

● *Original Contribution*

ACOUSTIC AND KINETIC BEHAVIOUR OF DEFINITY IN MICE EXPOSED TO HIGH FREQUENCY ULTRASOUND

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Abstract—Microbubble contrast agents have shown clinical potential for characterising blood flow using 1 to 10 MHz ultrasound; however, scaling their use for similar applications in the mouse with high frequency ultrasound (20 to 60 MHz) has not been addressed. The goal was to determine the utility of microbubbles for mouse imaging with 30 MHz ultrasound by investigating their attenuation and backscatter characteristics as a function of concentration *in vitro* and dose response *in vivo*. The agent was exposed to a 30 MHz, 20% bandwidth pulse with a peak negative pressure of 244 kPa. *In vitro* results showed that the attenuation and backscatter increased linearly for concentrations between 2.8×10^6 and 28×10^6 bubbles per mL of deionized water. *In vivo* experiments were performed in the jugular vein of CD-1 mice and time intensity curves were acquired for doses between 10 and 100 $\mu\text{L kg}^{-1}$. These doses corresponded to the range of concentrations used *in vitro*. *In vivo* results showed that the peak enhancement of the agent increased linearly for doses between 10 and 60 $\mu\text{L kg}^{-1}$, the duration of enhancement varied between 200 to 300 s and the integrated enhancement (area under the curve) increased linearly up to 100 $\mu\text{L kg}^{-1}$. A maximum enhancement of 13 dB over the blood pool was observed for a dose of 100 $\mu\text{L kg}^{-1}$. The intra- and inter-mouse variabilities were 10% to 40% and indicate that further optimisations are required. These results suggest that quantitative contrast flow studies in the mouse using high frequency ultrasound are possible for doses between 10 and 60 $\mu\text{L kg}^{-1}$. (E-mail: shawns@swri.ca) © 2009 World Federation for Ultrasound in Medicine & Biology.

Key Words: Ultrasound, High frequency, Ultrasound biomicroscopy, Microbubbles, contrast agent, Dose response, Quantification, Mouse, Small animal imaging, Tail vein catheter.

INTRODUCTION

The mouse has proven to be an invaluable research tool for the preclinical assessment of human diseases. Numerous laboratories have established genetic manipulation and gene targeting techniques to create novel mouse strains to study the pathogenesis of disease and efficacy of treatments. High frequency ultrasound has provided a valuable tool for the noninvasive investigation of such mice (Foster et al. 2000). There is growing interest in further developing high frequency ultrasound methods to investigate the role of the vascular system in disease progression and its response to treatment (Deng et al. 1998; Pavlin et al. 1998; Goertz et al. 2006a). An emerging and exciting technique to probe the circulation is

contrast enhanced ultrasound, where an exogenous agent composed of micron sized bubbles is introduced into the intravascular space and detected using ultrasound. Because these agents are strictly intravascular and mimic red blood cell rheology, they provide a window into the kinetics of blood flow (Lindner et al. 2002). Microbubbles have shown much promise clinically when exposed to conventional or low frequency (1 to 10 MHz) ultrasound (Wilson and Burns 2006); however their utility for the preclinical assessment of disease using high frequency ultrasound is currently being established.

It has only been within the last 10 years that the utility of microbubble contrast agents have been investigated for use with high frequency ultrasound (Deng et al. 1998; Pavlin et al. 1998; Moran et al. 2002; Goertz et al. 2001, 2003, 2006b, 2007). These studies have been dedicated to evaluating the acoustic properties of microbubbles exposed to high frequency ultrasound. For ex-

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ample, Goertz *et al.* (2005a, 2005b) demonstrated that the nonlinear echoes from microbubbles can be used to improve *in vivo* B-mode imaging and flow imaging in small animals using high frequency ultrasound. As well, Yeh *et al.* (2004) described a technique to quantify haemodynamics in the microcirculation using 1 MHz ultrasound to disrupt the bubbles and 25 MHz to detect the replenishment of the agent with high spatial resolution. While these studies have established the potential of contrast agents exposed to high frequency for the quantitative assessment of haemodynamics in a preclinical setting, no indication is given as to the optimal contrast agent dose.

The quantitative assessment of haemodynamics *in vivo* using high frequency ultrasound and microbubbles is dependent on the relationship between the backscattered energy from bubbles and the agent concentration. In the regime where the scattered energy from bubbles is proportional to the agent concentration, changes in haemodynamic properties, such as blood volume, will be reflected by proportional changes in the detected ultrasound energy. Microbubble-induced attenuation, multiple scattering and bolus spreading will affect the range over which the scattered energy from bubbles is proportional to the agent concentration (de Jong *et al.* 1992; Uhlendorf *et al.* 2000; Becher and Burns 2000). In addition, due to the small focal volume of a high frequency transducer and the resonant population of microbubbles exposed to high frequency ultrasound, a clinical concentration of microbubbles might not provide sufficient enhancement of the blood pool. In the case of mice, there is currently no consensus on a physiologically tolerable and acoustically relevant concentration of microbubbles to administer.

We have presented preliminary work toward establishing a range of contrast agent doses suitable for contrast flow studies in large vessels (~1 mm) of mice exposed to high frequency ultrasound (Stapleton *et al.* 2006). However, the variability of such measurements were not evaluated for repeat injections in the same and different mice. Based on this work, we have also performed a preliminary investigation of the dose response in the microcirculation of a mouse kidney exposed to high frequency ultrasound, using a different contrast agent (Stapleton *et al.* 2007). To accomplish a dose response in the microcirculation, we modified a high frequency imaging system developed by Visualsonics (Toronto, Canada) to perform nonlinear contrast imaging. We found only modest enhancement over the background signal for the small range of doses investigated and large inter-mouse variabilities. The work presented in this article represents a first step in determining the appropriate range of dose that are physiologically tolerable and provide sufficient enhancement for quantitative

contrast flow studies in mice using high frequency ultrasound.

This study is separated in two parts. In the first part, *in vitro* experiments demonstrating the scattering and attenuation characteristics as a function of agent concentration are presented. A suitable ultrasound contrast agent for quantitative contrast flow studies should provide a linear relationship between concentration and bubble backscatter with minimal attenuation. In the second part, the acoustic and kinetic behaviour of the contrast agent is characterised in the mouse when exposed to 30 MHz ultrasound. The clinical contrast agent Definity (Bristol-Myers Squibb Medical Imaging, New York, NY, USA) is used for both *in vitro* and *in vivo* experiments. A method to perform minimally invasive repeatable contrast injections into the tail vein of a mouse using a catheter is presented. The time course of enhancement for a bolus administration of contrast agent was analysed and a range of suitable doses which are both physiologically tolerable and acoustically relevant is identified. Finally, the variability of the dose response was evaluated for repeated injections in the same mouse and different mice.

METHODS AND MATERIALS

Contrast agent

The contrast agent used in this study was Definity, composed of octafluoropropane gas encapsulated in a lipid shell. As demonstrated by Goertz *et al.* (2007), the number and volume distribution is dependent on the method by which Definity is handled after activation. Therefore, the number and volume distribution, as well as the native concentration of Definity were measured based on the specific handling method used in this article. Definity was activated according to the manufacturer's guidelines and allowed to cool to room temperature before use. In all cases, withdrawals from the vial were accomplished by agitating the agent for 20 s, followed by inverting the agent for 10 s and gently extracting the agent from the inverted vial using an 18 gauge needle inserted with the base of its beveled tip just inside the rubber stopper while venting. Measurements were performed using a Coulter Counter Multisizer Z3 (Beckman Coulter Inc., Fullerton, CA, USA) with a 30 μm aperture tube, which permitted accurate sizing of bubbles between 0.87 and 18 μm in diameter. A background count was obtained by measuring the distribution of particles of the filtered ISOTON II solution and was subtracted from the measured distribution of Definity. Figure 1 shows that approximately 97% of the population of microbubbles have diameters between 0.87 and 3.00 μm , while the remaining 3% of the population have diameters between 3 and 18 μm . The native concentration of Definity for

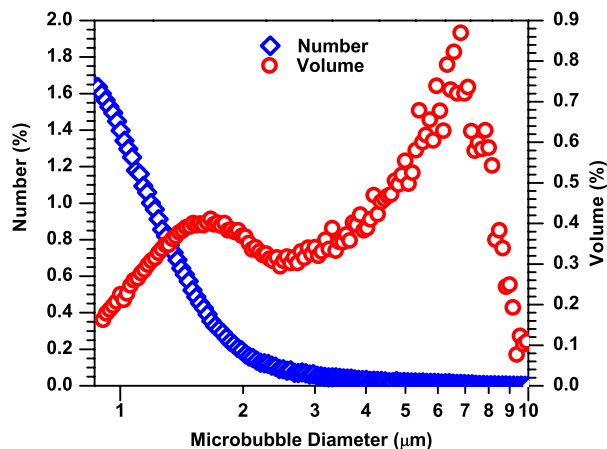


Fig. 1. The number (diamond) and volume (circle) distribution of native Definity. Definity contains a substantial number of submicrometer and micrometer sized bubbles. Microbubbles with diameters between 0.87 and 3.00 μm account for 97% of the population. The remaining 3% of the population have diameters between 3 and 18 μm .

bubbles with diameters between 0.87 and 18 μm was $1.6 \pm 0.1 \times 10^{10}$ bubbles per mL. The native concentration was used to determine the *in vitro* and *in vivo* concentrations of Definity for this study.

In vitro

Attenuation and backscatter versus concentration.

Attenuation and backscatter experiments were performed in a flow cell, which is illustrated in Fig. 2. Agent was flowed through the system at a rate of roughly 1 mL per s using gravity. A 30 MHz Gaussian shaped pulse with a -6 dB fractional bandwidth of 20% (~ 7 cycles) was generated using an arbitrary waveform generator

(AWG2020, Tektronix, Beaverton, OR, USA), amplified by a power amplifier (M3206, AMT, Anaheim, CA, USA) and transmitted using a 30 MHz transducer (RMV 707; f-number 2.1; aperture 6 mm) provided by Visual-Sonics, Canada. The transducer was characterised using a 40 μm diameter needle hydrophone (Precision acoustic LTD, Dorchester, Dorset, UK), which was calibrated from 5 to 60 MHz by the National Physical Laboratory (Teddington, Middlesex, UK). The peak negative pressure of the transmitted pulse was 244 kPa at the focus, which was previously determined to produce predominantly linear backscatter without disrupting the microbubbles (Stapleton et al. 2006). The -3 dB depth of field and beam width were 1390 μm and 104 μm , respectively. The received echoes from the agent were band-pass filtered from 10 to 70 MHz, amplified by a 40 dB preamplifier (AU-1313, Miteq, Hauppauge, NY, USA) and digitized at a sampling frequency of 500 MHz using a PC based 8-bit analog to digital PCI card (DP240, Acqiris, Geneva, Switzerland). For each measurement, 50 traces were acquired at a pulse repetition frequency (PRF) of 10 Hz to insure each trace was from a different population of bubbles. The power spectrum was calculated for each trace using a 0.6 μs window centered on the geometric focus. The integrated power spectrum was then calculated over the fundamental bandwidth (27 to 33 MHz) for each of the 50 traces and averaged. The experiments were repeated 10 times and the mean and standard deviation were calculated.

The experiments were conducted as a function of contrast agent concentration. The agent was diluted into 100 mL of deionized water and fed through the flow cell by gravity. Concentrations ranged from 15 to 170 μL of Definity per 100 mL of deionized water. This corresponds to 2.8×10^6 and 28×10^6 bubbles per mL of

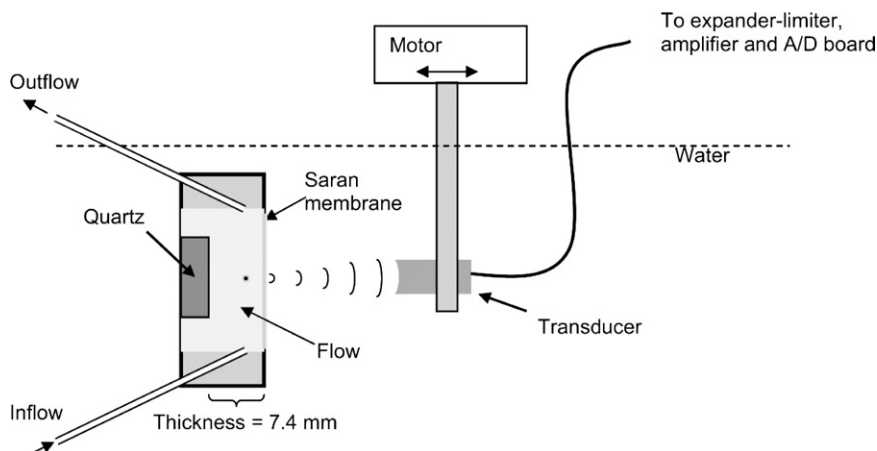


Fig. 2. Set-up for attenuation and backscattering experiments. For attenuation experiments the transducer is focused on the quartz reflector. For backscatter experiments the transducer is focused 1 mm inside the flow cell.

deionized water. Due to signal-to-noise ratio (SNR) issues, it was impossible to measure attenuation through the microbubbles above a concentration of 170 μL of Definity per 100 mL using this set-up. The attenuation of the transmitted beam through the contrast agent was determined by taking the ratio between the integrated power of the signal reflected off a quartz plate in the presence and absence of bubbles. The final result was corrected for the attenuation in water. The backscatter was determined by positioning the geometric focus approximately 1 mm inside the flow cell and calculating the integrated power from the bubble signals. The backscatter was normalised to the signal obtained from the highest concentration and reported in linear units. The linearity of attenuation and backscatter with respect to contrast agent concentration were determined by fitting the results with a straight line and calculating the r-squared value using OriginPro (v7.5, OriginLab Corp., Northampton, MA, USA).

In vivo

Tail vein preparation and injection. All animal experiments were conducted in accordance with protocols approved by the Sunnybrook Health Sciences Centre Institutional Animal Care and Use Committee (Toronto, Canada). Experiments were performed on nine female CD-1 mice of similar age (4 to 6 weeks) and mass ($23 \pm 1\text{g}$). Before the administration of 2% isoflurane mixed with oxygen, a lamp was used to gently warm the mouse in its cage for 10 min to dilate its blood vessels. Once anaesthetised, rubbing alcohol was immediately applied and a 26 G catheter (Abbocath-T, Venisystems, Hospira, Ireland) was inserted 0.25 to 0.5 cm into the lateral tail vein. Due to the large volume of the catheter hub (100 μL), it was filled with a mixture of sterile saline and heparin (0.3% heparin by volume) to remove any air. An injection cap (Strategic Applications International, IL, USA) was also filled with the solution and was screwed on to the catheter hub to facilitate the injections. The mouse was laid supine on a platform with all legs taped to ECG electrodes for heart rate monitoring. All hair was removed from the neck using a chemical hair remover (Nair; Carter-Horner, Mississauga, Ontario, Canada).

The administration of the agent proceeded as follows: two 1 mL tuberculin syringes with a 22 G needle where primed for injection; the first syringe contained the microbubble contrast agent diluted in saline and the second contained the saline-heparin mixture. Both syringes were inserted through the injection cap. Each injection consisted of 50 μL bolus of the microbubble-saline mixture immediately followed by a 100 μL flush of the saline-heparin mixture. Both injections were delivered in less than 5 s. Any changes in the population

distribution of Definity by pushing the contrast agent through the 26 G catheter were evaluated using the Coulter Counter Multisizer Z3 and no differences were observed.

Dosing

Definity was diluted in sterile saline to ensure the same volume of fluid was injected for each dose. Doses ranged from 10 to 100 $\mu\text{L kg}^{-1}$, which corresponds to 2.8×10^6 and 28×10^6 bubbles per mL of blood once the agent has fully mixed in the circulation. The concentrations were chosen to match those used *in vitro* and the volume of contrast in which to dilute in saline was calculated using the following equation,

$$V_{\text{Definity}}[\mu\text{L}] = \frac{D[\mu\text{L Kg}^{-1}]W[\text{kg}]}{V_{\text{Blood}}[\mu\text{L}]} * \frac{V_{\text{Total}}[\mu\text{L}]}{V_{\text{Injected}}[\mu\text{L}]} * V_{\text{Saline}}[\mu\text{L}] \quad (1)$$

where V_{Definity} represents the volume of native Definity that was mixed with a volume of saline given by V_{Saline} , both in $[\mu\text{L}]$. D represents the dose in $[\mu\text{L kg}^{-1}]$. W represents the mouse weight in $[\text{kg}]$. V_{Injected} represents the volume of the contrast-saline dilution injected in the mouse in $[\mu\text{L}]$ (50 μL). V_{Total} represents the total volume of fluids in the mouse circulatory system and includes the mouse blood volume, the injected volume and the flush volume (100 μL). V_{Blood} represents the volume of blood in the mouse in $[\mu\text{L}]$ and was estimated using eqn 2 which relates body weight in $[\text{kg}]$ to blood volume (Janssen and Smits 2002). It is important to note that eqn 1 does not account for any remaining fluid from the previous injection, the effects of which will be discussed.

$$V_{\text{Blood}}[\text{L}] = 0.055 \times W^{0.99} \quad (2)$$

Dose response

The transmit and receive system consisted of a VisualSonics Vevo770 scanner (VisualSonics, Toronto, Canada) equipped with a 30 MHz transducer (RMV 707; f-number 2.1; aperture 6 mm). The transmit path on the Vevo770 was modified by the addition of an eighth order band pass filter with -3 dB points at 25.8 MHz and 34.3 MHz, and -20 dB points at 24.4 MHz and 36.8 MHz. The transmit filter allowed for the generation of Gaussian shaped pulses and reduced energy outside of the desired transmit bandwidth. The transmitted pulse had a centre frequency of 30 MHz and a -6 dB fractional bandwidth of 20% at the focus. Agent detection was performed in the left jugular vein with the geometric focus positioned at the centre of the vessel. M-mode measurements were made with a PRF of 1 kHz. To obtain a peak negative pressure (PNP) of 244 kPa inside the jugular vein, the transmit amplitude was increased by 3 dB to compensate

for attenuation through tissue. The signal increase was approximated by taking into account 3 dB per mm attenuation at 30 MHz (Cobbold 2007) through 1 mm of skin. Received echoes from the blood pool were digitized at a rate of 500 mega samples per s using a PC based 8-bit analog to digital PCI card (DP240, Acqiris, Geneva, Switzerland). One thousand received traces were recorded for a period of 1000 ms, followed by a 150 ms dwell time to store the data. The sequence was repeated for a total duration of 700 s. The baseline was established by acquiring signal from blood 30 s before injection. Each 1000 trace data set was analysed manually using custom software to remove any breathing motion. The integrated power spectrum was then calculated for each trace over the fundamental bandwidth (27 to 33 MHz) using a $0.6 \mu\text{s}$ rectangular window centered around the focus. The rectangular window was chosen to encompass the majority of the jugular vein and was smaller than the -3 dB depth of field (~ 1.2 mm) of the transducer, thus, reducing effects of on axis pressure differences. The mean of each 1000 trace ensemble was calculated to give a time averaged enhancement. The resulting time intensity curves were reported in linear units and normalized to the baseline.

A total of nine mice were used for the study. Each mouse received five injections of contrast agent and the start of each injection was separated by 20 min. A total of seven doses were evaluated for dose response experiments and each of the doses were repeated five times in different mice to evaluate the inter-mouse variability. The seven doses were randomly distributed and a total of seven mice were used for the inter-mouse variability experiments. The intra-mouse variability was determined by repeating a dose of $40 \mu\text{L kg}^{-1}$ in a single mouse five times and calculating the coefficient of variation. A control experiment was performed by injecting a $150 \mu\text{L}$ bolus of saline in a single mouse five times. In total, each mouse received $750 \mu\text{L}$ of fluid over a period of 100 min. Time intensity curves (TIC) were generated for each injection and were used to determine the peak enhancement, duration of enhancement (defined as the time for the peak enhancement to drop to 2 standard deviations above the baseline) and the integrated enhancement (the area under the TIC curve). The heart rate was monitored using an ECG and postprocessing analysis of M-mode images gave an indication of anatomical changes of the jugular vein over the course of each examination.

RESULTS

In vitro

Attenuation and backscatter versus concentration. Figure 3 demonstrates the relationship between microbubble-induced attenuation (in dB mm^{-1}) of the ultra-

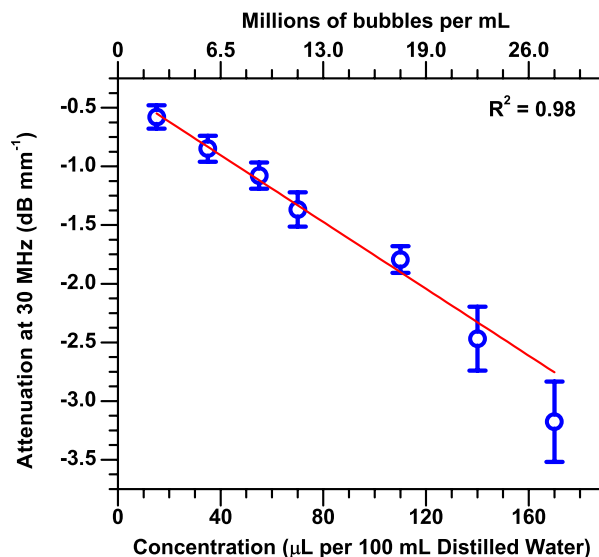


Fig. 3. *In vitro* attenuation as a function of concentration of Definity microbubbles measured with a 30 MHz transducer. The fit demonstrates a linear relationship. Error bars represent the standard deviation ($n = 10$).

sound wave and the concentration of the contrast agent. The top x-axis shows the theoretical number of bubbles per mL of deionized water, calculated based on diluting the measured native concentration of Definity. Regression analysis demonstrated a linear relationship between microbubble-induced attenuation and microbubble concentration. The attenuation at 30 MHz ranged from 0.5 to 3.1 dB per mm, which are significantly higher than those previously reported (Goertz et al. 2007). The attenuation measurements presented in this article are based on realistic imaging concentrations, while those reported by Goertz et al. (2007) focused on dilute concentrations.

Figure 4 demonstrates that the integrated backscattered power (in linear units, relative to quartz) of the ultrasound wave is linearly related to the concentration of the agent when exposed to a 30 MHz, 20% bandwidth, pulse with a PNP of 244 kPa. A straight line fit further confirmed this relationship. The integrated backscattered power improved by a factor of 5 (7 dB) when the concentration was increased from 2.8×10^6 and 28×10^6 bubbles per mL.

In vivo

The heart rate of each mouse was recorded before the injection of a bolus of contrast agent and again at the end of the trial. The initial and final heart rates were 455 ± 49 and 462 ± 63 beats per min, respectively. A paired two-tailed Student's *t* test (p value = 0.37) indicated that the initial and final heart rates did not significantly differ. Therefore, multiple injections of the contrast agent likely

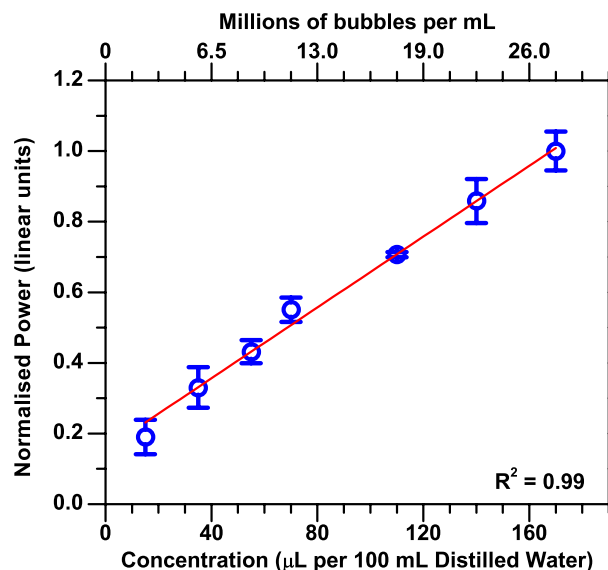


Fig. 4. *In vitro* integrated backscattered power as a function of concentration of Definity microbubbles exposed to 30 MHz ultrasound. The backscattered power is referenced to quartz, normalised and shown in linear units. The fit shows a linear relationship. Error bars represent the standard deviation ($n = 10$).

had little effect on cardiac function. Analysis of the vascular anatomy from M-mode data showed that the jugular vein is situated approximately 1 mm under the surface of the skin and has an average diameter of 0.95 ± 0.11 mm. Upon the first pass of the bolus, the jugular vein distended in response to the increased fluid volume, growing on average 0.17 ± 0.06 mm in the axial direction. The increase was found to be significant (two-tailed Student's t test; p value < 0.05). The vessel diameter subsequently decreased over time, in an exponential manner, taking on average 93 ± 58 s to return to its original size. These results contribute to establishing the physiological tolerance for a bolus injection of contrast agent in mice.

The acoustic and kinetic behaviour of i.v. bolus injections of Definity into the tail vein of CD-1 mice are shown in Fig 5a. Standard deviations were omitted for clarity but are reflected in the measurements of peak enhancement, duration of enhancement and integrated enhancement which were obtained from the dose response curves. Breathing motion was manually removed from the received signal using software to give an indication of the true kinetics of the agent. The TICs exhibited a rapid wash-in phase followed by a slower wash-out phase. The initial phase represents the first pass of the agent from the site of injection to the detection point in the jugular vein and can be better appreciated in Fig. 5b. Approximately 5 s after the first pass of the agent, the bolus has spread sufficiently and been partially elimi-

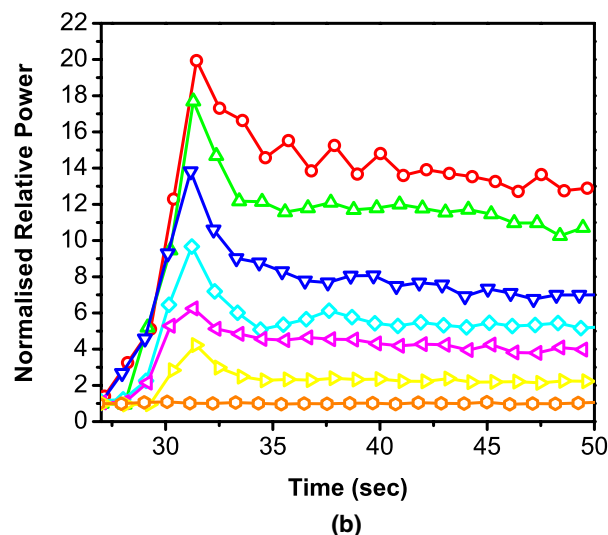
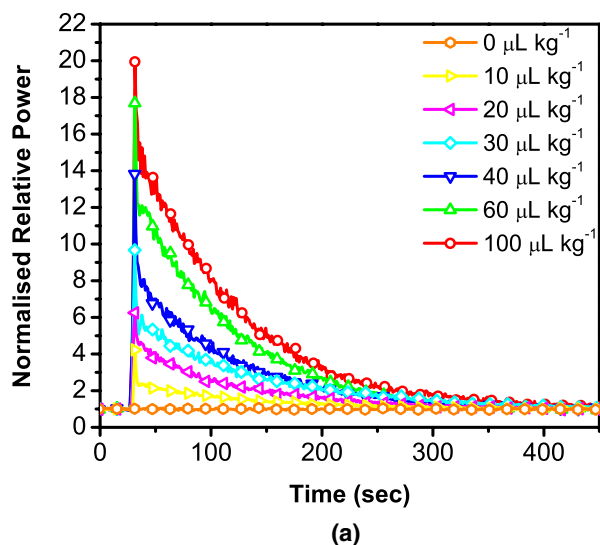


Fig. 5. (a) *In vivo* dose response curves for Definity exposed to 30 MHz ultrasound in linear units, normalised to the baseline. The enhancement is presented in linear units and normalised to the baseline power. Note that increasing the dose increases the peak enhancement and the duration of enhancement. Each time intensity curve is an average of 5 trials. (b) A zoomed in view of the dose-response curve showing the first pass kinetics of the agent.

nated such that the agents kinetic behaviour transitions to the second, slow wash-out, phase. The second phase represents the recirculation and subsequent elimination of the agent. The two phase kinetic behaviour of the agent was observed for all doses and is typical of a bolus injection of microbubble contrast agent (Schwarz *et al.* 1996). As the dose is increased from 10 to $100 \mu\text{L kg}^{-1}$, there was an increase in the peak enhancement, duration of enhancement and integrated enhancement.

The peak enhancement of the contrast agent echoes measured in the jugular vein as a function of micro-

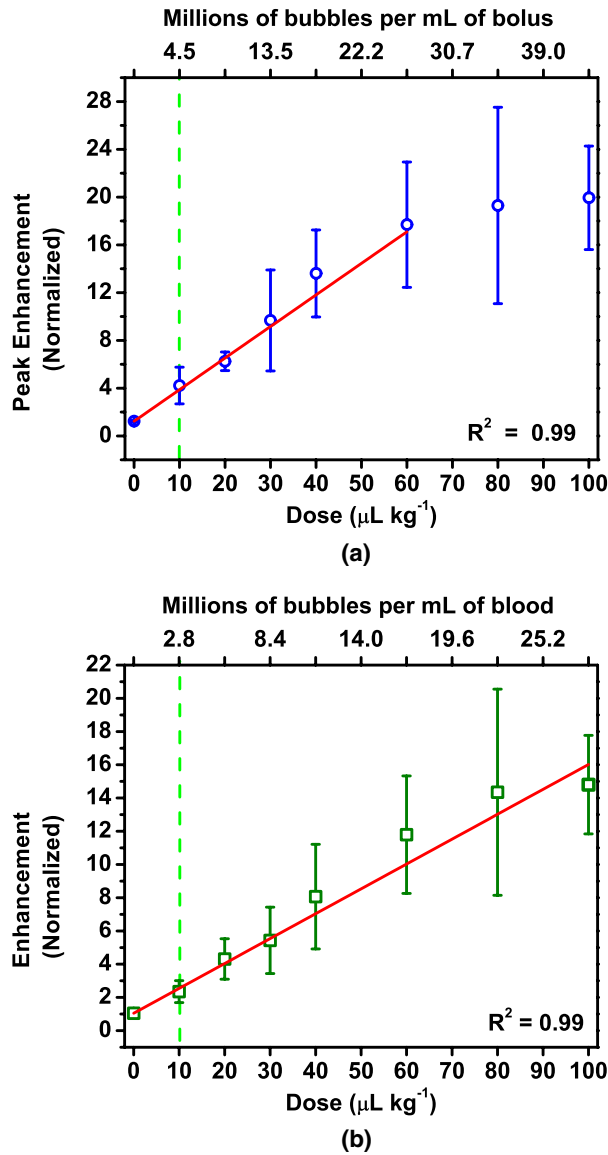


Fig. 6. (a) Peak enhancement as measured during the first pass of the bolus. Note that the peak enhancement increases nonlinearly for doses $>60 \mu\text{L kg}^{-1}$. Because there has been limited time for the bolus to mix with the circulation, the concentration of the agent is similar to the syringe concentration. The syringe concentration is an order of magnitude larger than when the agent is fully mixed in blood and is shown on the top y-axis. (b) The enhancement 8 s after first pass as a function of dose. After the agent has had sufficient time to mix with the circulating blood, the enhancement increases linearly for all doses. The dashed lines in both figures indicate the peak enhancement at 30 MHz for the suggested maximum clinical dose of Definity. The enhancement is presented in linear units and normalised to the baseline. Error bars represent the standard deviation ($n = 5$).

bubble concentration is given in Fig. 6a. Regression analysis was applied and a straight line fit with an r-squared value of 0.99 confirmed a linear relationship

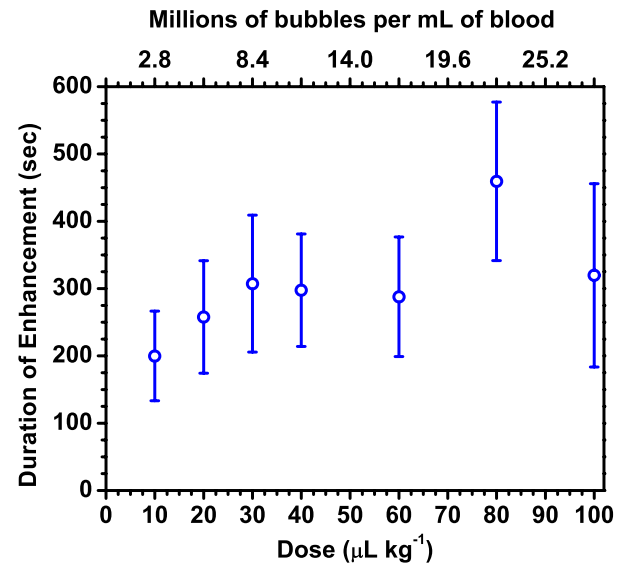


Fig. 7. Duration of enhancement as a function of dose. Note that the duration of enhancement increases for doses up to $30 \mu\text{L kg}^{-1}$. Error bars represent the standard deviation ($n = 5$).

between peak enhancement and microbubble concentration for doses between 10 and $60 \mu\text{L kg}^{-1}$. There was up to a 17 ± 5 times (12 dB) increase in integrated power over the baseline for doses in this range. For doses $> 60 \mu\text{L kg}^{-1}$, there was a plateau in the enhancement of 20 ± 8 (13 dB). However, a linear relationship between normalised power and doses up to $100 \mu\text{L kg}^{-1}$ was found 8 s post first-pass (Fig. 6b). After 8 s, the agent has had sufficient time to recirculate through the mouse's vascular system (Janssen and Smits 2002) and is presumably fully mixed in the circulation. The concentration of the first pass is likely similar to that in the syringe, which can be an order of magnitude higher than the fully mixed concentration. These concentrations are reflected on the top axis of Fig 6a and b and will be further discussed in the next section. The coefficient of variation was calculated from the five injections of the same dose in different mice. It was found to be independent of dose and, thus, a dose averaged coefficient of variation was used to represent the inter-mouse variability. The inter-mouse variability of the peak enhancement was 32%.

The wash-out time of the agent, reflected by the duration of enhancement, has been shown to be a result of various systemic elimination mechanisms including filtering through the microcirculation of the lungs (Bouakaz et al. 1998; Lindner et al. 2002) and natural dissolution in the blood stream (Schutt et al. 2003). The duration of enhancement increased monotonically from approximately 200 to 300 s for doses between 10 to $30 \mu\text{L kg}^{-1}$, at which point the duration of enhancement reached a plateau (Fig. 7). The inter-mouse variability of

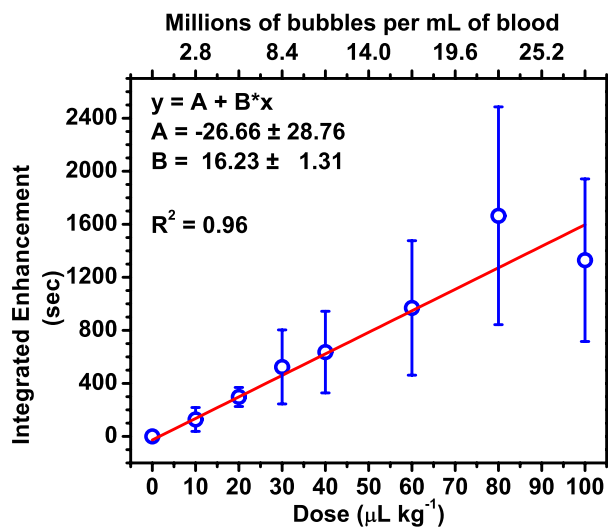


Fig. 8. Integrated enhancement as a function of dose. Error bars represent the standard deviation ($n = 5$).

the duration of enhancement was on average 32%.

The integrated enhancement was determined by calculating the area under the TIC for each dose. The integrated enhancement can be interpreted as the total enhancement from the administered contrast agent dose. Figure 8 is a plot of integrated enhancement as a function of dose. Regression analysis was applied and a straight line fit with an r -squared value of 0.96 confirmed a linear relationship between integrated enhancement and microbubble concentration for all doses. The inverse of the slope provides an estimate of the contrast agent clearance rate, indicating the decrease in enhancement from the agent per unit time, per unit mass. Contrast agent clearance was measured to be $6.16 \times 10^{-2} \pm 0.48 \times 10^{-2} \mu\text{L} \cdot \text{sec}^{-1} \cdot \text{kg}^{-1}$ and forms the basis for determining infusion rates. Inter-mouse variability of the integrated enhancement was 49%.

The intra-mouse variability gives an indication of the reproducibility of the acoustic and kinetic behaviour in the same mouse for repeat doses of the same concentration. The intra-mouse variability of peak enhancement, duration of enhancement and integrated enhancement were evaluated for a dose of $40 \mu\text{L kg}^{-1}$ of Definity and were found to be 10%, 18% and 34%, respectively (Fig. 9). There was no systematic trend in the acoustic and kinetic response between measurements in the same animal indicating that the previous injection had minimal effects on the subsequent injections. A comparison between injection 2 and injection 3 indicate that the peak enhancement and duration of enhancement are similar; however, the integrated enhancement for injection 2 is substantially lower than that for injection 3. It was observed from the time intensity curves for these two trials (not shown here) that while the rate of decay of

the curves were similar, the signal level immediately post peak enhancement was significantly less for injection 2 than injection 3. Overall, the peak enhancement and duration of enhancement exhibited minimal variability; however, the variability in integrated enhancement was comparatively large. The intra-mouse variability was consistently lower than the inter-mouse variability; however, a limitation is that it was only evaluated in a single mouse.

DISCUSSION

The *in vivo* time intensity curves for each contrast agent dose exhibited a two phase behaviour, which has been previously observed by several other investigators (Schwarz *et al.* 1996; Becher and Burns 2000; Correas *et al.* 2000). Phase 1 was characterised by a rapid increase in backscattered power as the agent first passes through the jugular vein. During the first pass of the bolus, the jugular vein diameter increased by 0.17 mm and this likely had an effect on the blood velocity and blood pressure (Li 2004). The distention is at least in part due to the increase in fluid volume; however, we are currently unsure as to how much of the distention can be attributed to the increase in fluid volume versus other effects such as an increase in pressure in the right side of the heart. Phase 2 was characterised by a slow mono-exponential decrease in backscattered power as the agent recirculates and is subsequently eliminated from the blood pool. During the second phase, the jugular vein returned to its original size as the excess fluid was cleared from the vascular system. There are several routes for the removal of excess fluid such as the kidney, lungs and skin. We have not tested through which combinations of these routes the excess fluid is being removed.

A linear relationship was observed between the peak enhancement and agent concentration during the first pass for doses between 10 and $60 \mu\text{L kg}^{-1}$, signifying that this range is suitable for quantitative contrast flow studies where the first pass kinetics are important. Doses between 60 and $100 \mu\text{L kg}^{-1}$ exhibited a nonlinear relationship with integrated power during the first pass. A marked shadowing of structures below the lumen of the jugular vein proximal to the transducer was observed, signifying that microbubble-induced attenuation within the contrast-filled lumen played a role in the saturation of the peak enhancement. Recall that at high concentrations, microbubble-induced attenuation can be as high as 3.1 dB per mm. Further contributing to the plateau is the spreading of the bolus. As the concentration is increased, the agent becomes increasingly dispersed in the vascular system as it travels from the injection site, through the pulmonary and upper body

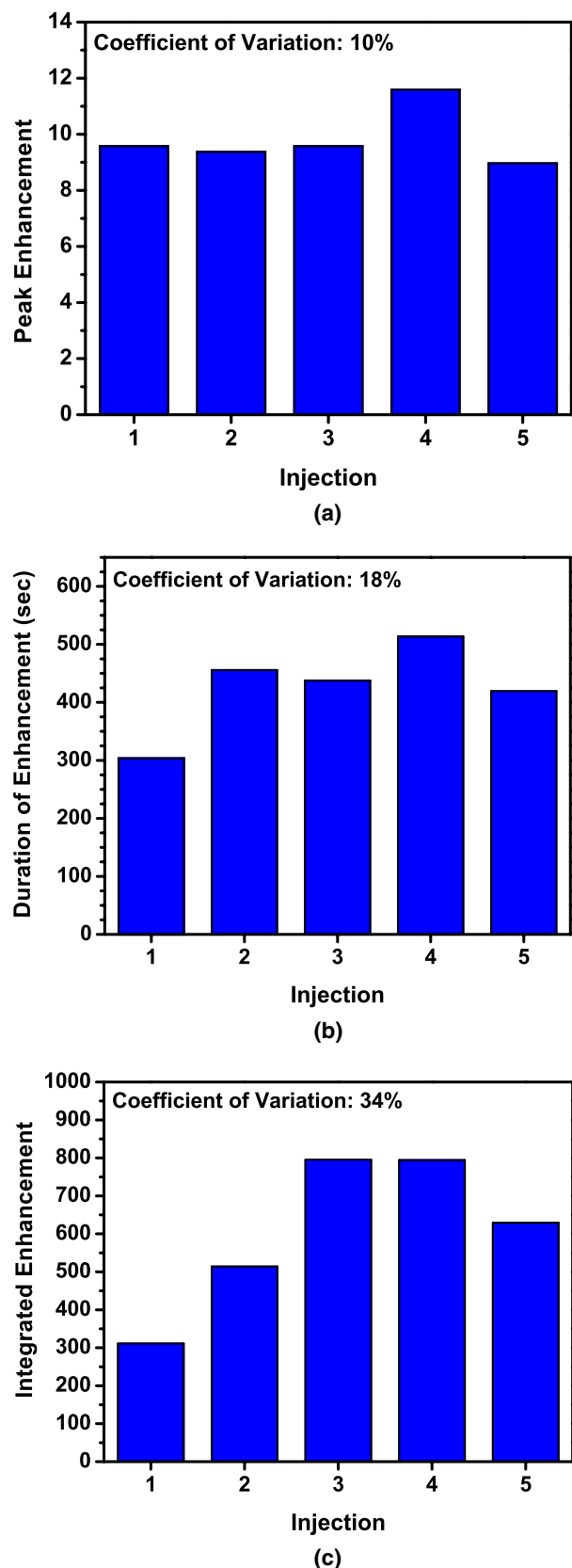


Fig. 9. The intra-mouse variability in (a) peak enhancement, (b) duration of enhancement and (c) integrated enhancement of microbubble kinetics for a bolus injection of $40 \mu\text{L kg}^{-1}$ of Definity measured in the jugular vein with 30 MHz ultrasound. The intra-mouse variability was evaluated in a single mouse. The enhancement is presented in linear units and normalised to the baseline power.

microcirculation, to the detection site. This has been shown to result in a plateau in signal and an increased duration of enhancement (Becher and Burns 2000). The enhancement 8 s after the first pass of the bolus did exhibit a linear relationship with concentration and is a result of the full mixing of the agent in the blood pool and elimination of some of the agent.

The duration of enhancement for a i.v. bolus of Definity ranged from 200 to 300 s and indicated the agent's stability in a mouse. A plateau was observed for doses $>30 \mu\text{L kg}^{-1}$ and it was speculated that it could be attributed to either: (1) increased activity of established elimination mechanisms in response to large doses; (2) the recruitment of additional elimination mechanisms designed to handle elimination of large doses; or (3) a combination of both; however, this has yet to be confirmed. This phenomenon was also observed for a bolus injection of Optison (GE Healthcare, Milwaukee, WI, USA) in rabbits (Becher and Burns 2000) and SonoVue (Bracco Inc, Geneva, Switzerland) in dogs (Correas et al. 2000).

The linear relationship between the integrated enhancement and agent concentration provides the basis for performing infusion measurements (Becher and Burns 2000; Correas et al. 2000). The slope is related to the contrast agent clearance which gives an indication of elimination of the detected agent as a function of dose, time and animal weight. For example, an infusion rate of $6.16 \times 10^{-1} \mu\text{L} \cdot \text{sec}^{-1} \cdot \text{kg}^{-1}$ would provide a steady 10 times enhancement over blood. It is important not to be confused with clearance as defined by pharmacokinetics. This is because the backscattered energy from bubbles is not an absolute measure of agent concentration.

A crucial portion of the present study was the analysis of variability in measurements of the acoustic and kinetic behaviour of Definity *in vivo*. Across mice, there was approximately a 30% variability in peak enhancement and duration of enhancement and almost 50% variability in integrated enhancement for all doses. A portion of the observed uncertainties was due to variability in the predicted blood volume based on allometry (Janssen and Smits 2002). A portion of variability was also due to the insertion of the catheter into the fragile tail vein, which is under a millimeter in diameter. There is a possibility that the catheter, which has an outer diameter of roughly 0.5 mm, could damage the tail vein or only be partially inserted. The variability in the jugular vein distention also provides a possible explanation for a component of the inter- and intra-mouse variabilities. It was found that the variability in distention was 18% and is representative of the variability of the injected volume of fluid. Furthermore, other technical factors such as the injection rate and biological factors such as respiratory rate, met-

abolic rate, heart rate and elimination rate between animals also contributed to the variability in the aforementioned metrics. The degree of biological and technical variability was assessed by observing differences between the inter- and intra-mouse variabilities. For repeat injections in the same mouse, the variabilities of the peak enhancement, duration of enhancement and integrated enhancement were less than their respective inter-mouse variabilities. The intra-mouse variability of the peak enhancement, duration of enhancement and integrated enhancement were reduced to 10%, 18% and 34%, respectively, indicating that a large portion of variability was due to biological differences between mice and the ability to perform reproducible injections.

The intra-mouse variability stems from a variety of sources, including the biological response to repeat injections, excess fluid volume and contrast agent from previous injections and the accuracy of extracting the agent from the vial and mixing it with saline. There was no clear systematic trend for repeat measurements of the same dose in the same animal indicating that excess fluid volume and contrast agent from previous injections were negligible compared to other sources of variability (Fig. 9). The intra-mouse variability of the peak enhancement was 10%, demonstrating the possibility of performing sensitive longitudinal studies of haemodynamic properties of the mouse using a *i.v.* bolus injections of Definity exposed to 30 MHz ultrasound. For example, peak enhancement has been demonstrated to be a valuable metric to distinguish cirrhotic from non-cirrhotic livers (Albrecht *et al.* 1999). Additionally, integrated enhancement has been demonstrated as a potentially sensitive indicator of treatment response in prostate cancer (Ekersley *et al.* 2002). In this study, the intra-mouse variability of the integrated enhancement was found to be 34%. This hinders the ability to perform longitudinal studies in mice where measurements of the integrated enhancement are important. Therefore, it is important to minimise the uncertainties in making repeat contrast measurements in the mouse which will require further investigation.

The kinetic behaviour of the contrast agent used in this study is similar to that observed in other studies using different agents, different animals and different ultrasound exposure conditions (Schwarz *et al.* 1996; Becher and Burns 2000; Correas *et al.* 2000). A comparison of these studies indicate the importance of the acoustic exposure conditions. The peak enhancement observed for the suggested maximum clinical dose of Definity ($10 \mu\text{L kg}^{-1}$ according to the Definity prescribing information) in the mouse and exposed to 30 MHz ultrasound was four times, or 2.5 dB (Fig. 6, dashed line). Comparatively, the enhancement in a rabbit for a similar dose when exposed to 5 MHz ultrasound can be over 100 times (Becher and Burns 2000). In light of the

differences in achievable enhancement between conventional and high frequency contrast detection, it can be confusing to determine the important scaling parameters needed to translate doses between animals exposed to different acoustic conditions. Contributing to this confusion is the representation of dose as a volume per unit mass. This implies that the acoustic and kinetic behaviour of the contrast agent are dependent solely on body weight, which appears not to be entirely true. Scaling the agent dose based on body weight inherently ignores the acoustic conditions under which the agent is exposed. Currently, there is no all encompassing metric to predict the acoustic and kinetic behaviour of the agent regardless of animal size and frequency. Furthermore, a metric that would be valid for all frequencies, tissues and animal sizes is unrealistic. Scaling the dose between species is affected by a combination of factors including: scaling of anatomy and physiology; scaling of the acoustic exposure conditions; and scaling of the acoustic properties of microbubbles. Scaling of the physiology of the body, such as the active and passive filtering mechanisms of the lungs, is likely nonlinear. It is conceivable that changes in the population distribution as the agent passes through the lungs is dissimilar between species; owing to factors such as the number of arteriovenous shunts which could differ greatly between species. Scaling of the acoustic properties from conventional to high frequency ultrasound is theoretically possible; however, in practice, maintaining the same focusing properties, sensitivity and bandwidth between conventional and high frequency ultrasound transducers is difficult. Scaling of the acoustic properties of microbubbles is nonlinear in part because the resonant frequency of a microbubble is nonlinearly related to the resting radius. It is important to note that scattering from large, nonresonant, bubbles will also contribute to the backscattered power at high frequencies; however, there are a small number of large bubbles in the population and these numbers could be further reduced through filtering by the lungs. Therefore, to be assured that results obtained from a high frequency contrast examination are quantifiable, a characterisation of the agents dose response for a given application is required.

The results presented in this study are limited to the low pressure 30 MHz ultrasound detection of Definity in a large vessel of the mouse. As previously discussed, the acoustic response is frequency, pressure and contrast agent dependent. High frequency ultrasound has increased resolution but suffers from poor contrast enhancement owing to: (1) the sparse number of bubbles in the focal volume of the transducer; (2) the increased level of backscatter from blood; and (3) the resonant population of microbubbles. Optimisation of contrast enhancement at higher ultrasound frequencies should be possible by increasing the exposure pressure, increasing

the contrast agent dose and using contrast agents with a resonant size distribution tuned to the ultrasound exposure conditions. Increasing the pressure will result in improved enhancement, but is limited by microbubble destruction (Stapleton et al. 2006). Increasing the microbubble concentration is limited by attenuation, multiple-scattering, bolus spreading and physiological tolerability. Perhaps the most promising avenue is choosing a contrast agent that contains a substantial number of resonant bubbles. This could be achieved by mechanical filtering and decanting current contrast agents or by developing an agent that is comprised solely of submicron to micron sized bubbles (Goertz et al. 2006b, 2007).

The translation of the optimal dose range found in the jugular vein to the calibre of vessels comprising the microcirculation requires further investigation. Complicating matters are the small scale of the geometric focus of a high frequency ultrasound beam, the sparse number of microbubbles in the microcirculation and clutter from the surrounding tissue. Therefore, the doses range examined in the present study might not provide adequate enhancement over tissue to detect the bubbles in the microcirculation. The detection of microbubbles in the microcirculation will also require exploiting nonlinear imaging techniques to suppress tissue signals. A preliminary investigation of the subharmonic dose response of the contrast agent MicroMarker (VisualSonics, Toronto, Canada) in the cortex of a mouse kidney exposed to 30 MHz ultrasound has highlighted these issues (Stapleton et al. 2007). Using concentrations similar to those presented in this study, it was found that the amount of subharmonic enhancement was only four times (2.5 dB) over the background and had large inter-mouse variabilities. Improving the subharmonic contrast enhancement in the microcirculation of a mouse will require further development of high frequency ultrasound systems capable of performing nonlinear contrast imaging with low noise characteristics, using contrast agent with strong nonlinear properties at high frequencies and characterisation of optimal acoustic exposure conditions. The work presented in this study has laid the foundation for future investigations of the microcirculation dose response and nonlinear dose response for contrast enhanced high frequency ultrasound flow studies in mice.

CONCLUSION

The acoustic and kinetic behaviour of the clinical contrast agent Definity exposed to 30 MHz ultrasound were characterised. A linear relationship between the peak enhancement and agent concentration was found for doses between 10 and 60 $\mu\text{L kg}^{-1}$. However, once the agent has had sufficient time to mix in the circulation a linear relationship was found for doses between 10 and

100 $\mu\text{L kg}^{-1}$. In this range of doses, changes in haemodynamic properties, such as blood volume, were reflected by proportional changes in the power of received microbubble echoes. An enhancement of up to 17 ± 5 times (12 dB) over blood was achieved and lasted up to 288 ± 89 s. The basis for infusion rates was determined by analysing the contrast agent clearance rate, however a large inter-mouse variability demonstrated the difficulty of translating infusion rates between animals. Inter-mouse variability in peak enhancement, duration of enhancement and integrated enhancement indicated that biological variations between animals contributes significantly to differences in the kinetic behaviour of the agent in different mice. The intra-mouse variability demonstrated the potential to perform sensitive longitudinal contrast enhanced high frequency ultrasound flow studies in mice. These results establish the utility of Definity for high frequency contrast-flow studies in large vessels such as the jugular vein and provides the basis for further high frequency dose response studies in smaller vessels of the mouse.

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