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● *Original Contribution*

IN VIVO CHARACTERIZATION OF ULTRASOUND CONTRAST AGENTS: MICROBUBBLE SPECTROSCOPY IN A CHICKEN EMBRYO

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Abstract—The dynamics of coated microbubbles was studied in an *in vivo* model. Biotinylated lipid-coated microbubbles were prepared in-house and were injected into a chick embryo chorioallantoic membrane (CAM) model on the fifth day of incubation. The microbubbles, ranging between 1.0 and 3.5 μm in diameter, were insonified in the frequency range of 4–7 MHz. Two amplitudes of acoustic pressure were applied: 300 kPa and 400 kPa. The fundamental and subharmonic responses were recorded optically with an ultra-fast camera (Brandaris 128) at 20 million frames per second. A subharmonic response was observed for 44% of the studied bubbles. From the data the frequency of the maximum fundamental and subharmonic response was derived for each individual bubble and resulted in the resonance curves of the microbubbles. All the bubbles showed shell (strain) hardening behavior for a higher acoustic pressure. We conclude that the subharmonic oscillations observed in this study belonged to the transmit at resonance (TR) regime. (E-mail: t.faez@erasmusmc.nl)

Key Words: **Chicken embryo, Ultrasound contrast agent, Microbubbles, Subharmonic response, *In vivo*.**

INTRODUCTION

Ultrasound contrast agents are used extensively in medical ultrasound imaging (Chang et al. 1995; Burns 1996; Shi et al. 1999; Dayton and Ferrara 2002; Bhagavatheeshwaran et al. 2004; Goertz et al. 2005, 2006, 2007a; Forsberg et al. 2007; Chérin et al. 2008; Masoy et al. 2008; Needles et al. 2010; Eisenbrey et al. 2011; Shen et al. 2011). An ultrasound contrast agent consists of small encapsulated microbubbles, which scatter the ultrasound very efficiently (De Jong 1993; Klibanov 2002). The contrast agent is introduced in the blood to increase the scattering properties from the blood pool. The fundamental understanding of the interaction of these bubbles with the ultrasound and its resulting nonlinear vibration dynamics, is an ongoing field of research, since the quantitative knowledge of characterization of the bubbles is essential for a better engineered and optimal agent for its use in the clinic.

In-depth studies have been performed to quantify the acoustic response of the microbubbles acoustically, both in a bubble population (De Jong et al. 1992; Gorce et al. 2000; Hoff 2000; Sarkar et al. 2005; Goertz et al. 2007b; Emmer et al. 2009a; Conversano et al. 2010; Faez et al. 2011a) and for single bubbles (Sijl et al. 2008, 2011), as well as optically also for single bubble (Morgan et al. 2000; Marmottant et al. 2005; De Jong et al. 2007; Emmer et al. 2007; van der Meer et al. 2007; Overvelde et al. 2010; Sijl et al. 2010; Faez et al. 2011b). Optical methods for acoustical characterization of contrast agent microbubbles have been a huge step forward in understanding the behavior of these bubbles in an ultrasound field. The physical properties of single bubbles reported for various contrast agents have shown that the acoustical response of a bubble is strongly size dependent (Emmer et al. 2009b; Faez et al. 2011b). However, until now the physical influence of biological parameters, (*e.g.,* blood flow, blood cells, vicinity or attachment to the vessel wall, floatation in the blood pool, *etc*.) on the dynamics of contrast agent microbubbles has been neglected or simplified. A genuine understanding of the acoustical behavior and resulting physical properties of the microbubbles can be

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achieved by investigating their acoustical response *in vivo*. For this purpose, we propose the chick embryo chorioallantoic membrane (CAM) model.

Chicken embryo is a well known animal model, which has been extensively used in various areas of research such as angiogenesis and anti-angiogenesis (Ribatti et al. 2000, 2001; Richardson and Singh 2003), wound healing (Ribatti et al. 1996), tissue engineering (Borges et al. 2003), biomaterials and implants (Zwadlo-Klarwasser et al. 2001; Valdes et al. 2002; Klueh et al. 2003), biosensors (Valdes et al. 2003) and drug delivery systems (Tartis et al. 2006; Vargas et al. 2007). The increasing interest in the chick embryo model and specifically its CAM is due to its simplicity, ease of visualization and low cost compared with mammalian models. The CAM of a developing chicken embryo is an extra-embryonic membrane and has a very dense capillary network, which makes it suitable for the intravenous injection of contrast agent microbubbles (Lange et al. 2001) and is easily accessible with standard optical microscopy. Moreover, the vascular network of a CAM is located in the chick mesoderm, a transparent matrix, which does not significantly absorb or scatter the incident visible light. This allows for high-contrast and high-resolution imaging.

It is reported that the vitelline network of a chicken embryo (extra-embryonic vessels, connecting the embryo to the yolk sac vasculature) is a good model for the human blood vessel network (Poelma et al. 2008). The CAM model is very often used in studying cardiac development and human birth defects because of similar structure and functionality between human and chicken embryonic hearts at early developmental stages (Antin 2004; Liu et al. 2011). The chick during its morphogenesis undergoes true growth similar to the situation in the human embryo, whereas their developing organs increase dramatically in size. Therefore, the CAM model is considered to be one of the best model embryos for numerous *in vivo* manipulations such as, overexpressing secreted proteins and viral gene constructions (Antin 2004; Vargas et al. 2007).

Another important aspect of using CAM models concerns studies on brain cancer tumors. Indeed, Hagedorn et al. (2005) have proven that tumor growth with key features of human gliblastoma can occur in a highly reproducible manner on a CAM model. This opens a new option for preclinical *in vivo* testing of anti-cancer drugs, which to date is mainly performed in adult rodents, raising major ethical concerns. There are, however, several significant anatomic differences between chick embryo cardiovascular anatomy and the human fetal cardiovascular anatomy, such as the orientation of the heart within the chest cavity, which are explained in details by Schellpfeffer and Kolesari (2012).

In vivo characterization of the microbubble dynamics is important for contrast-enhanced ultrasound imaging methods. The outcome of this type of studies defines the essential parameters for a suitable diagnostic imaging method such as the insonifying frequency and the acoustic pressure, in which microbubbles are more responsive to. So far, the results of *in vitro* experiments have been used as the reference. However, *in vitro* set-ups lack the complexity of a clinical environment and the physical effect of the major biological parameters such as blood plasma, red blood cells and proteins are simply neglected. Moreover, an *in vivo* study of the microbubble behavior has the advantage of testing the bioeffects, which contrast-enhanced diagnostic ultrasound can induce (*e.g.*, capillary damage, cell sonoporation and hemolysis) (Skyba et al. 1998; Miller et al. 2001; Li et al. 2004; Samuel et al. 2009).

To date, observations of bubble dynamics in actual vessels have been focused on the transient interaction of ultrasound-activated microbubbles and the blood vessel, by means of cavitation and microjetting phenomena (Caskey et al. 2007; Samuel et al. 2009; Chen et al. 2011a, 2011b). However, all these studies have been performed *ex vivo* and the blood in the vessels was replaced with another fluid (*e.g.*, saline mixed with ink), which does not represent a real clinical environment.

In the present study, the dynamics of ultrasound-activated microbubbles are studied in real-time. Home-made microbubbles are injected into a chick embryo CAM whilst, for the first time, investigating their *in vivo* fundamental and subharmonic responses at two different acoustic pressures. The dynamic response of a single bubble to pressure pulses driven at frequencies of 4–7 MHz is recorded optically using an ultra high-speed camera system. Microbubble spectroscopy techniques (van der Meer et al. 2007; Overvelde et al. 2010; Faez et al. 2011b) were applied at acoustic pressures of 300 and 400 kPa to characterize the physical properties of the bubbles. The results are compared with *in vitro* experiments reported in the literature using the very same experimental technique.

MATERIALS AND METHODS

Microbubble preparation

Biotinylated lipid coated microbubbles with a C_4F_{10} gas core were made by sonication as described by Klibanov et al. (2004). Biotinylation has no influence on the dynamics of microbubbles (Overvelde et al. 2011) and it is generally applied as a preparation step to functionalize the bubbles for targeting, which was not in the scope of this study.