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Modulation of the molecular arrangement in artificial and biological membranes by phospholipid-shelled microbubbles

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ABSTRACT

The transfer of material from phospholipid-coated microbubbles to cell membranes has been hypothesized to play a role in ultrasound-mediated drug delivery. In this study, we employed quantitative fluorescence microscopy techniques to investigate this phenomenon in both artificial and biological membrane bilayers in an acoustofluidic system. The results of the present study provide strong evidence for the transfer of material from microbubble coatings into cell membranes. Our results indicate that transfer of phospholipids alters the organization of molecules in cell membranes, specifically the lipid ordering or packing, which is known to be a key determinant of membrane mechanical properties, protein dynamics, and permeability. We further show that polyethylene-glycol, used in many clinical microbubble formulations, also has a major impact on both membrane lipid ordering and the extent of lipid transfer, and that this occurs even in the absence of ultrasound exposure.

1. Introduction

Gas-filled microbubbles (MBs) stabilized by a surfactant or polymer coating are routinely used in medical imaging as ultrasound (US) contrast agents, capable of enhancing US backscatter from blood by several orders of magnitude [1]. The MB core typically consists of a high molecular weight gas (e.g., perfluorocarbon or sulphur hexafluoride) to enhance sample stability both *in vivo* and during handling or storage. The outer coating may consist of a cross-linked protein, saturated phospholipid or lipid mixture. Composition strongly influences MB acoustic response and hence clinical utility [2]. Polyethylene glycol (PEG) chains covalently bonded to either phospholipids or fatty acids can also be integrated into the coating in order to reduce immunogenicity [3,4], and PEG has furthermore been reported to influence the physical characteristics of the end-product in MB production processes [3,5].

The surfactant coating can be employed as a scaffold for the attachment of biologically active compounds [6] and/or targeting agents [7]. This has paved the way for the use of MBs as vehicles in therapeutic applications such as drug delivery and/or gene therapy [8–10] [2]. Their responsiveness to US facilitates triggered release of the therapeutic material; and, even more importantly, the interaction between MBs and living cells in an US field has been observed to generate cell membrane permeabilization, through a process often referred to as 'sonoporation' [11]. Although the underlying mechanisms have not been clearly identified, it has been postulated that the mechanical action of US-activated MBs causes the formation of temporary pores within nearby cellular membranes [6], which can be exploited to effectively deliver compounds into the intracellular milieu. Pore formation has been revealed and characterized *in vitro* using confocal microscopy [11], membrane potential measurements [12] and scanning and transmission electron microscopy [13]. In some cases, however, pores do not reseal spontaneously and this can lead to cell death [11,14–16].

The mechanical perturbation of the cellular microenvironment

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during US exposure in the presence of MBs may be attributed to distinct physical phenomena, including the pushing-and-pulling effect of a volumetrically oscillating MB [17,18], acoustic radiation forces causing MB translation against or across the cell membrane [19,20], fluid shear stress fields generated by acoustic microstreaming [21], or shock waves and fluid jets produced by MB collapse [11,22]. Mechanistic studies have been conducted to investigate the mechanical interaction between US-activated MBs and either artificial [23] or biological membrane bilayers [18]. Giant unilamellar vesicles (GUVs) composed of unsaturated phospholipids were observed to undergo elongational or compressional deformation when approaching or receding from a MB respectively, under low-pressure US fields [17,23]. At sufficiently high shear rates and/or elongation amplitudes pore formation occurred, which was accompanied by vesicle rupture [23]. Importantly, the deformation and break-up dynamics of GUVs have been observed to depend on the mechanical properties of the membrane bilayer [24]. Cyclic elongational and compressional membrane deformation have also been observed on adherent cells *in vitro* [17], and a direct correlation between cell membrane strain and the resulting transient permeabilization has been demonstrated using fluorescent probes [17].

Alternative mechanisms have also been suggested to govern or enhance US-mediated intracellular delivery, including: (i) activation of clathrin-mediated endocytic pathways [2,19,25], (ii) alteration of reactive oxygen species (ROS) homeostasis [26] potentially due to increased intracellular H_2O_2 levels [27], (iii) influx of calcium ions (Ca^{2+}) [12], and (iv) potential exchange or fusion of the phospholipid MB shell with the phospholipid bilayer of a cell membrane [28–30]. In recent years significant efforts have been made to characterize the behaviour of biological membranes exposed to US-activated MBs and to identify the underlying interaction phenomena [31], particularly regarding membrane permeabilization and recovery dynamics [11,32]. Nevertheless, the lack of quantitative methods for investigating the biophysical perturbations at a sub-cellular level often impedes correlation of experimental observations with biochemical or biophysical events.

In the present study we employed acoustofluidic systems integrated with quantitative fluorescence microscopy techniques to measure changes in the physical properties of both artificial and biological membrane bilayers interacting with phospholipid-shelled MBs, both in the presence and absence of an US field. We have focused primarily on changes in the arrangement of membrane lipids, given the latter's relevance to a variety of biophysical properties and membrane-associated cellular processes, including mobility, function and organization of membrane proteins [33,34], formation and dynamics of membrane domains [35], membrane mechanical properties [36] and permeability [37].

2. Materials and methods

2.1. Methods rationale

In order to investigate the effect of US and/or MBs of different composition in model and cell membranes we studied several biophysical characteristics that indicate how tightly lipids are packed within the membrane, namely: membrane viscosity, lipid order and lipid mobility.

Initially we investigated material transfer from MBs to live-cell membranes using a fluorescent, lipid-mimetic membrane probe. Next, to investigate changes in biophysical membrane properties after transfer, we selected giant unilamellar vesicles (GUVs) as a membrane model [38]. GUVs composed of dioleoyl phosphatidylcholine (DOPC) were specifically employed, since the resultant membrane model was a highly disordered system in terms of lipid arrangement, in contrast to the well ordered lipid shell typical of MBs. In this way, we were able to maximise the sensitivity of our model system in order to verify the ability of microscopy-based techniques to quantify changes in membrane physical properties. Multiple techniques including fluorescence lifetime measurement of a viscosity-sensitive membrane probe, membrane order characterisation using spectral imaging with an environmentally sensitive probe, and lipid mobility using fluorescence-correlation-spectroscopy (FCS) on a fluorescent lipid analogue were employed to validate the biophysical membrane characteristics. Increased lipid order was found to be associated with reduced diffusion time and increased membrane viscosity in agreement with previous studies [39].

In view of these results, we opted for using spectral imaging in living cells, as a means to efficiently measure changes in lipid order over statistically significant numbers of cell membranes. This approach was preferred to point-measurements (i.e., FCS) or more laborious techniques, to allow for analysis of large data samples. In these experiments, we further characterized dependencies on MB formulation. For this reason, MBs were produced in-house so as to be able to control and vary their composition.

Ultrasound exposure was performed using an acoustofluidic device developed in house for integration with advanced microscopy techniques. The device was designed to operate at a frequency of ~1 MHz, which is an ultrasound frequency employed in many studies investigating ultrasound-mediated drug delivery [19]. The acoustic amplitude was selected to be sufficiently high to cause cavitation microstreaming, but low enough to avoid an undesirable increase in fluid temperature.

2.2. Formation of giant unilamellar vesicles (GUVs)

GUVs made of DOPC (from Avanti Polar Lipids, USA) were employed as cell models and produced by electroformation as initially proposed by Angelova et al. [40], but following a modified version of the original protocol [41]. Briefly, a custom built Teflon® electroformation chamber containing two platinum (Pt) wires parallel to each other was employed. The lipid solution (1 mg/mL in chloroform) was spread on the Pt wires (3 μL per wire) and left at room temperature for 2 h for the solvent to evaporate. The electroformation chamber was primed with approximately 350 mL of a sucrose solution (300 mM in deionized water), and the Pt wires were connected to a signal generator. A sinusoidal wave was generated at 2V RMS and 10 Hz for approximately 1.5 h, which resulted in GUVs formation over the wires surface. The signal frequency was subsequently reduced down to 2 Hz for approximately 20 min, allowing the GUVs to detach from the wires. The formed GUVs were removed from the chamber using a micropipette, and transferred to a chambered (8-well) coverslip for labelling (μ-Slide 8 Well, Ibidi GmbH, Germany). The GUV concentration in the final sample was equal to ~1×10^4 GUVs/mL.

2.3. Preparation and characterisation of microbubble suspensions

A phospholipid-coated MB formulation frequently employed in therapeutic applications consisting of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids, USA) and polyoxyethylene (40) stearate (PEG40S, Sigma Aldrich, UK) in a 9:1 M ratio was selected as the primary MB model in the present study [42,43]. Additional MB formulations investigated include DSPC-PEG40S MB with a 90:1 M ratio, DSPC MB with no PEG40S, and 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC, Avanti Polar Lipids, USA) MB with no PEG40S.

All MBs were produced using a previously reported batch sonication protocol [44]. Briefly, 1,2-Distearoyl-sn-glycero-3-