Journal of Controlled Release 238 (2016) 197–211

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Viability of endothelial cells after ultrasound-mediated sonoporation: Influence of targeting, oscillation, and displacement of microbubbles

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ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form 20 July 2016
Accepted 24 July 2016
Available online xxxx

Keywords:
Ultrasound contrast agents
Microbubble behavior
Sonoporation
Drug delivery
Therapy
Endothelial cells

ABSTRACT

Microbubbles (MBs) have been shown to create transient or lethal pores in cell membranes under the influence of ultrasound, known as ultrasound-mediated sonoporation. Several studies have reported enhanced drug delivery or local cell death induced by MBs that are either targeted to a specific biomarker (targeted microbubbles, tMBs) or that are not targeted (non-targeted microbubbles, ntMBs). However, both the exact mechanism and the optimal acoustic settings for sonoporation are still unknown. In this study we used real-time uptake patterns of propidium iodide, a fluorescent cell impermeable model drug, as a measure for sonoporation. Combined with high-speed optical recordings of MB displacement and ultra-high-speed recordings of MB oscillation, we aimed to identify differences in MB behavior responsible for either viable sonoporation or cell death. We compared ntMBs and tMBs with identical shell compositions exposed to long acoustic pulses (500–50,000 cycles) at various pressures (150–500 kPa).

Propidium iodide uptake highly correlated with cell viability; when the fluorescence intensity still increased 120 s after opening of the pore, this resulted in cell death. Higher acoustic pressures and longer cycles resulted in more displacing MBs and enhanced sonoporation. Non-displacing MBs were found to be the main contributor to cell death, while displacement of tMBs enhanced reversible sonoporation and preserved cell viability. Consequently, each therapeutic application requires different settings: non-displacing ntMBs or tMBs are advantageous for therapies requiring cell death, especially at 500 kPa and 50,000 cycles, whereas short acoustic pulses causing limited displacement should be used for drug delivery.

1. Introduction

Microbubbles (MBs) are ultrasound (US) contrast agents that consist of gas bubbles with diameters between 1 and 10 μm, which are encapsulated by a stabilizing coating. Non-targeted microbubbles (ntMBs) are clinically used as blood pool agents for contrast-enhanced US imaging in cardiology and radiology [1,2] and also have therapeutic potential [3,4]. Targeted microbubbles (tMBs) are promising agents for US molecular imaging and therapy; in particular for diseases that can alter the endothelium, such as cancer and inflammation. The tMBs can adhere to specific disease-associated intravascular biomarkers by the addition of targeting ligands to the MB coating [5,6].

When MBs are insonified by US, they oscillate due to the acoustic pressure wave [4]. Oscillating MBs can increase cell membrane permeability to facilitate intracellular drug uptake (sonoporation), stimulate endocytosis, and open cell-cell junctions [4,7]. Although the exact mechanisms of MB-mediated drug uptake still remain unknown, many studies have attempted to pinpoint the US settings that best stimulate intracellular drug uptake [4,8]. So far the key findings are: 1) cell membrane pores induced by oscillating MBs can be reversible or irreversible [9]; 2) a MB has to oscillate with sufficient amplitude to induce sonoporation [10]; 3) tMBs stimulate drug uptake better than ntMBs both *in vitro* [11–15] and *in vivo* [16–19]. Recently, it has also been shown that the cell membrane pore size and pore resealing coefficient can be mathematically obtained from real-time observed MB-mediated intracellular drug uptake [20].

While reversible sonoporation likely facilitates cellular drug uptake without causing lethal damage to the cell, irreversible sonoporation is thought to lead to significant cell damage and eventually cell death. Different therapeutic approaches may require reversible or irreversible sonoporation, and a balance is expected between therapeutic effectiveness and cell damage. Hu et al. [9] revealed the size of the created cell

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http://dx.doi.org/10.1016/j.jconrel.2016.07.037
0168-3659/

membrane pore to be a predictor for reversible or irreversible sonoporation: pores < 30 μm^2 successfully resealed within 1 min after insonification, while pores > 100 μm^2 had not resealed within 30 min. An established method to study drug uptake by sonoporation relies on the intracellular uptake of the model drug propidium iodide (PI) [10,20–23], because this molecule can only pass the cell membrane of a live cell when it has been disrupted. After entering the cell it binds to DNA and RNA and becomes fluorescent [24]. Fan et al. [20] showed that intracellular PI fluorescence intensity directly relates to the amount of PI-DNA and PI-RNA complexes that have formed in the cell. They proposed a model to relate intracellular fluorescence intensity to the size of the created pore and its resealing time, which corresponded well with their experimental *in vitro* results on kidney [20] and endothelial cells [25].

For MB-mediated drug uptake, MB dynamics also have to be considered. The frequency generally used for MB-mediated drug delivery is 1 MHz [4], which means that the MBs oscillate one million times per second. These MB oscillations can only be resolved using an ultra-high-speed camera, capable of recording at least two million frames per second (Mfps) to satisfy the Nyquist sampling criterion [26]. While our group used ultra-high-speed recordings to determine that the relative oscillation amplitude of tMBs had to be above 50% to successfully sonoporate a cell [10] (6× 10 cycles at 1 MHz and 80–200 kPa peak negative acoustic pressure), others used high-speed cameras (in the order of a few thousand fps) to reveal that MB displacement is an important contributor to sonoporation-mediated cell death (1.25 MHz, 60–600 kPa, pulse repetition frequency (PRF) 10–1000 Hz, duty cycles 0.016–20%) [27]. MBs displace due to acoustic radiation forces, especially when longer acoustic pulses are used [28,29]. Long acoustic pulses have sparsely been used in MB-mediated drug delivery studies [30–33], even though one of these studies reported that 7000 cycles resulted in significantly more luciferase activity than 1000 and 5000 cycles in endothelial cells *in vitro* (2.25 MHz, 330 kPa, PRF 20 Hz, 120 s treatment) [33]. On the other hand, no significant differences between 1000, 5000, and 10,000 cycles were observed; different US pulse lengths thus affected luciferase activity. So far, in depth sonoporation studies on the effect of longer acoustic pulses at different acoustic pressures are lacking, as is the relation between MB oscillation and sonoporation efficiency. In addition, the effect of the same type of ntMBs and tMBs on endothelial cells has never been directly compared *in vitro*. All prior studies comparing ntMBs and tMBs were performed on cancer [11,13–15] and smooth muscle cells [12], despite that MBs are primarily in contact with endothelial cells when injected intravenously [4,5].

In this study we used long US pulses (500–50,000 cycles) at various pressures (150–500 kPa) to investigate how these settings affect US-mediated endothelial cell membrane permeability and cell death. In order to properly compare ntMBs and tMBs, we used home-made MBs with identical shell compositions to investigate their effect. The real-time observed PI uptake patterns were fit to the previously proposed diffusion model of Fan et al. [20] and additional Principal Component Analysis was used to determine whether cells were reversibly or irreversibly damaged. In combination with high-speed optical recordings of MB displacement and ultra-high-speed recordings of MB oscillation, we aimed to identify MB behavior responsible for viable sonoporation or cell death.

2. Materials and methods

2.1. Microbubble preparation

Lipid-coated MBs with a C_4F_{10} gas core (F2 Chemicals, Preston, UK) were made by sonication as described previously [22,34]. The coating of the non-targeted MBs (ntMBs) consisted of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC; 59.4 mol%; P6517; Sigma-Aldrich, Zwijndrecht, the Netherlands), polyoxyethylene-(40)-stearate (PEG-40 stearate; 35.7 mol%; P3440; Sigma-Aldrich), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxy(polyethylene glycol) (DSPE-PEG(2000); MW 2000; 4.9 mol%; 880125P; Avanti Polar Lipids, Alabaster, AL, USA). Before the experiment, the ntMBs were washed three times using centrifugation for 1 min at 400*g*. After washing the ntMBs, the size distribution and concentration were measured using a Coulter Counter (n = 3; Multisizer 3; Beckman Coulter, Mijdrecht, the Netherlands). The mean (± standard deviation, SD) diameter of the ntMB was 2.54 (±0.02) μm.

The same components were used for the targeted MBs (tMBs), except 0.8% of DSPE-PEG(2000) was replaced with DSPE-PEG(2000)-biotin (MW2000; 880129C; Avanti Polar Lipids). This allows for adding targeting moieties to the MBs *via* biotin-streptavidin bridging as previously described [22,35]. Briefly, after three washing steps by centrifugation at 400*g* for 1 min, the concentration of the MBs was measured using a Coulter Counter (n = 3) and 1×10^9 biotinylated MBs were incubated with 20 μg of streptavidin (S4762; Sigma-Aldrich) on ice for 30 min. Following incubation, the streptavidin-conjugated MBs were washed once to remove non-bound streptavidin. Next, 5 μg of biotinylated anti-human CD31-antibody (BAM3567; R&D Systems, Abingdon, United Kingdom) were conjugated to the MB shell, during incubation for 30 min on ice. Following this, tMBs were washed once to remove non-bound antibodies. Directly afterwards the size distribution and concentrations were measured using a Coulter Counter (n = 3) and mean (±SD) diameter for the tMBs was 2.82 (±0.09) μm.

2.2. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs; C2519A; Lonza, Verviers, Belgium) were cultured in EGM-2 medium (CC-3162; Lonza) in T-75 flasks (353,136; BD Falcon Fisher Scientific, Breda, the Netherlands), and maintained in a humidified incubator under standard conditions (37 °C, 5% CO_2). Thereafter the cells were trypsinized using trypsin in EDTA (CC-5012; Lonza) and replated on one side of an OptiCell™ (Thermo Scientific, NUNC GmbH & Co, Wiesbaden, Germany). Experiments were performed two days later with 100% confluence of HUVECs in the OptiCell.

2.3. Experimental set-up

For visualization of the MBs and HUVECs, the microscopic set-up consisted of a fluorescence microscope (Olympus, Zoeterwoude, the Netherlands) equipped with a 5× objective (LMPlanFl 5X, NA 0.13, Olympus) for the sonoporation and cell viability assays or a 40× objective (LUMPlanFl 40XW, NA 0.80, water immersion, Olympus) to capture MB behavior. For bright-field imaging the sample was illuminated from below *via* an optical fiber using a continuous light source and for fluorescence imaging a mercury lamp and a suitable set of fluorescent filters were used for the detection of propidium iodide (U-MWG2 filter, Olympus), Hoechst 33342 (U-MWU2 filter, Olympus), and calcein (U-MWIB2, Olympus). On top of the microscope three different cameras were mounted: 1) a high sensitivity CCD camera (AxioCam MRc, Carl Zeiss, Germany) for fluorescence imaging, 2) a high-speed Redlake Motion Pro Camera (10K, San Diego, CA, USA), and 3) the ultra-high-speed Brandaris 128 camera [36]. The experimental set-up is illustrated in Fig. 1.

For the acoustical set-up, a 1 MHz single-element, focused transducer (focal distance 75 mm; V303; Panametrics-NDTTM, Olympus NDT, Waltham, MA, USA) was mounted in the water bath at a 45° angle below the sample (Fig. 1). Each OptiCell was divided into eight equally sized, acoustically non-overlapping sections (19 × 33 mm each; for schematic see Fig. 1), which covered the beam area (−6 dB beam width of 6.5 mm) at the focus of the transducer, as verified in advance with a calibrated 0.2 mm PVDF needle hydrophone (Precision Acoustics Ltd., Dorchester, UK). The acoustic focus was aligned with the optic focus.