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● *Original Contribution*

# MICROBUBBLE-ASSISTED ULTRASOUND-INDUCED TRANSIENT PHOSPHATIDYLSERINE TRANSLOCATION

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**Abstract—Microbubble-assisted ultrasound (sonopermeabilization) results in reversible permeabilization of the plasma membrane of cells. This method is increasingly used *in vivo* because of its potential to deliver therapeutic molecules with limited cell damage. Nevertheless, the effects of sonopermeabilization on the plasma membrane remain not fully understood. We investigated the influence of sonopermeabilization on the transverse mobility of phospholipids, especially on phosphatidylserine (PS) externalization. We performed studies using optical imaging with Annexin V and FM1-43 probes to monitor PS externalization of rat glioma C6 cells. Sonopermeabilization induced transient membrane permeabilization, which is positively correlated with reversible PS externalization. This membrane disorganization was temporary and not associated with loss of cell viability. Sonopermeabilization did not induce PS externalization *via* activation of the scramblase. We hypothesize that acoustically induced membrane pores may provide a new pathway for PS migration between both membrane leaflets. During the membrane-resealing phase, PS asymmetry may be re-established by amino-phospholipid flippase activity and/or endocytosis, along with exocytosis processes. (E-mail: jean-michel.escoffre@univ-tours.fr)** © **2016 World Federation for Ultrasound in Medicine & Biology.**

***Key Words:*** **Microbubble, Ultrasound, Membrane permeabilization, Phosphatidylserine externalization.**

## INTRODUCTION

In modern pharmacology, one of the main research goals is to design methods to efficiently increase gene and drug delivery without harming healthy cells and tissues. Among these methods, microbubble-assisted ultrasound (also known as sonoporation or sonopermeabilization) is receiving increasing attention as a physical method for the *in vitro* and *in vivo* delivery of various exogenous molecules, including nucleic acids, anti-cancer drugs, peptides and antibodies (Chang et al. 2013; Escoffre et al. 2013; Li et al. 2016). This method involves ultrasound-induced cavitation of gas-filled microbubbles in the vicinity of the biological barriers (*e.g.,* plasma membrane, endothelial barrier, blood–brain barrier) to transiently increase their permeability (Kooiman et al. 2014).

To this effect, microbubbles may stably oscillate on exposure to a low acoustic pressure, a process termed *stable cavitation*. The oscillations generate fluid flows surrounding the microbubbles, known as acoustic microstreaming (Doinikov and Bouakaz 2010; Wu 2002). At higher acoustic pressures, microbubbles oscillate with increasing amplitude, leading to their violent collapse and destruction, termed *inertial cavitation*. Microbubble disruption might be accompanied by generation of shock waves in the medium close to the microbubbles (Junge et al. 2003; Ohl and Wolfrum 2003). Microstreaming and shock waves induce shear stress on biological barriers, resulting in their enhanced permeability. The ultrasound-induced collapse of a microbubble can be asymmetric, leading to the formation of high-velocity jets that puncture biological barriers and thereby entail greater permeability (Ohl et al. 2006; Postema et al. 2005). Both cavitation regimes are exploited to create membrane pores and stimulate endocytosis and transcellular and paracellular pathways, thus enhancing the extravasation and intracellular uptake of exogenous molecules (Lentacker et al. 2014).

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Despite the use of microbubble-assisted ultrasound in drug delivery, there is still a lack of knowledge on acoustically mediated structural and dynamic changes of the plasma membrane. In addition to changes in the transmembrane potential (Tran et al. 2007) and membrane permeability (Derieppe et al. 2013), the main structural membrane alterations of sonopermeabilized cells are bleb formation (Leow et al. 2015) and phosphatidylserine (PS) externalization (Van Ruijssevelt et al. 2013). Even though the former membrane changes are reversible and not related to a loss of cell viability, the latter can be permanent and is usually associated with cell death.

For most healthy eukaryotic cells, the outer leaflet of the plasma membrane is composed mainly of neutral lipids, including phosphatidylcholine and sphingolipids, whereas anionic lipids such as PS, phosphatidylethanolamine, phosphatidylinositol and phosphoinositides are preferentially restricted to the inner leaflet of the plasma membrane (Hankins et al. 2015). This asymmetric distribution generates two membrane surfaces with highly different electrostatic potentials and has a tremendous impact on membrane protein activity (Hankins et al. 2015). Membrane asymmetry is maintained by two enzymes acting in concert with membrane synthesis and recycling: (i) $Ca^{2+}$-inhibited, ATP-dependent amino-phospholipid translocase moves PS from the outer to the inner leaflet of the plasma membrane; (ii) $Ca^{2+}$-dependent amino-phospholipid scramblase facilitates the randomization of PS distribution between the two leaflets of the plasma membrane. Regulated PS externalization is a physiologic event involved in several biological processes including thrombosis (Balasubramanian and Schroit 2003), intra-membrane signal transduction in lymphocytes (Elliott et al. 2005) and the phagocytosis of apoptotic cells (Schlegel and Williamson 2001). Although the net energy required to move charged phospholipid head groups, for example, PS, from one leaflet of the lipid bilayer to the other, is only of the order of the thermal energy of $k_BT$ (where $k_B$ is Boltzmann's constant and $T$ is the thermodynamic temperature), the hydrophobic membrane core constitutes a high-energy barrier (Vernier et al. 2004a, 2004b). Homan and Pownall (1988) reported that activation energies for translocation are on the order of 100 kJ/mol, and rate constants for spontaneous transverse phospholipid migration are on the order of a few hours. However, a number of biological (*e.g.,* membrane-spanning peptides, calcium release from internal storages) and physical (*e.g.,* temperature, electric field) stimuli may lower this activation energy or facilitate membrane restructuring (*e.g.,* membrane defects or pores) and PS translocation (Vernier et al. 2004a, 2004b; Wu and Hubbell 1993). In gene and drug delivery (*i.e.,* gene therapy, DNA vaccination, chemotherapy), PS externalization would lead to phagocytic clearance of PS-exposed cells, thus preventing gene expression or drug activity.

The objective of the present study was to further investigate the effects of microbubble-assisted ultrasound on the plasma membrane. We report on the influence of microbubble-assisted ultrasound on the asymmetric distribution of phospholipids, in particular on the transverse mobility of PS. Our experiments, which included optical imaging and biological assays, address two questions: (i) Does microbubble-assisted ultrasound induce PS externalization in viable cells? (ii) Is there a biophysical link between membrane permeabilization and PS externalization? Finally, we discuss a putative molecular model(s) that can support sonopermeabilization-induced PS translocation.

## METHODS

### *Cell line and culture conditions*

The glioma cell strain C6 was cloned from a rat glioma tumor induced with *N*-nitrosomethylurea (European Collection of Cell Cultures, Salisbury, UK). This cell line has been frequently used to develop and optimize sonoporation for drug and gene delivery (Burke et al. 2011; Wang et al. 2009), as well as to understand the mechanisms involved in sonoporation (Lepetit-Coiffe et al. 2013; Van Ruijssevelt et al. 2013). Cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) with 1 g/L glucose, 0.584 g/L L-glutamine and 3.7 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich) and 1× penicillin/streptomycin (Sigma-Aldrich). The cells were routinely subcultured every 4 d and incubated at 37°C in a 5% $CO_2$ incubator with a humidified atmosphere.

For the ultrasound experiments, $2 \times 10^6$ C6 cells were seeded into OptiCell culture chambers (Thermo Fischer Scientific, Waltham, MA, USA), and grew as a monolayer to cell confluency (Van Ruijssevelt et al. 2013). These culture chambers have two parallel gas-permeable polystyrene membranes of 50 $cm^2$ that are attached to a rectangular frame with two access ports. The membranes are 75 $\mu$m thick, and the inner walls are 2 mm apart. Compatible with optical imaging (Derieppe et al. 2013), the polystyrene membranes attenuate less than 2% of the ultrasound beam (Lammertink et al. 2015). The water surface was 10 cm above the culture chamber, which, in combination with the short duration of the ultrasound pulses, prevented the buildup of standing waves.

### *SonoVue microbubbles*

SonoVue microbubbles (Bracco Research, Geneva, Switzerland) were prepared according to the