# ATTACHED ULTRASOUND CONTRAST AGENTS

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## 1 INTRODUCTION

Ultrasound contrast agents are microbubbles which respond to medical ultrasound and are used to enhance contrast in medical ultrasound images. They are a blood pool agent which will travel with the blood throughout the body enhancing the backscatter of ultrasound in organs such as the heart and liver as well as in blood vessels[1]. Ultrasound contrast agents comprise gas microbubbles encapsulated by a shell, typically the shell is lipid, polymer or albumin and the gas is air or a perfluorocarbon. Fluorocarbon gases dissolve slower in blood than air thus making contrast agents with a longer lifetime.

In addition to contrast agents enhancing the blood pool microbubbles can be targeted to specific markers within the body. For example, at areas of inflammation specific markers are expressed, significantly these markers are expressed at around vulnerable plaque within the coronary arteries[2]. Vulnerable plaque is soft, unstable plaque which can break up leading to thrombus within the artery and subsequent myocardial infarction. It is not currently possible to use ultrasound to distinguish between this unstable plaque and an intact stable plaque, although this is an area of significant ongoing research interest. Microbubbles targeted to markers associated with inflammation would bind to areas of unstable plaque and not to areas of stable plaque, and thus be highlighted in an ultrasound image allowing differentiation between the two. Microbubbles have been successfully targeted to cells made to express markers of inflammation[3].

Other applications of targeted microbubbles include local delivery of drugs and genetic material to specific sites. Enhanced uptake of drugs has been demonstrated in the presence of microbubbles through a process known as sonoporation, although the mechanisms behind the process at not fully understood[4]. Microbubbles can be loaded with the material to be delivered, or placed in solution with the material, and targeted to specific sites. When disrupted at specific areas within the body, the material would be released and delivered directly to the site of interest.

For the development of targeted applications, a contrast agent made from lipids has been developed in-house (IP Title: Improved Microbubble Composition and method for making the same. Patent No. 0625382.7 (Ross and Moran)). The agent can be modified as necessary to bind to specific cells and has been imaged with intravascular ultrasound [(5)]. The agent has been attached to agar and imaged under flow conditions, remaining attached when subjected to shear stresses of up to 3.4 Pa[6]. In order to further determine feasibility of use of the contrast agent, a study into the process of attaching the agent under flow conditions was undertaken.

The mechanisms behind the transfer of material from microbubble to cell are not fully understood, neither is the behaviour of microbubbles when attached to a surface. In order to improve this understanding a system has been developed which allows the interrogation of single, attached microbubbles with ultrasound. It has been shown that the acoustic behaviour of a population of microbubbles is often an aggregate of different microbubble behaviours, and as a result knowledge generated by microbubble suspensions can not infer to single microbubble behaviour [(6,7)] In addition, a large number of newly emerging imaging applications utilise very small concentrations of contrast as they are aiming at differentiating subtle differences of vasculature. The need to develop systems for the study of single microbubbles is therefore imperative.

# 2 METHODS

## 2.1 Attachment of contrast agent under flow conditions

A chamber allowing intravascular ultrasound imaging of agar samples which can be subjected to flow has been previously described[6]. Laser Doppler anemometry (LDA) was used to determine point velocities and thus flow profiles within the chamber for flow rates of 110-395 ml $min^{-1}$, which in turn were used to determine the shear rate and the shear stress experienced by the surface of the agar within the flow chamber. This calibrated system was utilised to determine the feasibility of attaching contrast agents to a surface under flow conditions.

Lipid based in-house contrast agents were made using a solvent method, a full description of the manufacture process and characterisation for high frequency ultrasound has been previously published[5]. Agar samples prepared with a streptavidin coating were placed in the flow chamber. In-house contrast agent made containing 1%, 3% and 5% biotin was circulated through the flow chamber for up to two hours at a concentration of 0.5 ml bubbles/ 50 ml saline at flow rates of 1.65 ml $min^{-1}$. A control of unbiotinylated contrast agent was circulated through the flow chamber. Steady flow was used throughout and the images were captured using Intelicam software (Matrox Intelicam Software, Matrox VITE Ltd, UK).

## 2.2 Single attached bubble system

The system described in section 2.1 was not suitable for detailed study of individual attached contrast agents thus a system was developed which permitted attachment and insonation of individual microbubbles. The experimental system, designed and constructed, in-house comprised a Perspex tank which had two separate sections as shown in Figure 1 and is described in more detail elsewhere [9]. One section of the tank was positioned over an inverted Leica fluorescence microscope for optical imaging and alignment procedures; the other was lined with acoustic absorber (Aptflex F28 Precision Acoustics) allowing insonation with a known acoustic field. A calibrated 0.5 mm active element membrane hydrophone (Precision Acoustics) was used to determine the acoustic field at the area of interest within the tank. The depth of interest was 7.5 cm from the transducer. An assessment of reflections from surfaces within the tank was also made to ensure the lack of significant reflections. The tank was filled with 20 litres of purified and degassed water.

BiSphere™ (Point Biomedical) microbubbles comprising an albumin-coated polymer shell and filled with air were attached to a polyester membrane using poly-L-lysine. The microbubbles were attached in small patches and singly using a dilution technique [10]. Initially patches were used to remove the need for fine alignment of the ultrasound field. A Philips Sonos5500 clinical ultrasound scanner was used throughout with an S3 transducer with frequency range of 1.2-3.55 MHz. The unprocessed RF data could be captured from the ultrasound scanner. The transducer was placed at an angle of 20° to the attached microbubbles to remove the echo from the membrane. A 6-cycle incident pulse was used throughout with the scanner running in M-mode. Acoustic peak negative pressures of up to 1.6 MPa were used. An attached copper sphere of diameter approximately 90 µm was centred in the optical field of view and used to align the acoustic field with the optical field. Perspex blocks were secured in place to act as a breaking point for the membrane holder, ensuring the copper sphere and hence attached microbubble could accurately exchange location between the calibrated ultrasound field position and the optical microscope field of view. Attached single biSphere™ microbubbles were then insonated at acoustic pressures up to 1.6 kPa.

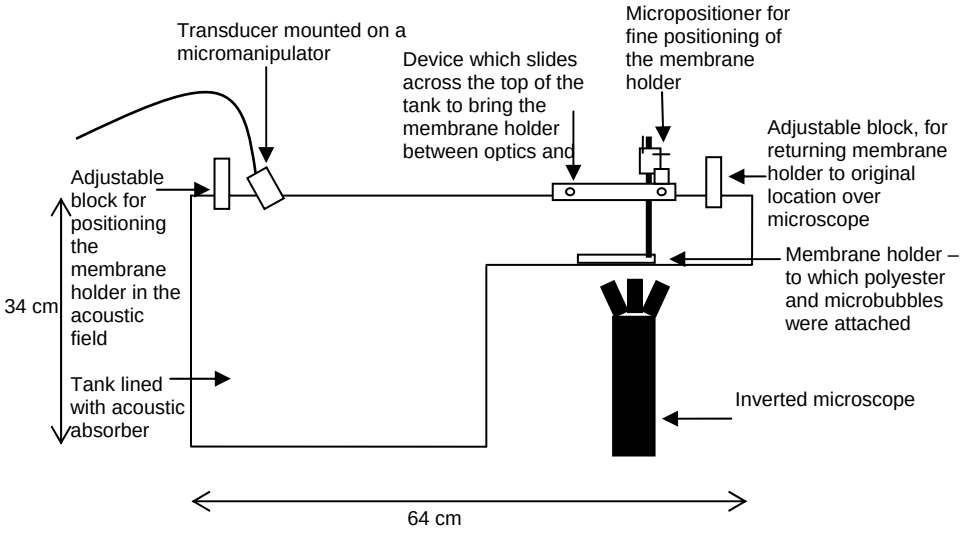


**Figure 1: Schematic diagram of system designed for the investigation of attached, single microbubbles .**

# 3 RESULTS

## 3.1 Attachment under flow conditions

No enhancement and thus no contrast agent attachment was seen when microbubbles containing 0% biotin were circulated as shown in Figure 2. Figure 3 shows IVUS images of the surface of the agar before and after circulation of the contrast agent. There is a line of enhancement seen when microbubbles made containing 3 and 5% biotin were circulated. Attachment of contrast agents was seen after circulation around the flow chamber for up to 1.5 hours

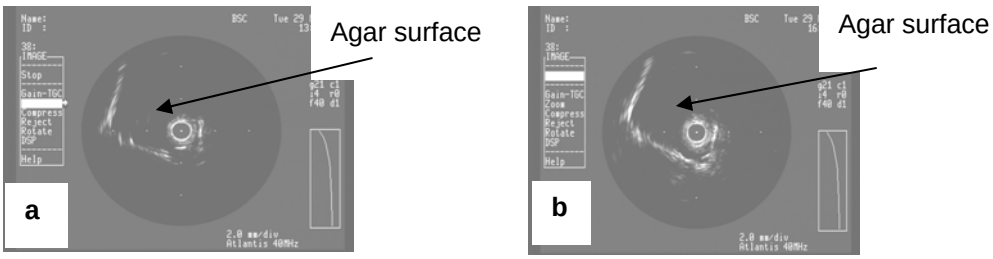


**Figure 2: IVUS image of streptavidin-coated agar in flow chamber (a) before and (b) after circulation of contrast agent containing 0% biotin, no change in backscatter from the agar surface was noted.**

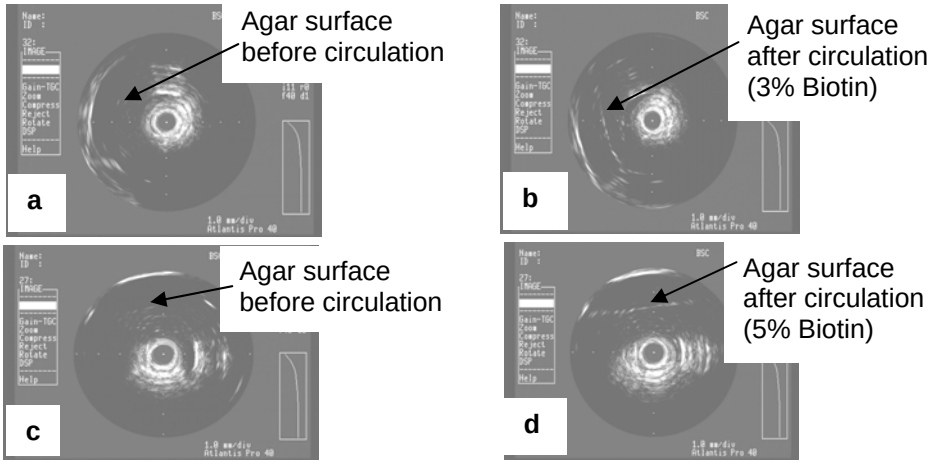


**Figure 3: IVUS images of streptavidin coated agar before (a,c) and after (b,d) after the circulation of contrast agent containing 3% and 5% biotin.**

## 3.2 Single microbubbles

A microscope image of attached biSphere™ microbubbles before and after insonation is shown in Figure 4. The area where most microbubbles are attached is circled. The fluorescent shells of the microbubbles can be seen attached to the membrane before and after insonation, however, in the brightfield images, it was not possible to visualise the microbubbles after insonation as the cracked microbubble shells released the gas contained within thus allowing the surrounding water to fill the shell. The acoustic backscatter from the patch of attached microbubbles where the incident acoustic pressure was 1.6 MPa is given in Figure 5. The amplitude of the backscattered signal decreases with time, as the released air dissolved in the water. The backscattered signal from an individual attached microbubble is shown in Figure 6, as with Figure 5 the signal decreases with time.

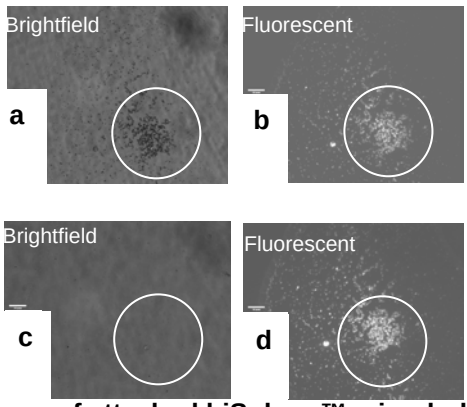


**Figure 4: Microscopy image of attached biSphere™ microbubbles (circled), fluorescent and brightfield microscopy images a) brightfield before insonation, b) fluorescent before insonation, c) brightfield after insonation, d) fluorescent after insonation.**

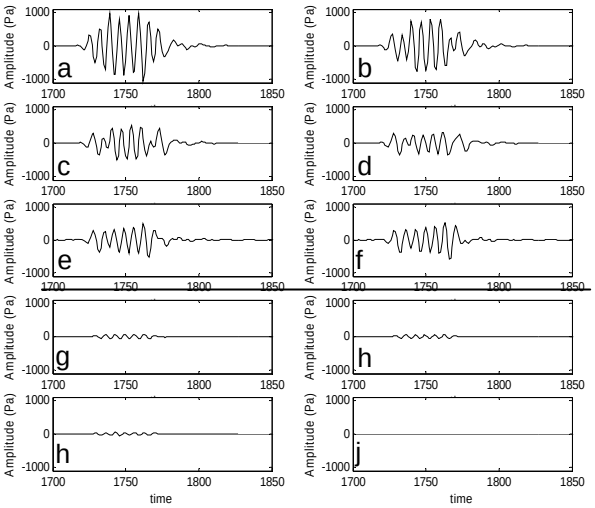


**Figure 5: RF backscatter from attached biSphere™ insonated with 1.6 MPa, a-j backscattered acoustic signal decreasing as the gas is released from the microbubbles. Images a-f are the first 30 pulses in five pulse intervals with the g-j being pulses 120-135 in five pulse intervals**

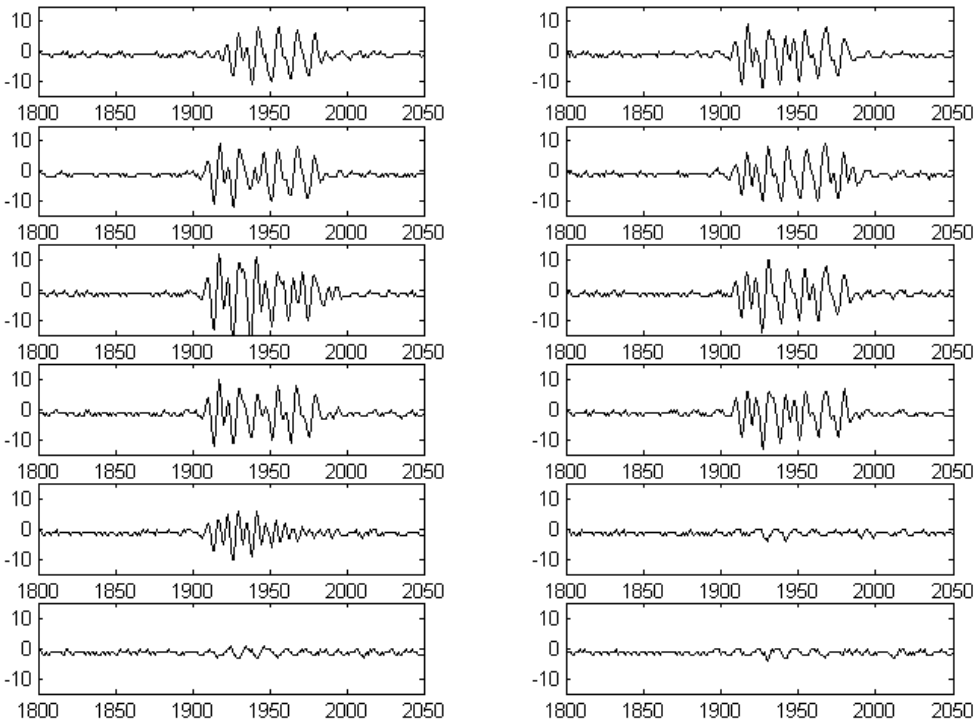


**Figure 6: Unprocessed RF data backscattered from single attached biSphere™ insonated with 1.6 MPa. First 12 consecutive pulses.**

# 4 DISCUSSION

## 4.1 Attachment under flow conditions

Previously, attachment of the in-house agent had been under static (no flow) conditions and while attachment under static conditions is significantly different to that under flow conditions, the principle that longer exposure to the surface results in more attachment events was followed thus the contrast agent was left to circulate for up to 2 hours.

Figure 3 shows an increase in the echo from the agar surface after circulation of contrast agent containing 3% and 5% biotin, the line is more evident than for that of microbubbles containing 1% biotin (not shown) suggesting that more binding events occurred when more biotin was incorporated into the microbubble shell. When in solution there is no significant difference in backscatter from agents containing different amounts of biotin.

Current research on attaching targeted microbubbles under flow conditions proposes the radiation force as a mechanism to push the microbubbles towards the vessel wall, increasing the number which make contact, hence, increasing the number of microbubbles which bind [(10)]. This is relevant in larger vessels with high flow rates as few microbubbles will make contact with the vessel wall. Dayton et al have demonstrated the use of the radiation force to move contrast microbubbles towards a vessel wall, in this instance, vessels of 200 μm and 50 μm were used. The acoustic radiation force has also been used to enhance adhesion of lipid shelled microbubbles which were targeted to P-selectin (a cell adhesion molecule) [(11)]. Attachment studies of contrast agents have been completed under controlled shear rates [(12)]. Takalkar et al assessed the binding and detachment of lipid based contrast agent targeted to P-selectin where the targeted microbubbles were drawn through a P-selectin coated flow chamber. The maximum number of microbubbles was found to attach to the chamber wall at a shear stress of 0.06 Pa and 50% of the bubbles had detached by the time the shear stress was increased to 3.4 Pa. The in-house contrast agents remained attached, when bound with streptavidin-biotin linking at shear stresses up to 3.4 Pa which is higher than experienced *in vivo* [(6)]. Currently the in-house agent has been shown to attach to agar when subjected to a shear stress of 0.012 Pa, which is less than experienced *in vivo*, however a range of shear stresses have not yet been investigated for attachment studies.

The flow rate through the flow chamber described here was limited by the method used. The flow rate was 1.65 ml $min^{-1}$ for which the shear stress was calculated to be 0.012 Pa. Methods of improving attachment of contrast agent under flow conditions as mentioned earlier include utilizing the radiation force to push the contrast agent towards the vessel wall, thus increasing the number of contact events between agent and wall. For targeted contrast agents to be successfully utilised *in vivo* the achievement of attachment under flow conditions is essential. In addition to being able to obtain attachment under flow conditions it is also necessary to increase understanding of the behaviour of attached contrast agents. However to use targeted contrast agents to their full potential, it is also necessary to have a more fuller understanding of the behaviour of attached microbubble, hence the development of a system for the study of single attached microbubbles.

## 4.2 Single attached microbubbles

A technique has been developed which allows single attached microbubbles to be insonated in a known acoustic field. When insonated at high acoustic pressures, 1.6 MPa, the microbubble shell is cracked, releasing the air contained within. At such high acoustic pressures a number of cavitation phenomena may occur that have been registered using high speed acquisition microscopy. The free air bubbles formed have been detected and the backscattered acoustic signal seen to decrease with time as the air bubbles eventually dissolve in the water or move out of the acoustic field.

On insonating a patch comprising of hundreds of attached biSphere™ microbubbles, it was demonstrated that the fluid filled microbubble remains attached to the surface after cracking (Figure 4) although no significant echo was detected from the shell remaining on the surface. Similar patches of biSphere™ were used during development of the experimental system and demonstrated the feasibility of detecting acoustic signals from attached microbubbles.

Figure 5 shows the backscatter signal from consecutive frames of ultrasound from a single attached biSphere™, the maximum backscattered amplitude is significantly lower for the single microbubble than for the patch, as is the duration of the backscattered signal due to the smaller volume of gas released from one microbubble. As with the patch of biSphere™ microbubbles, the fluorescent shell of the single microbubble has been seen to remain attached to the membrane after insonation.

A new technique has been demonstrated which can be used to study the behaviour of single attached microbubbles. The study of single attached microbubbles is necessary to develop further understanding of sonoporation and delivery mechanisms. A more thorough study will now be undertaken to investigate the response of single attached microbubbles with bubble type, acoustic pressure and frequency. To date microbubbles insonated at high acoustic pressures have been investigated. The facility is also in place to compare the response of attached microbubbles with unattached microbubbles when insonated with identical acoustic fields [13].

# 5 CONCLUSIONS

Targeting of ultrasound contrast agents is an evolving application. A contrast agent has been designed in-house which can be modified to target specific cell markers, the agent has been demonstrated to remain attached under flow conditions and further more has been attached to an agar surface while experiencing flow conditions. A system has been developed which allows the capability of the study of individual, attached microbubbles and has been used to investigate attached biSphere™ microbubbles at acoustic pressures up to 1.6 MPa. The system will be further utilised to investigate acoustic responses over a range of acoustic pressures and frequencies and the data compared to that of free, unattached microbubbles.

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