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● *Original Contribution*

# INTERACTIONS BETWEEN INDIVIDUAL ULTRASOUND-STIMULATED MICROBUBBLES AND FIBRIN CLOTS

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**Abstract—The use of ultrasound-stimulated microbubbles (USMBs) to promote thrombolysis is well established, but there remains considerable uncertainty about the mechanisms of this process. Here we examine the microscale interactions between individual USMBs and fibrin clots as a function of bubble size, exposure conditions and clot type. Microbubbles (n = 185) were placed adjacent to clot boundaries ("coarse" or "fine") using optical tweezers and exposed to 1-MHz ultrasound as a function of pressure (0.1–0.39 MPa). High-speed (10 kfps) imaging was employed, and clots were subsequently assessed with 2-photon microscopy. For fine clots, 46% of bubbles "embedded" within 10 μm of the clot boundary at pressures of 0.1 and 0.2 MPa, whereas at 0.39 MPa, 53% of bubbles penetrated and transited into the clots with an incidence inversely related to their diameter. A substantial fraction of penetrating bubbles induced fibrin network damage and promoted the uptake of nanobeads. In coarse clots, penetration occurred more readily and at lower pressures than in fine clots. The results therefore provide direct evidence of therapeutically relevant effects of USMBs and indicate their dependence on size, exposure conditions and clot properties. (E-mail: chrisacc@sri.utoronto.ca)** © **2014 World Federation for Ultrasound in Medicine & Biology.**

*Key Words:* **Sonothrombolysis, Thrombolysis, Ultrasound, Microbubbles, Fibrin clot, Contrast agent.**

## INTRODUCTION

The occlusion of blood vessels by thrombi is a major cause of mortality and morbidity in the context of stroke and myocardial infarction. The challenge of resolving thrombotic occlusions to restore blood flow is therefore a high clinical priority, but it is in many circumstances poorly addressed by current methods. In stroke, for example, the thrombolytic enzyme recombinant tissue plasminogen activator (rt-PA) is the sole approved medical therapy; however, due to safety issues, it can be used only in a small fraction of patients, and in these cases it has only limited effectiveness (Go et al. 2013; National Institute of Neurologic Disorders and Stroke rt-PA Stroke Study Group 1995). There is therefore significant interest in developing improved methods for breaking down blood clots, and one of the most promising of these is sonothrombolysis. A range of ultrasound-based approaches have been investigated over the past several decades (Alexandrov et al. 2004; Pfaffenberger et al. 2005; Tachibana and Tachibana 1995), a prominent one being the use of ultrasound-stimulated microbubbles (USMBs), in either the presence or the absence of thrombolytic enzymes (*e.g.*, rt-PA). There is now extensive evidence of the thrombolytic activity of USMBs *in vitro* (*e.g.*, Culp et al. 2001; Datta et al. 2008; Hitchcock et al. 2011; Petit et al. 2012; Tachibana and Tachibana 1995; Xie et al. 2011), *in vivo* (*e.g.*, Birnbaum et al. 1998; Brown et al. 2011; Culp et al. 2004) and in initial clinical trials (*e.g.*, Molina et al. 2006, 2009). In these studies, the effectiveness of thrombolysis is generally determined by either the reduction in mass (or volume) of clots or the extent to which flow has been restored.

Despite this substantial body of work, there remains considerable uncertainty about the specific mechanisms by which USMBs achieve thrombolytic effects. A number of mechanisms have been proposed that are linked to stable and/or inertial microbubble cavitation (Cintas et al. 2004; Collis et al. 2010; Porter et al. 2001; Prokop et al. 2007). Clot mass loss and rt-PA penetration in whole blood clots has been reported to be higher in the presence of stable cavitation compared with inertial cavitation (Datta et al. 2008). It has been proposed that microbubbles oscillating in the vicinity of clots will act to promote the uptake of lytic agents, and that microstreaming associated with their oscillations may facilitate the convective

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removal of clot degradation products. Such processes could, in principle, be facilitated by the persistent presence of stably oscillating bubbles. It has also been hypothesized that shear stresses induced by microstreaming and jets (in the case of inertial collapse) may induce mechanical damage to the clot surface, which, if employed in conjunction with lytic agents, may expose new sites for enzymatic action. There is, however, little direct evidence of how specifically USMBs cause the degradation of clots at a microscale. Indeed, there is paucity of information about how USMBs interact with blood clots, which differ from other boundaries encountered by bubbles, such as vessel boundaries, in that clots are complex porous viscoelastic media composed of platelets and red blood cells enmeshed in a fibrin network.

Because the effectiveness of USMB-mediated thrombolysis may be governed predominantly by the interaction between microbubbles and clots at a microscale, gaining a mechanistic understanding at this scale is of relevance to the development of improved sonothrombolysis methods. With this perspective, we have undertaken studies (Acconcia et al. 2012, 2013) looking at microscale interactions between USMBs and fibrin clots that, because of their near-optical transparency, permit assessment with white light transmission microscopy. Fibrin clots are composed of a fibrin network, which is formed from purified fibrinogen in the absence of erythrocytes and platelets. The fibrin network is largely responsible for the mechanical integrity of whole blood clots and is the target of lytic agents. Fibrin clots are widely employed in rt-PA studies (Collet et al. 2005; Meh et al. 2001), and they have also been used extensively in ultrasound-mediated fibrinolysis research (Blinc et al. 1993; Everbach and Francis 2000; Francis et al. 1995; Siddiqi et al. 1995; Spengos et al. 2000), albeit in the absence of microbubble contrast agents.

In Acconcia et al. (2012, 2013), we investigated the interaction of dilute populations of USMBs (Definity) with fibrin clots. Fibrin clot phantoms were constructed that incorporated a 0.28 mm diameter flow channel though which a microbubble suspension was introduced and then exposed to 1-MHz ultrasound (1 ms pulse length). A high-speed camera (10 kfps) was employed to observe the microbubbles during insonation, followed by a post-sonication assessment of the fluorescently labeled fibrin network with two-photon (2P) microscopy. It was found that microbubbles were first brought to the boundary by primary radiation force and could then produce clot boundary deflections up to 80 $\mu$m. Complex bubble behavior was indicated, such as the coalescence of bubbles under secondary radiation force both at the boundary and within clots, as well as the generation of smaller "daughter" bubbles. Bubbles were observed to penetrate into clots, depending on insonation pressure, and could locally disrupt the fibrin network and induce the uptake of fluid present adjacent to the clots. These results therefore provided direct evidence of the possible mechanisms by which USMBs achieve fibrinolytic effects. However, a limitation of that study was that bubbles entered the optical field of view while rapidly translating and undergoing oscillations, which precluded determining a relationship between their resting radii and the resulting clot interactions.

The objective of the present study was to investigate the effect of microbubble size and acoustic pressure amplitude on the interaction between individual microbubbles and fibrin clots. This was achieved using optical tweezers to isolate individual bubbles, which were sized and then manipulated to be adjacent to the clot boundary before ultrasound exposure. Fast frame imaging was then carried out to observe the bubble interactions with clots during insonation. Subsequently, 2P microscopy was employed to evaluate the potential for damage to the fibrin networks induced by microbubbles, as well as to assess potential for the uptake of fluorescent nanobeads as a marker for fluid transport. Previous studies of fibrin clots have found that the fibrin network architecture has an effect on thrombolysis efficiency with lytic agents (Collet et al. 2000). To investigate the possible influence of network architecture in the context of thrombolysis with USMBs, experiments were carried out on two types of clots with different degrees of coarseness.

## METHODS

### *Experimental overview*

This subsection provides a general overview of the experimental approach, which is then followed by a more detailed description of individual procedures.

*Apparatus overview.* Fibrin clots with either "coarse" or "fine" network architectures were formed within a Mylar-bounded chamber and were constructed to have a flat boundary adjacent to a fluid-filled channel (see later). The clot chamber was located within a water tank (Fig. 1a) under a microscope (Olympus BXFM, 100 × objective; LUMPLFL100 XW, 1.5 mm working distance, Olympus, Tokyo, Japan) equipped with a fast frame camera (Photron, APX-RS, Tokyo, Japan). To enable position control of the clot relative to the microscope field of view, the chamber was mounted to a linear XYZ translation stage (460 p Series, Newport, Irvine, CA, USA) with motorized stepper motors (XY: ZST25 B and Z: ZST13 B, Thorlabs, Newton, NJ, USA).

The optical field of view was co-registered with the transducer (IL0108 HPSF1.0, Valpey Fisher, Hopkinton, MA, USA) focus using a glass bead (Sigma-Aldrich, St. Louis, MO, USA, G1277, diameter: ~200 $\mu$m). The transducer (diameter: 2.54 cm, *f*-number: 1) had a center