. The PNP was confirmed using a membrane hydrophone with a 0.2mm diameter active element made of Polyvinylidene Fluoride (PVDF) (Precision Acoustics Ltd., Dorchester, UK). The PNP of -0.56 MPa was chosen as we have previously demonstrated that microbubbles were not disrupted at this PNP and frequency at RT [29].

2.6 Data analysis

The attenuation and contrast to tissue ratio (CTR) of the MB suspension were measured in this study to evaluate the fundamental acoustic signature of UCAs. The measurements of attenuation coefficient, α, and CTR as a function of frequency are described in more detail in Sun et al. [29]. In this study, both the attenuation and CTR were averaged over the 3dB bandwidth of the transducer giving a mean value.

The normalised subharmonic intensity (NSI) of backscatter is measured to characterise the nonlinear property of the UCAs using Equation 1.

$$NSI = 10\log_{10}\frac{\overline{\sum_{2MHzBW} Subharmonics}}{\sum_{2MHzBW} Fundamental_{in-vitro}} \quad \text{(Eq.1)}$$

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where the numerator is the subharmonic component of the mean backscattered signal measured over a 2MHz bandwidth centred over the subharmonic frequency (15MHz) and the denominator is the mean backscattered signal measured over a 2MHz bandwidth over the fundamental frequency (30MHz) of the *in vitro* case (i.e., 19G needle, 3ml·min^{-1} at RT).

The data analysis used the radio-frequency (RF) data acquired from a pre-selected region of interest (ROI) (1.05 mm × 1.6 mm) centred over the focal position of the transducer. The RF data was output and calculations were performed offline using MATLAB software (MATLAB 2009A, The MathWorks Inc., Natick, MA, USA). Each experiment was repeated three times and 900 independent samples (300 consecutive frames on 3 lines) in each ROI were collected per experiment. The error bars were the standard deviation calculated from three independent measurements. For the measurement of attenuation and CTR, a single cycle signal was used as the driving pulse. For the measurement of NSI, modified VisualSonics software was used to generate a 25-cycle pulse (3% power, PNP equal to -0.67 MPa) and the subharmonic response was isolated from the receiving signals.

2.7 Statistics

A student's T test was performed between the *in vitro* case and all other combinations of needle gauge size, injection rate and temperature to investigate whether there is a significant difference in the mean diameter and acoustic properties of UCAs in the frequency range 17-31MHz due to the variation in the needle gauges, injection rate and temperature. The significance level was set at 0.05. The results of the student's T

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test are marked on the top of each group using the *in vitro* result as control in Figures 1 - 5.

2.8 Pressure drop and shear stress in the syringes and needles

The pressure drop from the syringe to the needle and the shear stress, τ, in the syringes and needle were calculated using the equations found in [21].

3 RESULTS

3.1 Pressure drop and shear stress in the syringes and needles

Table 3 lists the calculated shear stress in the syringes and needles and the pressure drop from the piston in the syringe to the outlet of the needle at different settings. At certain injection rates (0.85 ml/min using 1ml-syringe, 3ml/min using 2-ml syringe), the pressure drop and the shear stress in the needle increase with increasing needle gauge (i.e., decreasing I.D.).

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Table 3 *The calculated velocity and shear stress in the tested syringe (1ml I.D.: 4.78mm, 2ml I.D.: 8.66) and needles (19G, 27G) and the pressure drop (Ps - Pn) from syringe to needle (Ps: the pressure in the liquid near the piston in the syringe, Pn: the pressure in the liquid at the outlet of the needle)*

Injection rate (ml/min)	**Syringe volume (ml)**	**Syringe ID (mm)**	**Mean velocity in syringe (mm/s)**	**Needle Gauges**	**Needle ID (mm)**	**Mean velocity in needle (mm/s)**	**Pressure drop Ps-Pn (Pa)**	**Shear stress in syringe (mPa)**	**Shear stress in needle (Pa)**
0.85	1	4.78	0.79	19G	0.686	38.35	0.73	1.32	0.45
				27G	0.21	409.22	83.73	1.32	15.59
3	2	8.66	0.85	19G	0.686	135.35	9.16	0.78	1.58
				27G	0.21	1444.31	1043.02	0.78	55.02

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3.2 The variation in diameter, concentration and acoustic characterisation

In the following figures the headings of the groups are such that 'Needle Gauge' group indicates that only the needle gauge changed, 'Injection Rate' group indicates that only the injection rate changed and 'Temperature' group indicates that only temperature changed with respect to the *in vitro* data. For the cases of *in vivo* data, all three parameters changed.

Figure 1 shows the mean diameter of Definity, SonoVue and MicroMarker as a function of different injection regimes. The mean diameters in *in vitro* case are larger than the values of Definity and SonoVue shown in Table 1 as the mean diameter in this study was from the volume-based size measurement by Mastersizer, while the values from the manufacturer use the number-based size measurement. Compared with the *in vitro* case, the mean diameter of Definity decreases with increasing needle gauge, decreasing injection rate and increasing temperature. The individual impact of needle gauge makes no significant difference in the mean diameter of SonoVue, but lower injection rate, and the combined effect of higher temperature and lower injection rate show a significant decrease in the mean diameter of SonoVue. MicroMarker exhibits the smallest mean diameter of the three UCAs. Unlike the mean diameters of Definity and SonoVue, MicroMarker shows no significant variation between *in vitro* and *in vivo* experimental regimes.

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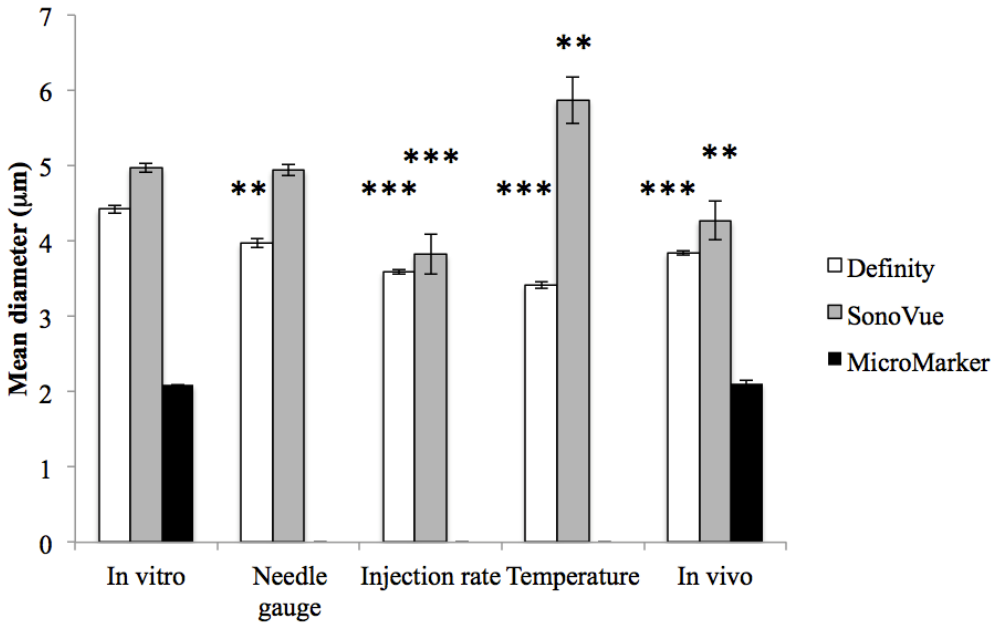

Fig.1: Volume-based mean diameter of Definity, SonoVue and MicroMarker under different experimental regimes. Needle gauge group indicating that only the needle gauge changed, injection rate group indicating that only the injection rate changed and temperature group indicating that only temperature changed with respect to the *in vitro* data. *In vivo* data, all three parameters changed as detailed in text. Significance determined between '*in vitro*' data set and other experimental regimes so that no mark indicates no significant difference, **P<0.01, ***P<0.001

The concentration of each of the three UCAs significantly decreases with increasing needle gauge in Figure 2. Only SonoVue presents a significant increase in concentration with decreasing injection rate. Both SonoVue and Definity show a decrease in concentration with temperature. In a comparison between *in vivo* and *in vitro* applications, MicroMarker concentration displays no significant variation but both SonoVue and Definity display a reduction in concentration.

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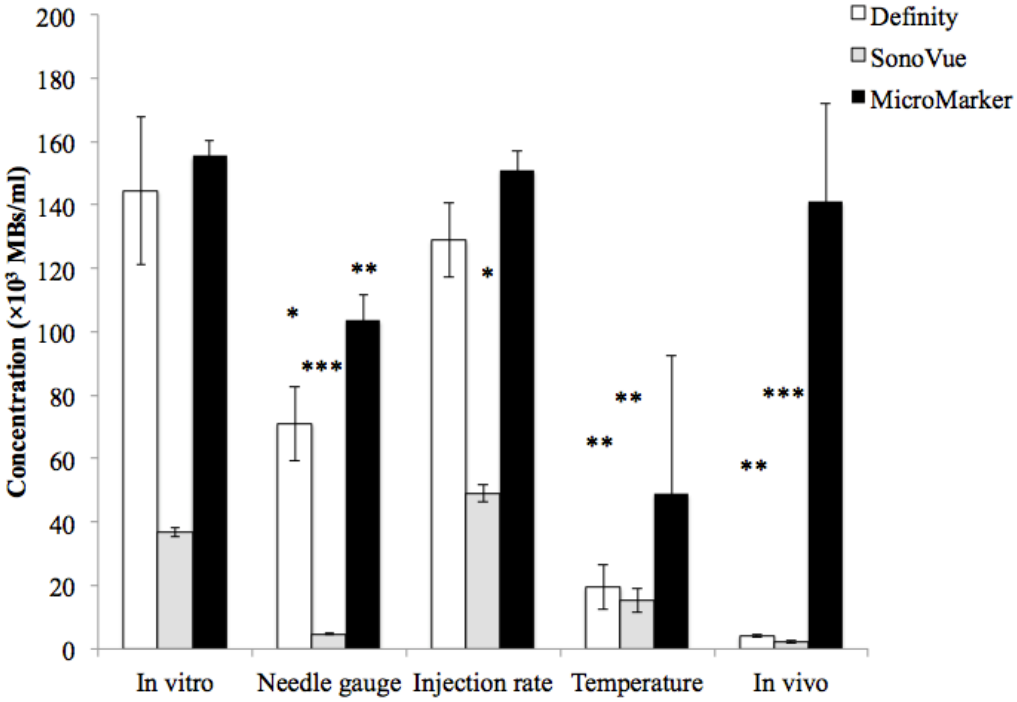

Fig.2: Concentration of Definity, SonoVue and MicroMarker under different experimental regimes, * P<0.05, **P<0.01, ***P<0.001. Group headings similar to that displayed in Figure 1

In Figure 3 the attenuation of ultrasound through the suspensions of Definity, SonoVue and MicroMarker in the frequency range 17-31 MHz is presented. No significant variation in the attenuation of Definity was found with changing needle gauge and injection rate. Compared to the *in vitro* data SonoVue exhibited significant decreases in attenuation with decreasing injection rate, increasing needle gauge and temperature. Conversely, for MicroMarker, the attenuation significantly increases from RT to BT, but neither increasing needle gauge nor injection rate makes a significant difference to attenuation.

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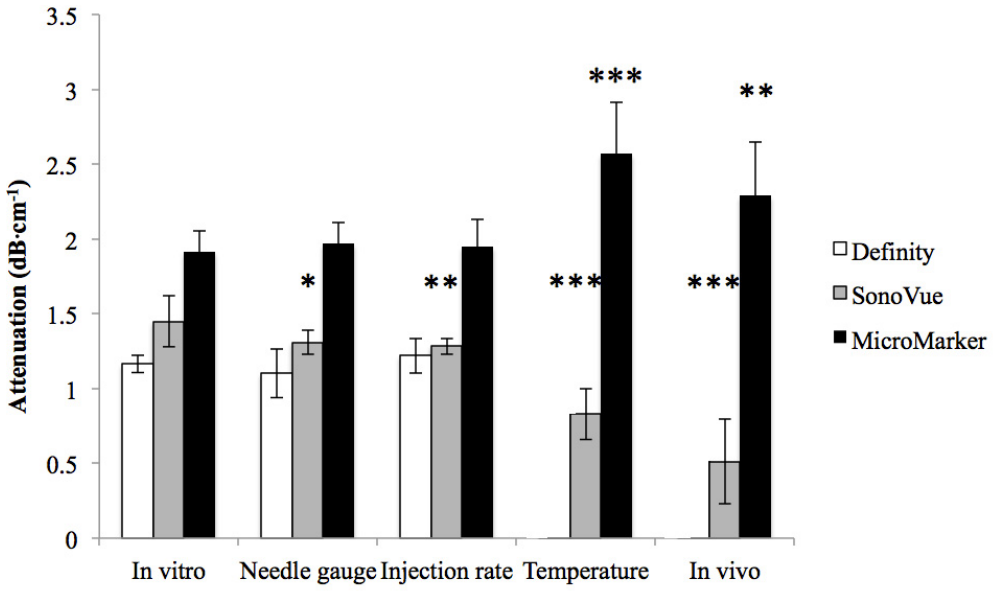

Fig.3: The attenuation comparison of Definity, SonoVue and MicroMarker, No mark: no significant difference, * P<0.05, **P<0.01, ***P<0.001. Group headings similar to that displayed in Figure 1.

From Figure 4, increasing gauge size needle only shows a significant, but small, impact on the CTR of SonoVue. Using a lower injection rate decreases the CTR of Definity and SonoVue significantly but increases the CTR of MicroMarker. The variation in temperature increases the CTR of SonoVue and MicroMarker. Overall a change from *in vitro* to *in vivo* conditions results in a small but significant decrease in CTR for SonoVue and a highly significant increase for MicroMarker.

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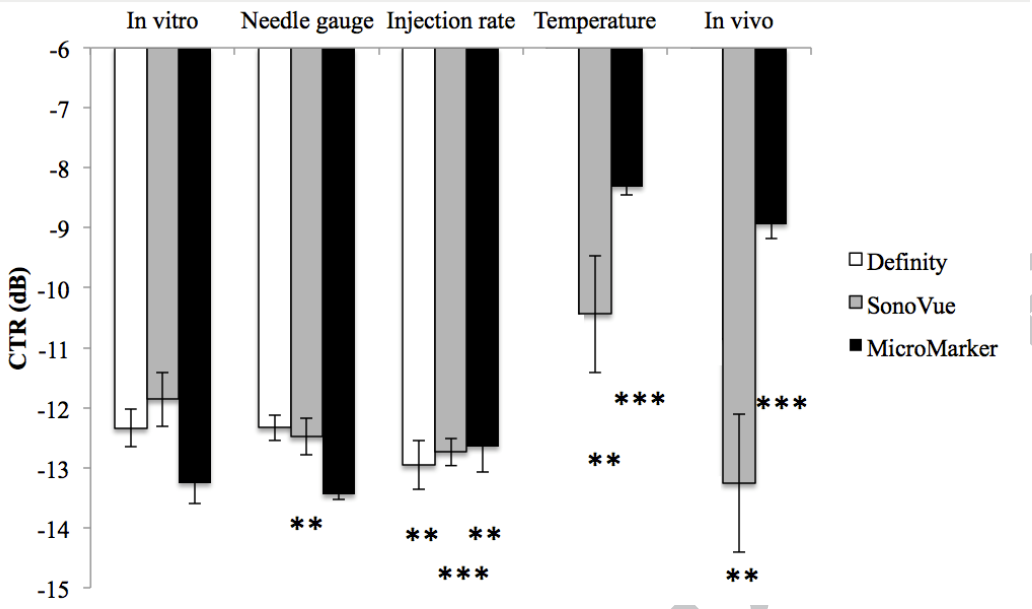

Fig.4: The CTR comparison of Definity, SonoVue and MicroMarker, no mark: no significant difference, ** P<0.01, ***P<0.001. Group headings similar to that displayed in Figure 1

The NSI of Definity, SonoVue and MicroMarker are shown in Figure 5. For Definity, NSI shows no significant variation with needle gauge and injection rate. Significant decrease in NSI of SonoVue was found with increase in temperature. The variation in the trend of SonoVue is consistent with the variation of MicroMarker in the temperature and *in vivo* cases. MicroMarker had the largest NSI of the three agents.

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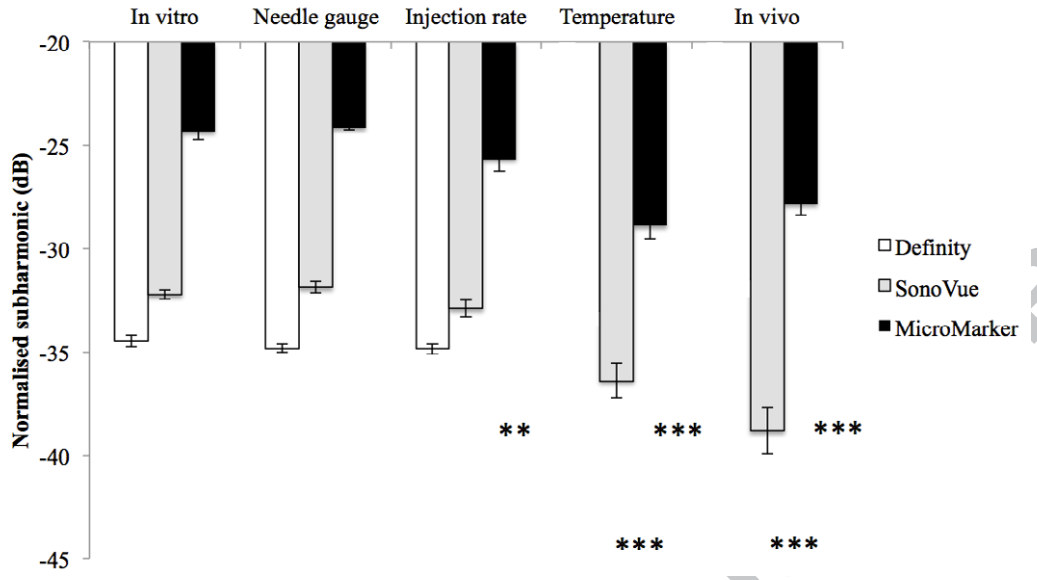

Fig.5: The normalized subharmonic intensity comparison of Definity, SonoVue and MicroMarker, No mark: no significant difference, **P<0.01, ***P<0.001. Group headings similar to that displayed in Figure 1

4 DISCUSSION

4.1 The impact of needle gauge on the mean diameter, concentration and acoustic properties of the MBs at RT

The concentration of Definity, SonoVue and MicroMarker obtained from the study of needle gauge size between 19G and 27G at RT agrees with Talu's study [20] which show that increasing needle gauges (i.e., narrower ID) reduces the concentration of the MBs because of the aggravated diffusion of the MBs due to an increment in the forced compression named as shear stress (Table 3). The concentration of SonoVue was found to decrease by far the most in the three UCAs. This may also be the reason for the decrease in mean diameter of Definity agreeing with previous studies [20, 21].

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However, no significant difference was found in the mean diameter for SonoVue MB. Definity, SonoVue and MicroMarker are poly-disperse MBs of concentration 1.2×10^{10}MBs/ml [10], 2-5$\times10^{8}$ MBs/ml [24] and 2×10^{9} MBs/ ml [26] respectively. Talu et al. [20] showed less than 10% variation in mean diameter of in-house MBs when using 27G needle at an injection rate of 0.1 ml/sec (3ml/min = 0.05 ml/sec). Although the structure and the size distribution of SonoVue MBs are comparable to the in-house MBs described in the Barrack et al study, they are different from the small MBs (0.95 ± 0.75 μm) with a narrow distribution in Talu et al. [20] study. However the concentration of MBs used in this study is similar to Talu's study (0.1/0.5/1$\times10^{9}$ MBs/ml) and approximately a factor of 500 greater than that used in the Barrack's study. Therefore it is likely that the differences between the tested agents are caused by differences in concentration and size distribution.

For Definity in a frequency range of 17-31MHz and at RT, only the measured NSI was found to decrease with increasing gauge needle. However a significant decrease was found in the attenuation and CTR of SonoVue at RT with increasing needle gauge that may be due to the reduction in mean diameter and concentration of MBs.

4.2 The impact of injection rate on the mean diameter, concentration and acoustic properties of the MBs

The reduction in injection rate from 3 ml/min to 0.85 ml/min at RT is found to decrease the mean diameter of both SonoVue and Definity MBs. This is not in agreement with the trend from previously reported studies [20, 21]. However in our study, in addition to the difference in the structure, concentration and size distribution

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between the tested MBs, from Table 3 it can be seen that at RT the decrease in mean diameter from high to low injection rate may be attributed to a lower shear stress (0.78 mPa) within the 2ml syringe used for high injection rates compared to a higher shear stress (1.32mPa) experienced within the 1ml syringe used for low injections rates. The results in Talu's and Barrack's studies support these observations as the change in mean diameter of MBs in Talu's study is less than 5% using a 23G needle [20]; while in this study using a 19G needle (larger I.D.) a smaller reduction in the variation in mean diameter would be expected. The syringe was placed horizontally on the syringe pump during injection so it is important to note that at a low injection rate there was sufficient time for large MBs to float towards the wall of the syringe and therefore be exposed to the increasingly higher shear stress [21, 22, 30]. However, the total time of the needle being held horizontally was a maximum of 1 minute from set-up to the UCA being output into an Eppendorf. From calculations based upon Goertz et al [23], it can be shown that even a bubble of as large a diameter of 5 micron requires approximately 3 minutes to float from the center of the syringe to the syringe wall, which is significantly longer than the limited time of bubble passage through the syringe. Unlike Definity, MicroMarker showed no significant variation in concentration with changing injection rate. However, the concentration of microbubbles decreases for SonoVue with increasing injection rate similar to the results in Barrack's study [21].

For Definity and SonoVue in the frequency range of 17-31 MHz the measured CTR was found to decrease significantly with decreasing injection rate with respect to the *in vitro* data. From Table 3, using 19G needle, the pressure drop and the shear stress in the needle at an injection rate of 3 ml/min is 10 times and 5 times greater

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respectively than the corresponding values at 0.85 ml/min. Additionally, as analysed in section 4.2 that the mean diameter of Definity and SonoVue decreased at lower injection rate, i.e., large MBs are more likely to be disrupted at a higher injection rate than at a lower injection rate. This may explain the significant reduction in CTR observed for SonoVue and Definity at lower injection rates. Conversely a significant increase in CTR with injection rate was observed for MicroMarker. The mean diameter of MicroMarker was smaller (*in vitro* case in Figure 1) than that of Definity and SonoVue, thus the floatation of large MBs of MicroMarker towards the wall of syringe during the slow injection process is likely to have less impact on the size distribution and concentration for Definity and SonoVue.

4.3 The impact of temperature on the mean diameter, concentration and acoustic properties of the MBs

The mean diameters of SonoVue MBs was shown to increase from RT to BT which agreed with previous results [16]. The process of MBs dissolution was previously found to be in 3 phases: (1) an initially quick growth, (2) steady dissolution [31], (3) phase changes from low vapour pressure to vapour-to-liquid under Laplace pressure [32, 33]. In the first growth stage, it was proposed that the increase in temperature enabled the expansion of SonoVue MBs by weakening the chemical bonds between lipid molecules and changing the shell elasticity and surface tension [16]. However in our studies, Definity showed a significant decrease in mean diameter form RT to BT. Because of the different shell components of the two UCAs, when close to the phase transition temperature, Definity may tend to dissolve faster than SonoVue and lead to

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the overall reduction in mean diameter. The concentration of both Definity and SonoVue decreases significantly at BT.

The results from this study show that the attenuation of SonoVue reduces from RT to BT in the frequency range 17-31MHz. Previously published data of SonoVue measured at 3.5 MHz and at 100kPa peak negative pressure found attenuation increased with temperature below 40^{o}C [19]. However, the frequency of insonation in this study is much greater than the reported resonance frequency range of SonoVue MBs (1-3 MHz) [25] and we have shown that the concentration of SonoVue MBs reduces significantly at BT. In comparison, in our study the attenuation of MicroMarker was found to increase significantly with temperature suggesting that at higher temperatures there may be an increase in mean diameter of the MB population giving a larger attenuation. Previous studies at RT have shown that MicroMarker demonstrated an increase in attenuation up to 21MHz [34]. In addition the higher values of attenuation exhibited by MicroMarker in BT measurements compared to RT measurements in our studies are consistent with similar measurements made by Raymond et al [14] at lower frequencies. The variation of acoustical properties for Definity as a function of temperature is not included in this paper.

The CTRs of SonoVue and MicroMarker were found to significantly increase at BT compared with RT. For SonoVue, although the number of microbubbles decreased at BT, there was an increase in mean diameter. The increased CTR may be caused by the larger scattering cross-section that can be attributed to an increase in mean diameter. The NSIs of SonoVue and MicroMarker decrease from RT to BT. de Jong et al (2000) has shown that maximal subharmonics are generated by microbubbles resonating at

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half of the driving frequency [35], which is fixed at 15MHz in this study. As we have shown in Figure 1 that the diameters of SonoVue and MicroMarker bubbles increase with temperature they will therefore resonate at lower frequencies potentially outwith the frequency bandwidth of the transducer used in this study.

4.4 The combined effect of needle gauge, injection rate and temperature and future research

Based on the above discussions of the individual impact of the three factors (needle gauge, injection rate and temperature), no clear trend is evident in the changes in the mean diameter, concentration and acoustic parameters of the three UCAs. However, for SonoVue and MicroMarker, significant variation was found in both size and acoustic properties when needle gauge, temperature and injection rate were changed from a typical *in vitro* to a typical *in vivo* experimental design. The differences between the *in vivo* and *in vitro* settings are the cumulative result of variations in the parameters with one or more factors predominating and effectively determining the measured outcome. In the future, studying the variation in single MB of different diameters at distinct environments might be of interest to give a quantitative answer to this question. Details of this variation will allow researchers to estimate the response of MBs under different environments and explain the discrepancy between *in vitro* and *in vivo* experiments.

5 CONCLUSION

In this paper, the influence of needle gauge and injection rate on the mean diameter, concentration and acoustic properties of Definity, SonoVue and MicroMarker over the

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frequency range of 17-31MHz and the influence of temperature on SonoVue and MicroMarker over the same frequency range were investigated. In particular, the influence of these parameters on typical *in vitro* settings (19G needle, 3ml/min at RT) and *in vivo* settings *(*27G needle, 0.85ml/min at BT) were compared. Although no consistent trend was found for each individual experimental set-up change, we found that the mean volume-based diameter and concentration of both SonoVue and Definity reduced significantly with a tenfold reduction in concentration when measured under typical *in vivo* experimental conditions compared to *in vitro* conditions. For MicroMarker, neither the mean volume-based diameter nor concentration changed significantly. With respect to the acoustic properties of the UCAs we found that the attenuation, CTR and NSI of SonoVue significantly decreased when moving from typical *in vitro* to *in vivo* experimental set-ups. From our limited measurements of Definity, we found no significant change in attenuation, CTR and NSI with needle gauge. For MicroMarker we found that, while the attenuation and CTR increased, the NSI decreased when changing from typical *in vitro* to *in vivo* experimental set-ups. These results suggest that in similarity to results obtained at lower frequencies, care must be taken in translating results obtained *in vitro* to preclinical *in vivo* imaging studies. Moreover based on these results, it is likely that other variables such as choice of diluent (saline or blood) and the gas saturation level of the testing environment may have significant effect on the acoustic measurements and thus are worthy of further exploration at high frequencies.

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CONFLICT OF INTEREST

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Research highlights

- When changing from typical *in vitro* to *in vivo* experimental conditions, Definity and SonoVue demonstrated a significant reduction in mean diameter. MicroMarker did not demonstrate any significant change in diameter.
- When changing from typical *in vitro* to *in vivo* experimental conditions, Definity and SonoVue demonstrated a significant tenfold reduction in concentration. Micromarker did not demonstrate any significant change in concentration.
- When changing from typical *in vitro* to *in vivo* experimental conditions, SonoVue demonstrated a significant reduction in attenuation, contrast to tissue ratio (CTR) and normalized subharmonic intensity (NSI).
- When changing from typical *in vitro* to *in vivo* experimental conditions, MicroMarker demonstrated a significant increase in attenuation, CTR but a reduction in NSI.
- Care must be taken in translating results obtained *in vitro* to preclinical imaging studies.

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