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● *Technical Note*

THE EFFECT OF SHORT DURATION ULTRASOUND PULSES ON THE INTERACTION BETWEEN INDIVIDUAL MICROBUBBLES AND FIBRIN CLOTS

Christopher Acconcia,*† Ben Y. C. Leung,† Anoop Manjunath,† and David E. Goertz*†
*Department of Medical Biophysics, University of Toronto, Toronto, Canada; and †Physical Sciences Platform, Sunnybrook Research Institute, Toronto, Canada

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Abstract—In previous work, we examined microscale interactions between microbubbles and fibrin clots under exposure to 1 ms ultrasound pulses. This provided direct evidence that microbubbles were capable of deforming clot boundaries and penetrating into clots, while also affecting fluid uptake and inducing fibrin network damage. Here, we investigate the effect of short duration (15 μs) pulses on microscale bubble-clot interactions as function of bubble diameter (3–9 μm) and pressure. Individual microbubbles (n = 45) were placed at the clot boundary with optical tweezers and exposed to 1 MHz ultrasound. High-speed (10 kfps) imaging and 2-photon microscopy were performed during and after exposure, respectively. While broadly similar phenomena were observed as in the 1 ms pulse case (*i.e.,* bubble penetration, network damage and fluid uptake), substantial quantitative differences were present. The pressure threshold for bubble penetration was increased from 0.39 MPa to 0.6 MPa, and those bubbles that did enter clots had reduced penetration depths and were associated with less fibrin network damage and nanobead uptake. This appeared to be due in large part to increased bubble shrinkage relative to the 1 ms pulse case. Stroboscopic imaging was performed on a subset of bubbles (n = 11) and indicated that complex bubble oscillations can occur during this process. (E-mail: chrisacc@sri.utoronto.ca) © **2015 World Federation for Ultrasound in Medicine & Biology.**

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INTRODUCTION

It is well established that ultrasound stimulated microbubbles (USMBs) can facilitate the degradation of blood clots, both in the presence and absence of lytic agents (Brown et al. 2011; Culp et al. 2001; Petit et al. 2012; Xie et al. 2011). This approach holds considerable promise as a means to restore blood flow in the context of acute ischemic stroke, myocardial infarction and deep vein thrombosis. Due to the potential clinical impact of this technique, there is a high level of interest in its optimization, which has resulted in the accumulation of a substantial body of work (de Saint Victor et al. 2014) examining the influence of a range of exposure parameters on lytic efficiency. While this research has provided very valuable information, the potential exposure parameter space is vast and the interplay between different parameters can be complex. The development of improved sonothrombolysis techniques would therefore be significantly aided by a more basic, mechanistic understanding of the manner in which bubbles act to degrade thrombus, which at present is very limited.

Thrombolytic effects have been linked to both stable and inertial bubble cavitation, as inferred from "bulk" cavitation signals detected from suspensions of bubbles in the vicinity of clot boundaries (Datta et al. 2008; Leeman et al. 2012). Based on general principles of the behavior of bubbles in acoustic fields, several hypotheses have been advanced relating to what could be occurring during this process. Fluid flow around oscillating bubbles may facilitate the transport of lytic enzymes (*e.g.,* tissue plasminogen activator [tPA]) to and within clots (Prokop et al. 2007), a speculation that is consistent with histologic examinations of treated clots (Datta et al. 2008). In addition, fluid flow could be involved in the removal of clot degradation products, thus exposing fresh binding sites for tPA (Hitchcock et al. 2011). It has also been proposed that mechanical damage to clots could result from the fluid flow effects associated with microstreaming and the occurrence of microjets (Chen et al.

Address correspondence to: Christopher Acconcia, Sunnybrook Research Institute, 2075 Bayview Avenue, M4 N 3 M5, Toronto, Canada. E-mail: chrisacc@sri.utoronto.ca

2014; Petit et al. 2012). Though these hypotheses relate to microscale effects, sonothrombolytic effectiveness has generally been assessed *via* macroscopic metrics such as clot mass loss, clot erosion profiles (Petit et al. 2012; Sutton et al. 2013) or flow restoration. A thrombus is comprised of blood cells (primarily erythrocytes) that are enmeshed in a fibrin network, which is the primary structural element of the clot. The spatial scale of the network pores are on the order of the bubble size, which creates a complex physical situation that is distinct from that of a homogeneous solid boundary.

With the perspective that direct observations of the interactions between microbubbles and the fibrin network are necessary to fully understand this process, we undertook high frame rate microscopy studies of microbubbles interacting with fibrin clots (Acconcia et al. 2012, 2013, 2014). Fibrin clots are nearly optically transparent and are frequently employed in the context of examining the activity of lytic enzymes that act to degrade the fibrin network. In Acconcia et al. (2012, 2013), we examined the interaction between dilute suspensions of bubbles and clot boundaries during exposure to ultrasound. The results provided direct evidence related to the lytic process, demonstrating that bubbles could penetrate into clots, disrupt the fibrin network and transport with them fluid from outside the clot. Both primary and secondary radiation forces were prominent, inducing boundary strain patterns and resulting in the coalescence of nearby bubbles both outside and inside the clots. In Acconcia et al. (2014), individual bubbles interacting with clot boundaries were examined using optical tweezers to place bubbles adjacent to the boundaries where they were subsequently exposed to ultrasound. It was demonstrated that the degree of penetration and network damage was strongly dependent on the bubble size, pressure and network composition. This work yielded a number of novel, direct observations of bubble interactions with clots and indicated the power of this type of approach for gaining insight into the lytic process and the effect of different exposure parameters.

A question of significant interest in microbubble-mediated sonothrombolysis is the influence of pulse length on the generation of therapeutically relevant effects. A variety of pulsing schemes have been examined in previous studies, ranging from short duration (*e.g.,* 5–20 μs) pulses (Molina et al. 2006, 2009; Wu et al. 2014; Xie et al. 2011) to millisecond-scale pulses (Borrelli et al. 2012; Brown et al. 2011; Datta et al. 2008) and intermittent continuous wave transmission (Culp et al. 2003; Hitchcock et al. 2011; Petit et al. 2012; Sutton et al. 2013). Previous studies investigating the effect of pulse length on thrombolysis efficiency have observed longer pulses to produce improved thrombolysis (Leeman et al. 2012; Wu et al. 2014), though they were conducted such that duty cycle and/or pressure was also varied, which leaves a degree of ambiguity about the origins of the differences in results.

In our previous studies (Acconcia et al. 2012, 2013, 2014) we employed 1 ms pulses. In the present study, we investigate the interaction between individual USMBs and fibrin clots using exposures with a short (15 μs) pulse length. This length is within the range of several previous studies (Molina et al. 2006, 2009; Wu et al. 2014; Xie et al. 2011) that have employed shorter pulses, in part due to their compatibility with implementation on current diagnostic ultrasound platforms.

METHODS

The majority of methodologies employed here were nearly identical to those reported in Acconcia et al. (2014) to enable a comparison between the results obtained with these two pulse lengths. As such, the common methods are only described briefly here. For further details pertaining to the apparatus, microbubble handling procedures, fibrin clot preparation and optical tweezers, the reader is referred to Acconcia et al. (2014).

Apparatus overview, microbubble handling procedures and fibrin clot preparation

Fibrin clots were formed within a mylar-bounded chamber that was placed in a degassed and de ionized water tank 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). Clots were formed from purified native fibrinogen (Hyphen-BioMed, Neuville-Sur-Oise, France) and fluorescently tagged fibrinogen (AlexaFluor-488 tagged human fibrinogen; Invitrogen Canada Inc., Burlington, ON, Canada), according to the method described in Acconcia et al. (2014). Combining these two reagents, the final fibrinogen concentration was 3 mg/mL. Data were acquired from a total of 22 clots, each one prepared for a given experimental day. Definity (Lantheus Medical Imaging, North Billerica, MA, USA) bubbles (dilution ratio of ∼1:200,000) and fluorescent nanobeads (Fluospheres, carboxylate modified, 200 nm diameter, excitation/emission: 540/560 nm, dilution ratio of 1:500; Invitrogen) were diluted in Tris buffered saline (50 mM Tris-base, 100 mM NaCl, adjusted to a pH of 7.4 with HCl) and introduced into the chamber. Individual bubbles were then optically trapped with an infrared laser (YLR-10–1064-LP, IPG Photonics, Oxford, MA, USA) and brought to within 10 μm of the clot boundary, where it was then exposed to pulsed ultrasound. A vial of Definity was activated for each experimental day. Agent