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● *Original Contribution*

INVESTIGATION OF MICROBUBBLE CAVITATION-INDUCED CALCEIN RELEASE FROM CELLS *IN VITRO*

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Abstract—In the present study, microbubble (MB) cavitation signal analysis was performed together with calcein release evaluation in both pressure and exposure duration domains of the acoustic field. A passive cavitation detection system was used to simultaneously measure MB scattering and attenuation signals for subsequent extraction efficiency relative to MB cavitation activity. The results indicate that the decrease in the efficiency of extraction of calcein molecules from Chinese hamster ovary cells, as well as cell viability, is associated with MB cavitation activity and can be accurately predicted using inertial cavitation doses up to 0.18 V × s ($R^2 > 0.9$, $p < 0.0001$). No decrease in additional calcein release or cell viability was observed after complete MB sonodestruction was achieved. This indicates that the optimal exposure duration within which maximal sono-extraction efficiency is obtained coincides with the time necessary to achieve complete MB destruction. These results illustrate the importance of MB inertial cavitation in the sono-extraction process. To our knowledge, this study is the first to (i) investigate small molecule extraction from cells via sonoporation and (ii) relate the extraction process to the quantitative characteristics of MB cavitation acoustic spectra. (E-mail: Saulius.Satkauskas@gmf.vdu.lt) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Sonoporation, Calcein, Extraction, Inertial cavitation dose, Attenuation, Microbubble.**

INTRODUCTION

Sonoporation is a novel technique developed to deliver non-invasively bioactive molecules such as drugs (Iwanaga et al. 2007; Lentacker et al. 2010; Tamosiunas et al. 2012a, 2012b), DNA (Duvshani-Eshet et al. 2006; Escoffre et al. 2013a; Tsai et al. 2009) and RNA (Dewitte et al. 2014; Inoue et al. 2014; Juffermans et al. 2014) into cells and tissues. The sonoporation phenomenon is generally associated with ultrasound (US)-induced microbubble (MB) cavitation. Linear and periodic MB oscillations around a constant diameter are termed *stable cavitation*, whereas rapid increases in the size of MBs followed by eventual collapse are termed *inertial cavitation* (Fan et al. 2013a; Forbes et al. 2008; van Wamel et al. 2006). Cavitating MBs create microstreaming and/or liquid jets, which induce mechanical shear stress (Marmottant and Hilgenfeldt 2003; Ohl et al. 2006; van Wamel et al. 2004; Wu and Nyborg 2008; Zhou et al. 2012) leading to transient permeabilization of cell membranes by creating pores and/or inducing endocytosis (Derieppe et al. 2015; Juffermans et al. 2014; Mehier-Humbert et al. 2005; Meijering et al. 2009; Zeghimi et al. 2015). Thus, sonoporation provides a site-specific and spatiotemporally controllable strategy for delivery of therapeutic agents to the region of interest and is uniquely suitable for clinical applications (Fan et al. 2012; Hu et al. 2013). Nevertheless, US-MB-mediated intracellular delivery is accompanied by undesirable systemic side effects: (i) strong cellular stress, resulting primarily in membrane and intracellular lipid rearrangements (Chen et al. 2013; Juffermans et al. 2009; Leung et al. 2014; Zeghimi et al. 2015); (ii) increased level of oxidative stress (Hauser et al. 2009; Juffermans et al. 2006, 2009; Leung et al. 2014; Lionetti et al. 2009; Paula et al. 2011); (iii) cytoskeletal reorganization (Chen et al. 2014; Fan et al. 2013b; Juffermans et al. 2009); (iv) endoplasmic reticulum stress (Zhong et al. 2013); (v) membrane blebbing (Leow et al. 2015; Schlicher et al. 2010); and (vi) a

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delay in cell cycle progression (Chen et al. 2013; Zhong et al. 2011). However, if the cell damage is too severe to be repaired, the cell eventually dies as a result of apoptosis (Ando et al. 2006; Frampton et al. 2013; Honda et al. 2002, 2004; Leung et al. 2014; Zhong et al. 2011) or necrosis (Miller and Dou 2009; Schlicher et al. 2010). To achieve the desired level of cell permeabilization and diminish violent inertial cavitation effects, directly or indirectly leading to cell death, US energy must be precisely quantified and controlled—dosed. Thus, it is very important to develop a uniform, precise cavitation dosimetry system that would enable the control and monitoring of sonoporation outcome.

At low acoustic pressures, MB stable cavitation produces an US field that contains harmonic, sub-harmonic and ultra-harmonic components of the fundamental frequency. At higher driving pressures the components are detected in a broad frequency range; thus, the phenomenon termed *broadband noise* is witnessed as MBs violently collapse (Ammi et al. 2006; Shankar et al. 1999; Sundaram et al. 2003; Tezel et al. 2002).

Beginning with the early work by Everbach et al. (1997, 1998), it has been found that ultrasonic MB cavitation signals can be used for average root mean square (RMS) calculation and are associated with biological effects like hemolysis and platelet sonolysis. Later, Chen et al. (2003a, 2003b) successfully employed MB cavitation signals to quantify cumulated RMS, termed, inertial cavitation dose (ICD), which strongly correlated with erythrocyte hemolysis. Subsequent studies have reported ICD to be a suitable metric for calcein (Hallow et al. 2006), doxorubicin (Maciulevicius et al. 2015), and DNA (Lai et al. 2006; Qiu et al. 2010) intracellular delivery and cell viability (Hallow et al. 2006; Lai et al. 2006; Qiu et al. 2010), as well as pore size prognostication (Qiu et al. 2010).

Another way to examine MB behavior in the US field is to monitor the attenuation of the US wave that it undergoes while traveling through the suspension containing MBs (Chen et al. 2002; Jurkonis et al. 2015; Tang and Eckersley 2007). MB-induced US attenuation has already been used to evaluate MB concentration during sonoporation (Dicker et al. 2010; Lamanauskas et al. 2013), determine MB sonodestruction kinetics (Chatterjee et al. 2005), characterize different MB types (Emmer et al. 2009; Guo et al. 2013) and relate the rate of molecular delivery to the degree of attenuation (Escoffre et al. 2013b).

Small molecules pass across the plasma membrane barrier through transient non-specific pores and specific endocytotic pathways, whereas large molecules access the cell interior mostly via endocytosis (Derieppe et al. 2015; Juffermans et al. 2014; Meijering et al. 2009; Zeghimi et al. 2015). The origin of sonoporation as a bidirectional molecule diffusion-based process has been clearly exposed by van Wamel et al. (2006) and Fan et al. (2010). Some studies have reported small molecule release via sonoporation (Fan et al. 2012; Shamout et al. 2015; van Wamel et al. 2006); however, this was performed only for methodical purposes to determine the state of membrane integrity at the cellular level. Very consistent research with the main goal of investigating the efficiency of molecular sono-extraction was performed by Kaddur et al. (2010), who reported that enhanced green fluorescent proteins (EGFPs) were able to be extracted from cells in large amounts while maintaining high cell viability. The sono-extraction studies performed by D'Souza et al. (2009) and Forbrich et al. (2013, 2014) paved the way for new promising sono-extraction applications—namely, the liberation of blood biomarkers, carcinoembryonic antigen as well as mRNA and micro-RNA. The results have indicated that the amount of blood biomarkers used for cancer diagnostics, clinically usually available at low concentrations, can be increased with sonoporation recruitment.

As the new sonoporation applications for molecular extraction are emerging for both practical and clinical purposes, an implicit dosimetry model describing sono-extraction bio-effects has to be developed. In addition, sono-extraction has not been investigated from the physical point of view as in relation to MB cavitation activity. The development of a well-defined model relating cavitation metrics to bio-effect efficiency would allow accurate molecular release and cell viability prognostication. Successively, this leads to a real-time feedback method designed to adjust experimental conditions to obtain the desired biological outcome.

Thus, in this study we focused on MB cavitation response relative to the extraction of small molecules via sonoporation. For this purpose, Chinese hamster ovary (CHO) cells were loaded with the fluorescent molecule calcein acetoxymethyl ester (calcein AM) to perform subsequent extraction experiments *in vitro*. We used a passive cavitation detection system to simultaneously measure both MB scattering and attenuation signals for subsequent sono-extraction efficiency relative to MB cavitation activity on both acoustic pressure and exposure duration scales.

METHODS

Cell line

CHO cells are epithelial-like cells broadly used as a mammalian model cell line (Kumon et al. 2007). These cells have been well studied in sonoporation (Forbes et al. 2008; Kumon et al. 2007; Rahim et al. 2006) and electroporation (Dermol and Miklavcic 2015; Rols and