International Journal of Pharmaceutics 482 (2015) 92–98

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Duration of ultrasound-mediated enhanced plasma membrane permeability

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ARTICLE INFO

Article history:
Received 6 October 2014
Received in revised form 8 December 2014
Accepted 9 December 2014
Available online 11 December 2014

Keywords:
Sonoporation
Ultrasound
Microbubbles
Drug delivery
Membrane permeabilization
Fluorescent model drug

ABSTRACT

Ultrasound (US) induced cavitation can be used to enhance the intracellular delivery of drugs by transiently increasing the cell membrane permeability. The duration of this increased permeability, termed temporal window, has not been fully elucidated. In this study, the temporal window was investigated systematically using an endothelial- and two breast cancer cell lines. Model drug uptake was measured as a function of time after sonication, in the presence of SonoVue™ microbubbles, in HUVEC, MDA-MB-468 and 4T1 cells. In addition, US pressure amplitude was varied in MDA-MB-468 cells to investigate its effect on the temporal window. Cell membrane permeability of HUVEC and MDA-MB-468 cells returned to control level within 1–2 h post-sonication, while 4T1 cells needed over 3 h. US pressure affected the number of cells with increased membrane permeability, as well as the temporal window in MDA-MB-468 cells. This study shows that the duration of increased membrane permeability differed between the cell lines and US pressures used here. However, all were consistently in the order of 1–3 h after sonication.

1. Introduction

Efficient and controlled drug delivery to tumor tissue remains one of the major challenges in pharmaceutical research. To achieve drug delivery to diseased tissue, drugs need to overcome several biological barriers. For drugs with intracellular targets, one of these barriers is the plasma membrane. Ultrasound (US) can be applied to overcome this barrier, improving cellular uptake of drugs and genes (Frenkel, 2008). US has been observed to increase, for example, the anti-tumor effectiveness of anticancer chemotherapeutics including bleomycin (Lamanauskas et al., 2013; Iwanaga et al., 2007), cisplatin (Sasaki et al., 2012; Heath et al., 2012), methotrexate (Mei et al., 2009) and gemcitabine (Kotopoulis et al., 2014), both *in vitro* and *in vivo*.

US has some major advantages over other intracellular drug delivery techniques, *e.g.*, electroporation, since it can control drug delivery non-invasively in a spatial and temporal manner (Deckers and Moonen, 2010). Microbubbles (MBs), initially developed as ultrasound contrast agents for diagnostic imaging, are now widely studied to enhance the effectiveness of therapeutic ultrasound (Hernot and Klibanov, 2008). MBs oscillate when exposed to US, and at low pressure amplitude, this can result in stable cavitation over a longer period of time. MB-mediated cavitation induces normal stresses and, through micro streaming, shear stresses to their environment. Higher pressure amplitudes may lead to inertial cavitation, in which the microbubbles collapse, and high velocity jets may occur (Kooiman et al., 2014). When in close proximity to cells, these effects can induce transient permeabilization of the plasma membrane (Van Wamel et al., 2006; Zhou et al., 2012), which, in turn, allows the intracellular delivery of non-permeant agents (Cavalli et al., 2013).

Plasma membrane permeabilization can be the result of oscillating microbubbles (Deng et al., 2004). Initially, this enhanced membrane permeability has been ascribed to pores in the membrane and was therefore termed sonoporation (Lentacker et al., 2014). Recently, it has been shown that these pores are not the only mechanism responsible for US induced drug uptake. Meijering et al. (2009) observed that endocytosis was up-regulated

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http://dx.doi.org/10.1016/j.ijpharm.2014.12.013
0378-5173/

after US exposure and that the contribution of membrane pores and endocytosis to ultrasound induced uptake depended on the molecular size of the dye.

Another aspect of US induced membrane permeabilization under discussion, is the duration of increased cell membrane permeability, *e.g.,* for hydrophilic low molecular weight drugs. This "temporal window" may influence future clinical protocols using sonoporation. The degree of US induced membrane damage leading to permeability has been reported to depend on exposure conditions, such as US pressure (Keyhani et al., 2001; Karshafian et al., 2009), duty cycle (Pan et al., 2005) and sonication time (Karshafian et al., 2009). To remain viable, cells need to recover from US induced plasma membrane damage. It has been shown that membrane pore resealing is influenced by pore size, ATP, extracellular [Ca^{2+}] and presence of intracellular vesicles (Schlicher et al., 2006; Hu et al., 2013). Under normal physiological conditions *in vitro*, membrane recovery after US exposure was reported to take seconds (Mehier-Humbert et al., 2005; Zhou et al., 2009), minutes (Schlicher et al., 2006; Hu et al., 2013), or even hours (Zhao et al., 2008).

The duration of membrane permeability after sonication can also be measured by the internalization of a membrane impermeant model drug. A temporal window has been demonstrated in the order of seconds in bovine endothelial cells (Van Wamel et al., 2006), minutes in prostate cancer cells (Schlicher et al., 2006), or even 24 h in a glioma cell line (Yudina et al., 2011). These studies used similar small, hydrophilic dyes, *i.e.*, propidium iodide (668 Da; Van Wamel et al., 2006), calcein (623 Da; Schlicher et al., 2006) and SYTOX® Green (600 Da; Yudina et al., 2011), all impermeable to viable cells. Since propidium iodide and SYTOX® Green are DNA intercalating agents, they are considered as model drugs for small hydrophilic chemotherapeutics, based on their physicochemical properties and target site.

Until now, no study, to our knowledge, reported the temporal window of different cell lines with identical experimental settings (*i.e.*, US parameters, type of microbubble, model drug), and it remains unclear what causes the discrepancies in the reported temporal windows. Previous studies investigated the temporal window in either a cancer cell line or an endothelial cell line. Looking at future treatment strategies involving microbubbles and ultrasound, two routes of administration can be discriminated. The first involves intravenous administration of microbubbles and drugs, where US-activated microbubbles primarily affect endothelial cells. A second strategy implies intratumoral administration of microbubbles and drugs, where relative high local concentrations of MBs are in direct contact with tumor cells (Iwanaga et al., 2007; Matsuo et al., 2011; Watanabe et al., 2008).

From these perspectives, the objective of the present study was to investigate the temporal window of model drug uptake after US exposure in an endothelial and two cancer cell lines. Sonovue™ MBs were chosen since they are commonly used in the clinic as ultrasound contrast agents. The three cell lines were sonicated with identical US exposure conditions in the presence of MBs and the duration of model drug uptake was assessed. In addition, US pressure during sonication was varied to investigate its effect on the temporal window of uptake.

2. Materials and methods

2.1. Cell culture

MDA-MB-468 human breast cancer cells (ATCC® HTB-132™, LGC Standards GmbH, Wesel, Germany) were maintained in high glucose – Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich®, St. Louis, MO, USA). 4T1 mouse breast cancer cells (ATCC® CRL-2539™) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, and human umbilical vein endothelial cells (HUVEC; Lonza, Basel, Switzerland) were cultured in Endothelial Basal Medium-2 (EMB-2; Lonza) supplemented with Endothelial Growth Media-2 Microvascular complements (EGM™-2MV SingleQuots™ kit; Lonza). HUVEC cells were used between passages 6–9. All cell lines were cultured in a humidified incubator at 37 °C and 5% CO_2, in standard cell culture flasks.

Two days prior to ultrasound experiments, cells were seeded into OptiCells™ (Thermo Fisher Scientific Inc., Walthman, MA, USA). In the case of HUVEC cells, OptiCells™ were coated with collagen I (Sigma–Aldrich®) prior to cell seeding to minimize US induced cell detachment.

2.2. Ultrasound contrast agent

SonoVue™ (Bracco, Milan, Italy), a lipid shelled microbubble containing sulfur hexafluoride gas (SF6), was used as a cavitation inducing agent in US experiments (Schneider, 1999). The MB suspension was prepared according to the manufacturer's protocol, yielding a mean bubble diameter of 2.5 μm, and a concentration ranging between 1 and 5×10^8 microbubbles/mL. Before US experiments, 700 μL of fresh microbubbles was mixed with 9.5 mL medium, giving a suspension with a concentration of around 2×10^7 MBs/mL, which was then injected into the OptiCell™.

2.3. Chemicals

SYTOX® Green (Life Technologies™ Europe BV, Bleiswijk, Netherlands; Excitation (Ex)/Emission (Em) wavelengths = 504/523 nm), a nucleic acid stain unable to penetrate into

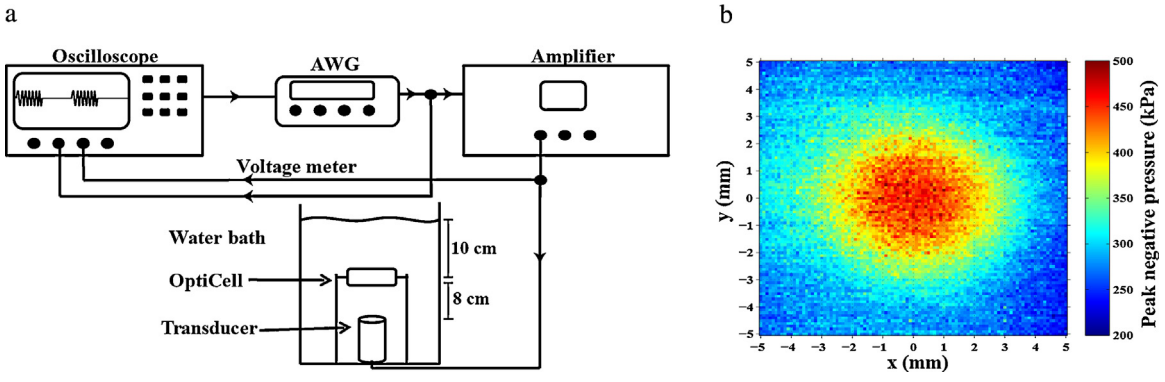

Fig. 1. (a) Schematic presentation of the ultrasound setup. (b) Horizontal XY plane of the ultrasound field measured at 80 mm perpendicular to the transducer. AWG = arbitrary waveform generator.