

Microbubble Agents: New Directions

Eleanor Stride^a, Tim Segers^b, Guillaume Lajoinie^b, Samir Cherkaoui^c, Thierry Bettinger^c, Michel Versluis^b, Mark Borden^d

^a Institute of Biomedical Engineering, Department of Engineering Science, University of Oxford, Oxford, UK

^b Physics of Fluids Group, Technical Medical (TechMed) Centre, MESA*+ Institute of Nanotechnology, University of Twente, 7500 AE Enschede, The Netherlands

^c Bracco Suisse SA – Business Unit Imaging, Global R&D, Address: Route de la Galaise 31 - 1228 Plan-les-Ouates, Switzerland

^d Mechanical Engineering Department, University of Colorado, Boulder, CO 80309-0427, USA

Corresponding author:

Eleanor Stride

Institute of Biomedical Engineering,

Department of Engineering Science,

University of Oxford,

Oxford,

UK

Email: eleanor.stride@eng.ox.ac.uk

Phone: +441865617747

Abstract

Microbubble ultrasound contrast agents have now been in use for several decades and their safety and efficacy in a wide range of diagnostic applications has been well established. Recent progress in imaging technology is facilitating exciting developments in techniques such as molecular, 3D and super resolution imaging and new agents are now being developed to meet their specific requirements. In parallel, there have been significant advances in the therapeutic applications of microbubbles with recent clinical trials demonstrating drug delivery across the blood brain barrier and into solid tumours. New agents are similarly being tailored towards these applications, including nanoscale microbubble precursors offering superior circulation times and tissue penetration. The development of novel agents does however present several challenges particularly with regard to the regulatory framework. This article reviews the developments in agents for diagnostic, therapeutic and “theranostic” applications, novel manufacturing techniques and the opportunities and challenges for their commercial and clinical translation.

Keywords: ultrasound contrast agents; microbubbles; cavitation nuclei; nanodroplets; theranostic

BACKGROUND

Discovery and development of ultrasound contrast agents

The first published report of an ultrasound contrast agent was by Gramiak and Shah who described observations made by themselves and Dr. Claude Joyner of enhanced echogenicity of the aortic root following rapid injection of saline (Gramiak and Shah 1968). They hypothesised that this effect was due to the presence of microbubbles either produced during injection or pre-existing in the liquid. Subsequent studies by Kremkau et al. supported this proposal by demonstrating that no contrast enhancement was observed when the ambient pressure was increased in order to suppress bubble formation (Kremkau et al. 1970). Similar observations were also reported with other liquids, including indocyanine green (Feigenbaum et al. 1970), ether and even whole blood (Ziskin et al. 1972). The duration of the contrast enhancement was, however, extremely short due to the instability of the bubbles. This challenge was addressed by the discovery that a stabilising coating of cross-linked serum albumin could extend the bubble lifetime by inhibiting gas diffusion and reducing surface tension. This in turn gave rise to the first commercial ultrasound contrast agent, Albunex (Feinstein et al. 1984). The following decades have seen the development of numerous alternative agents, offering improvements in echogenicity and circulation time and with exciting demonstrations of the potential use of microbubbles in molecular imaging (Klibanov 2007) and drug delivery (De Cock et al. 2015). Perhaps surprisingly, however, only a small number of agents have so far been successfully translated into clinical use. The aim of this article is to review the state of the art and recent developments in agents for ultrasound imaging, therapy and

their combined or “theranostic” applications; and to discuss the opportunities and challenges for their clinical translation¹.

Classes of agents

The key requirements for an imaging contrast agent are that it should be non-toxic, generate a strong and unique imaging signal and have a sufficient circulation time for diagnostic procedures. For certain applications it is also desirable to be able to target the agent to specific sites and to control whether or not it remains in the bloodstream or can diffuse into the surrounding tissue. For therapeutic or theranostic applications, the generation of an imaging signal may be of less importance but there may be the further requirement for attachment of a payload, e.g. drug molecules, and/or generation of mechanical, thermal or chemical effects.

Microbubbles

All of the contrast agents currently available clinically consist of suspensions of gas microbubbles, having diameters in the range 1-10 μm (Figure 1) and vial concentrations of approximately 10^8 to 10^9 microbubbles/mL. They are administered typically by intravenous injection of 1-2 mL, giving an average blood concentration of $\sim 10^5$ to 10^6 microbubbles/mL which is sufficient to produce

¹ The inclusion criteria for the pre-clinical and clinical studies cited were respectively that IACUC approval was obtained if animals were studied or that informed consent was obtained from each study participant and that each study was approved by an ethics committee or institutional review board. The majority of studies cited were published in journals for which these criteria were conditions of publication. For those studies for which this was not the case, a check was made that appropriate statements were included. There was a small number of studies for which ethical approval could not be confirmed due to their being published prior to the widespread requirement for inclusion of a statement of ethics board approval. The authors, however, have no reason to doubt that the work was not compliant with the 1964 Declaration of Helsinki.

strong contrast enhancement even at low ultrasound intensities ($<1 \text{ Wcm}^{-2}$). The original Albunex microbubbles contained air, but the so called “second generation” of contrast agents all contain a high molecular weight, low solubility gas such as perfluoropropane (C_3F_8) or sulphur hexafluoride (SF_6) which significantly enhances bubble stability and hence prolongs the duration of contrast enhancement. Alternative coatings to serum albumin have also been extensively investigated, including cyanoacrylates, biopolymers such as PLA and PLGA (Straub et al. 2005), palmitic acid (Goldberg et al. 1993) and, most successfully, phospholipids (Unger et al. 1994); which offer a favorable compromise between microbubble stability and echogenicity. Microbubbles do, however, have limitations for certain applications. They are too large to extravasate prior to ultrasound exposure and can thus only be used to image the vasculature. In addition, their circulation half-life is relatively short (<5 mins) on account of their size (Schneider 1999). For many conventional imaging procedures, these limitations do not present significant problems; indeed, for applications in which only vascular imaging is required and high patient throughput is desirable they are advantageous. They are, however, restrictive for more complex imaging and therapeutic procedures. Pegylation has been shown to improve circulation times but only by a relatively modest amount (Hyvelin et al. 2017). Microbubbles also can be rapidly destroyed once exposed to ultrasound which again limits their utility for prolonged imaging or therapeutic delivery.

Phase-change liquid droplets

One of the early second generation contrast agents, EchoGen® (Grayburn 1997) consisted of an emulsion of sub-micrometer perfluorocarbon liquid droplets that could be vaporised to form microbubbles following injection. The liquid-liquid emulsion offered improved storage stability

and was supposed to enable increased *in vivo* microbubble stability and hence contrast persistence. EchoGen® was not ultimately commercially successful but a similar principle has been adopted in the development of so-called “phase shift emulsions” or “acoustically activated vaporisation agents” with the aim of addressing the size limitations of microbubbles. A key difference is that the creation of microbubbles is initiated by exposure to ultrasound rather than by activation during injection. The droplets retain their sub-micrometer size whilst in the bloodstream, enabling them both to circulate for longer and potentially to extravasate for example in the leaky vasculature surrounding a tumour (Figure 2). Both imaging and therapeutic delivery have been demonstrated using droplets in animal models for multiple applications (Kripfgans et al. 2000; Rapoport et al. 2007; Sheeran et al. 2011). Droplets also offer potential advantages for more recent imaging techniques such as super-resolution by enabling localized activation and destruction; thereby reducing the need for high microbubble concentrations that both degrade the image and pose a potential safety risk (Zhang et al. 2018). Apart from the phase of the core, their composition is very similar to that of microbubbles and indeed it has been shown that stable droplets can be generated through condensation of commercial perfluorocarbon microbubbles (Sheeran et al. 2017). The main disadvantages of droplets are that, unlike microbubbles, they cannot be imaged prior to vaporisation using ultrasound; and relatively high pressures and/or long pulses are typically required to initiate vaporisation. One approach to reducing the requirement for high pressures has been to combine microbubbles and droplets into “acoustic clusters.” It is hypothesized that the shockwave produced by the inertial collapse of the microbubbles at moderate pressure amplitudes facilitates droplet vaporization (Sontum et al. 2015).

Solid cavitation nuclei

The significant difference between the theoretical tensile strength of a liquid and the pressures at which cavitation is observed has long been attributed to the presence of particles capable of stabilising pockets of gas within hydrophobic cavities (Atchley and Prosperetti 1989). Consequently, a variety of solid particles has also been investigated as agents for bubble generation, primarily for therapeutic applications. These have included particles already used in other applications such as carbon nanotubes (Delogu et al. 2012) and mesoporous silica (Zhao and Zhu 2014) and deliberately engineered particles such as polymeric “cups” (Kwan et al. 2015) and gold cones (Mannaris et al. 2018). Particles that generate gas bubbles through a chemical reaction that can be triggered at a specific site have also been explored (Kang et al. 2010). Similar to liquid droplets, these particles can be in the sub micrometer size range, enabling the use of relatively high concentrations, long circulation times and the potential for extravasation. Moreover solid particles have been shown to offer improved duration of cavitation activity, potentially because unlike either microbubbles or droplets the particles are not destroyed by ultrasound exposure. Unfortunately, they also suffer from the same limitations as droplets in that they cannot be imaged prior to activation and require the use of relatively high ultrasound pressures and/or low frequencies compared to microbubbles (Mannaris et al. 2019). Furthermore, they may be more difficult to functionalise than microbubbles or droplets for targeted imaging/therapeutic applications on account of the need to maintain sufficient surface hydrophobicity. There are also some safety concerns over the use of certain classes of nanomaterial.

Other novel agents

There have been a number of other sub-micrometer scale agents investigated for both imaging and therapeutic applications. These include echogenic liposomes (ELIPs) (Hitchcock et al.

2010), “nanobubbles” (Cai et al. 2015) and protein coated gas vesicles extracted from bacteria (Shapiro et al. 2014). Studies of these agents all report similar advantages in terms of circulation time and extravasation potential as phase-change liquid droplets and solid cavitation nuclei but without the high activation pressure thresholds. There is some controversy in the current literature as to the mechanisms of contrast enhancement by these agents (Hernandez et al. 2018; Raymond et al. 2016). They are theoretically too small to resonate at the imaging frequencies typically used in human imaging and ELIPs and nanobubbles must therefore be used in significantly higher concentrations in order to produce comparable contrast to noise ratios (Kopechek et al. 2011). Moreover, it is an open question whether there are any intrinsic differences between ELIPs, nanobubbles and the large quantity of sub-micrometer particles present within a microbubble contrast agent suspension. This controversy is as yet unresolved and, given the currently poor understanding of how bubble populations evolve in a biological environment and/or following ultrasound exposure, a detailed discussion is outside the scope of this review. Interestingly, the concentrations reported for contrast enhancement by bacterial gas vesicles are significantly lower than for nanobubbles and further investigation is required to explain this apparent discrepancy. A further potentially important feature of these vesicles is their potential to be engineered to enable cell labelling e.g. for tracking of micro-organisms in vivo (Bourdeau et al. 2018).

MICROBUBBLE STABILITY

Static

Microbubbles tend to thermodynamic equilibrium by converting from a particle suspension to completely separate gas and liquid phases. This process happens by two mechanisms: coalescence and dissolution. The outer coating or shell inhibits the rates of both processes by imposing repulsive surface forces and diminishing surface tension (Borden and Song 2018), thereby kinetically trapping the microbubbles and stabilizing them for a useful time period. Thus, static microbubbles are relatively stable when the surrounding aqueous medium is saturated with the encapsulated gas (Duncan and Needham 2004). In the sealed vial, which is open to energy (heat) transfer but closed to mass transfer, a microbubble suspension can be stable on the shelf for days to months. Once the vial is opened, however, exchange of mass with the atmosphere tends to destabilize the microbubbles, leading to ripening and a decrease in concentration. Indeed, static microbubbles are highly unstable to a degassed medium (Borden and Longo 2002), or to gas exchange between the bubble cores and the surrounding milieu (Kwan and Borden 2010; Kwan and Borden 2012). In general, microbubbles with stiffer, less permeable shells, such as those comprising long acyl-chain lengths, tend to yield more stable microbubbles; the role of emulsifiers is also important in this regard (Borden 2018; Segers et al. 2016b).

Acoustic

Microbubbles that are otherwise stable under static conditions can be altered and destroyed by exposure to ultrasound. Two mechanisms have been identified for acoustic destruction of microbubbles: dissolution and fragmentation (Chomas et al. 2000). Fragmentation is the acoustically driven breakup of a bubble into two or more daughter bubbles (Chomas et al. 2001). High-speed video microscopy has shown that fragmentation likely occurs owing to harmonic shape oscillations (Dollet et al. 2008a; Postema et al. 2004b). Acoustic dissolution, on the other

hand, is a process whereby the microbubble diameter shrinks with each pulse, sometimes stabilizing against further acoustically driven dissolution at a small size (1-2 μm diameter) (Borden et al. 2005). Mechanisms proposed for acoustic dissolution include lipid shedding and fragmentation of a very small daughter bubble (Cox and Thomas 2013; O'Brien et al. 2011; Thomas et al. 2013). The onset of these instabilities occurs with increasing mechanical index. Microbubbles are stable when insonified at a very low MI, but they become unstable to acoustic dissolution and then fragmentation as the MI is increased (Chomas et al. 2001). Interestingly, microbubbles may also attract one another through secondary radiation force (Kokhuis et al. 2013; Leighton 1994), and then coalesce owing to insonification (Postema et al. 2004a).

in vivo

Microbubbles injected into the bloodstream are unstable and circulate for only a few minutes before being cleared. Two primary mechanisms have been identified for microbubble clearance: dissolution and phagocytosis. Upon being injected into the bloodstream, freely circulating microbubbles exchange their gas core with the respiratory gases (mainly N_2 , O_2 and CO_2) (Kabalnov et al. 1998) and then completely dissolve owing to ventilation/perfusion mismatch (Mullin et al. 2011). Additionally, freely circulating microbubbles may be tagged by complement proteins (e.g., C3b) or other opsonins and then engulfed by phagocytic cells (e.g., macrophages, neutrophils, Kupffer cells). Microbubble phagocytosis has been observed to occur primarily in the lung, liver and spleen (Tartis et al. 2008). Interestingly, phagocytosed microbubbles, or those adhered to the endothelium through ligand-receptor bonds, are more stable than freely circulating

microbubbles (Dayton and Rychak 2007), perhaps owing to the more steady dissolved gas content in the surrounding microenvironment.

TARGETED AGENTS

Microbubbles as well as droplets and solid particles can be targeted to a specific cell phenotype, such as inflamed or angiogenic endothelium, by decorating their surfaces with ligand molecules that bind avidly and specifically to a target receptor molecule (e.g., selectins or integrins). Collision of the microbubble with the target cell leads to multiple ligand-receptor interactions, which in turn arrests microbubble motion and adheres it to the cell surface. This enables ultrasound molecular imaging (Dayton and Rychak 2007), whereby a combination of pulse sequencing and image analysis is used to detect, visualize and measure the extent of microbubble binding. This method is amenable for receptor molecules that are expressed on the luminal surface of the vascular endothelium. Recently, human clinical trials of ultrasound molecular imaging for detecting tumor angiogenesis were reported for peptide-bearing microbubbles (BR55, Bracco Suisse SA, Geneva, Switzerland) targeted to vascular endothelial growth factor receptor 2 (VEGFR2) (Smeenge et al. 2017; Willmann et al. 2017). Additionally, targeted microbubbles may be used to facilitate targeted drug delivery by incorporating chemical specificity in addition to the spatial specificity afforded by ultrasound focusing.

Surface modification

In most cases, the targeting ligand is bound to the microbubble surface via a poly(ethylene glycol) (PEG) tether (Klibanov 1999). The flexible PEG tether allows the ligand molecule to diffuse and orient itself to interact favorably with the receptor binding pocket (Kim et al. 2000). In some cases,

the ligand is attached to the lipid prior to microbubble production, as is the case for BR55. However, much of the ligand may be wasted by this approach as with some manufacturing techniques, as little as 10% of the precursor lipid used to generate microbubbles actually incorporates into the microbubble shell. Additionally, the targeted microbubbles must be “washed” by centrifugation and resuspension to remove free ligand molecules and thereby prevent competitive binding and blocking of the receptor molecules (Stieger et al. 2008). Conjugation chemistries have therefore been developed to attach the ligand directly onto preformed microbubbles (Klibanov 2005). The microbubbles can be washed to clear out free functional groups not attached to the microbubble surface, thereby increasing conjugation yield and reducing cost. Biotin-Avidin-Biotin conjugation chemistry was one of the first approaches to be tested and commercialized (Targestar, Targeson Inc, San Diego, CA, USA; Micromarker, Bracco Suisse SA, Geneva, Switzerland). In this method, the microbubbles are generated comprising PEG-biotin, then decorated with avidin, and finally conjugated to a biotinylated ligand (typically an antibody). Unfortunately, avidin is immunogenic, which limits the potential of this approach for clinical translation. Therefore, covalent conjugation chemistries with biocompatible linkers have been developed (Klibanov 2005), most notably maleimide-thiol (Geers et al. 2011). In this approach, microbubbles formed with PEG-maleimide are washed and then conjugated to thiolated ligand molecules, such as proteins that comprise exposed cysteine groups. Unfortunately, this scheme has unwanted side reactions in aqueous media, and exposed maleimide groups following conjugation must be “capped” by free cysteine to prevent nonspecific binding to blood proteins. Recently, biorthogonal click chemistry has been developed to conjugate ligand molecules to microbubbles rapidly and efficiently, thereby removing the need to cap unreacted functional groups on the microbubble surface (Slagle et al. 2018).

Buried ligand

One often overlooked concern with targeted microbubbles is the potential for complement protein fixation onto the microbubble surface and complement activation of the immune system. In particular, complement protein C3b is ubiquitous and has an unstable thioester group that can covalently bind to nucleophilic groups on the targeting ligand, such as hydroxyls (Janssen et al. 2010). This means that virtually all targeting ligands can be tagged with C3b, which has corresponding receptors (C3R) on nearly every cell of the mononuclear phagocyte system. Microbubble capture and phagocytosis reduces circulation persistence and target specificity, rendering the targeting strategy unreliable unless the target phenotype is inflammation or ischemia. Most ultrasound molecular imaging studies have ignored this important biological phenomenon, or perhaps it was implicitly assumed that complement fixation is kinetically slow enough in comparison to the microbubble pharmacokinetics and desired ligand-receptor interaction. This assumption is problematic, however, because results have shown that complement fixation of microbubbles can occur in less than 5 min (Borden et al. 2006; Chen and Borden 2011), and this can significantly reduce microbubble circulation persistence (Chen et al. 2012). Complement activation can also lead to hypersensitivity reaction that may be fatal (Szebeni 2014). Therefore, a buried-ligand approach has been developed to block complement C3b fixation, prolong microbubble circulation persistence and preserve the microbubble targeting specificity (Borden et al. 2006; Borden et al. 2008; Borden et al. 2013). The approach employs a tiered PEG brush layer with the ligand tethered by short PEG chains and surrounded by methyl-terminated long PEG chains that protect the ligand against complement attack. The ligand is then revealed for binding to the receptor by the primary ultrasound radiation force.

Magnetic targeting

An alternative and potentially complementary approach is to encapsulate magnetic material within the microbubble coating, thereby making the microbubbles responsive to an externally applied magnetic field. Magnetic targeting of microbubbles has been shown to enable enhanced delivery of multiple therapeutics *in vitro* and *in vivo* (Stride et al. 2009; Vlaskou et al. 2010), accelerated thrombolysis (de Saint Victor et al. 2019), improved contrast enhancement and also to provide magnetic resonance image contrast enhancement (Crake et al. 2016).

THERANOSTIC AGENTS

The term “theranostic” was originally coined to describe screening techniques to predict a patient’s suitability for a particular treatment (Pene et al. 2009). Its meaning has been extended however to describe methods and technologies that integrate diagnostic and therapeutic procedures. Microbubbles have multiple features that make them highly effective as theranostic agents. Their echogenicity provides a convenient means of real time treatment monitoring as both their location and amplitude of oscillation can be determined using conventional ultrasound imaging equipment. The same oscillatory behavior that makes microbubbles echogenic, also gives rise to a number of biological effects that can be exploited for therapeutic purposes. Specifically, when a microbubble or cloud of microbubbles oscillate(s), the surrounding fluid can be set into motion. This “microstreaming” can significantly enhance convection of drugs from the bloodstream into tissue (Mannaris et al. 2019). This is particularly important for the treatment of solid tumours and blood clots where diffusion is often insufficient to produce therapeutic drug concentrations throughout the target tissue. Microstreaming is also thought to be one of the mechanisms by which

microbubbles are able to promote reversible opening of the endothelium, including the blood brain barrier and enhance cellular uptake of drugs (sonoporation). To date, clinical studies have utilized existing commercial contrast agents (Carpentier et al. 2016), but pre-clinical studies have demonstrated that therapeutic efficacy can be enhanced, e.g. by refining the microbubble agent size distribution (Chen and Konofagou 2014) or exploiting the same targeting strategies as described in the previous section to increase microbubble concentration at the treatment site. At higher ultrasound pressures, microbubbles and their precursors can also produce both mechanical and thermal ablation of tissue. For a more detailed review of therapeutic applications please see the companion review in this special issue of *Ultrasound in Medicine and Biology* by Kooiman et al.

Microbubble agents can also be used to localize the release and/or activation of drugs. For example, Bezagu et al. utilized a specially designed form of composite droplet to encapsulate a prodrug of chemotherapeutic agent and demonstrated that its release was confined to the droplet activation site (Bezagu et al. 2017). Certain classes of drug become functional only when exposed to a physical stimulus. Their activity can thus be confined to a target tissue volume which significantly reduces the risk of systemic toxicity. It has been shown that ultrasound and microbubbles can provide a suitable stimulus (McEwan et al. 2015; Yumita et al. 1989) to enable so-called sonodynamic therapy (SDT). The underpinning mechanisms are still the subject of some debate but may relate to the production of light by oscillating microbubbles (sonoluminescence) (Beguin et al. 2019).

Microbubbles can be used for the delivery of therapeutic gases such as oxygen and nitric oxide (McEwan et al. 2015; Sutton et al. 2014) and their outer coating also provides a versatile platform for attachment or encapsulation of drug molecules. This facilitates targeted release as microbubbles can be stimulated to release the drug at the target site using focused ultrasound. This, combined with the enhanced tissue penetration discussed above, typically also significantly reduces the quantity of drug that needs to be injected. A detailed review of the different conjugation/encapsulation methods may be found in (Mulvana et al. 2017). An overview summary is shown in Figure 3.

MULTI-MODALITY IMAGING AGENTS

Microbubbles can also be functionalized to enable them to be visualized with other imaging techniques to facilitate multi-modality imaging (Figure 4).

Optical

Particularly for *in vitro* and pre-clinical studies, it is often helpful to visualize microbubbles with optical microscopy using a fluorescent dye molecule incorporated into the microbubble shell, for example intravital microscopy to view microbubble circulation dynamics (Unger et al. 2014). Dye molecules can be conjugated onto the microbubble surface (Slagle et al. 2018; Upadhyay et al. 2017), or they can be loaded into the microbubble shell (Klibanov 2002). Lipophilic cell labeling dyes (DiI, DiO, DiD, etc.; Vybrant, Thermo-Fisher, USA) are particularly useful for tagging lipid-coated microbubbles. Fluorescent molecules can quench their fluorescence at high concentration, so one must be careful not to overload the microbubble with dye. Some dyes can also photo-

quench more rapidly than others, so it is useful to investigate photo-stability during the microbubble design phase. Fluorescent nanoparticle dyes, such as quantum dots (Ke et al. 2009), have also been conjugated to microbubbles to enhance optical visualization. Optical coherence tomography (OCT) produces images from reflected light using interferometry to reject multiple scattering. Similar to ultrasound imaging, contrast can be improved by the introduction of strong scatterers such as microbubbles (Lee et al. 2003). Methods such as ultrasound-modulated fluorescence (UMF) have also been developed to combine optical and ultrasound imaging to provide fluorescence contrast at ultrasound resolution in optically scattering media (Liu et al. 2014).

Photoacoustics

In addition, optically absorbing plasmonic gold nanoparticles can be conjugated onto microbubbles for dual modality ultrasound and photoacoustic imaging (Dove et al. 2013). Plasmonic nanoparticles absorb photons and dissipate heat, leading to localized photo-thermal expansion and production of an acoustic wave. Such pulsed heating can also drive microbubble oscillations when the plasmonic nanoparticles are arrayed around a microbubble gas core (Dove et al. 2014a). Gold nanoparticle-coated microbubbles (AuMBs) focus the photoacoustic response at the microbubble resonance frequency, rather than the usual broadband response, thereby providing a more efficient energy conversion for sensing with medical ultrasound probes. Gold nanoparticles can also be loaded into phase-change emulsion droplets, where vaporization leads to an enhanced photoacoustic effect (Meng et al. 2019; Wilson et al. 2012) (Figure 4a). The vaporization event can be tailored to specific optical wavelengths, such as the near infrared, by selection of the nanoparticle structure (Dixon et al. 2015), or to laser fluence

by choice of the fluorocarbon core (Dove et al. 2014b). Alternatively, the microbubbles can be coated with a dye-loaded liquid layer (Lajoinie et al. 2017), which offers both an easier way to tune the absorption wavelength, and removes the long-term biocompatibility concerns of gold nanoparticles.

Magnetic resonance imaging (MRI)

Microbubbles can be used as contrast agents for MRI without modification on account of their ability to produce a difference in magnetic susceptibility when injected into the bloodstream (Ueguchi et al. 2006). To increase the contrast to noise ratio, microbubbles can also be loaded with hyperpolarized gases (Callot et al. 2001) or particulate MRI contrast agents for dual modality imaging. In one example, polymer shelled microbubbles were loaded with the T2 contrast agent, iron oxide (Yang et al. 2009). In another example, iron oxide particles were incorporated into the shell of lipid-coated microbubbles (Owen et al. 2018) (Figure 4b). The T1 contrast agent, Gd(III), has also been loaded onto microbubbles with polymer shells (Ao et al. 2010) or lipid shells (Feshitan et al. 2012a). Interestingly, it was found that Gd-DOTA did not enhance the T1 image for the intact microbubble, but it did enhance the signal for a fragmented bubble (Feshitan et al. 2012b). This brought up the tantalizing possibility of using MRI to guide ultrasound-targeted microbubble destruction, but unfortunately the signal-to-noise ratio has been too low for *in vivo* validation. Owing to the growing interest in MRI-guided focused ultrasound therapy, there remains strong interest for novel MRI-detectable microbubbles and nanodrops (Koshkina et al. 2019).

X-ray

Whilst invisible to conventional X-ray imaging, microbubbles can be loaded with X-ray opaque material to provide contrast. They can also be imaged directly using X-ray phase contrast imaging (XPCi). Individual microbubbles act as lenses, refracting the incoming X-rays producing a large phase difference when radiation is propagated through a microbubble population (Millard et al. 2013) (Figure 4c). The comparative safety of microbubbles compared to iodine or other X-ray contrast agents makes them a potentially attractive alternative.

PET/SPECT

Microbubbles can be radio-labelled and imaged with SPECT or PET isotopes to determine bio-distribution. This can be useful for assessing targeting efficiency and impact of acoustic exposure Tartis et al. 2008 (Figure 4d).

MICROBUBBLE SYNTHESIS

Sonication

Sonication was the first method described to generate microbubbles for ultrasonics (Feinstein et al. 1984). Sonication involves the high-frequency vibration (typically 20 kHz) of a horn tip at the gas/water interface. This vibration leads to entrainment and secondary breakup through cavitation in the bulk aqueous phase (Li and Fogler 1978a; Li and Fogler 1978b). Unfortunately, there is very little research into the details of bubble entrainment via sonication. It is generally known that low-power sonication with the tip submerged inside the aqueous medium leads to microbubble destruction (clarification) and break-up of the lipid structures from multi-lamellar vesicles (MLVs)

into small uni-lamellar liposomes (SUVs). This is often a preparation step in generating microbubbles. Microbubbles are then generated by moving the probe tip to the gas/water interface and turning the system to full power. Sonication generates many microbubbles very rapidly: for example, 1 L volume of 10^{12} bubbles/L can be generated within 1 minute. The stochastic processes of entrainment and break-up lead to a fairly polydisperse size distribution, which can be refined using sorting techniques as outlined below. Thus, sonication is a simple and economical method of generating microbubbles in high yield.

Shaking

Shaking is another process of mechanical agitation that is used to create microbubbles. Typically, a small volume (~1 mL) of lipid solution is sealed in a small vial with a gas headspace and placed in a dental amalgamator or similar mixing device. The device vibrates along the long axis of the vial at ~4000 Hz. This method is used to generate Definity® microbubbles (Lantheus, USA), for example. The benefit of the shaking method is that it can produce microbubbles rapidly (~ 10^9 bubbles in less than one minute) on demand. The lipid suspension in the vial can be made at a central facility, sterilized and then shipped to the end user, who simply places the vial into the shaking device to generate the microbubbles, e.g. at the bedside. Thus, it is a very simple and economical method for multiple uses of small quantities of microbubbles. As with sonication, the bubble entrainment and break-up processes during shaking are poorly understood, but it is known that they lead to a polydisperse size distribution. Coincidentally, the size distribution of microbubbles formed by shaking tends to be remarkably similar to that of microbubbles formed by sonication, despite the very different geometry and characteristic frequency. More research is

necessary to better understand the bubble formation process during shaking, as well as how the shaking parameters affect the microbubble size distribution.

Microfluidics

The classic microbubble production methods discussed above result in inherently polydisperse microbubble size distributions, typically ranging from 1 to 10 μm in diameter (Stride and Saffari 2003). A microbubble of a given size and coating properties will resonate, and thus generate the strongest echo, at a specific frequency (Minnaert 1933). Hence this polydispersity can be advantageous in the case of a multi-purpose imaging contrast agent used for a range of different anatomical targets and consequently with a range of different imaging frequencies. It does, however limit the contrast to noise ratio that can be achieved for a given microbubble concentration under any one set of imaging conditions. Consequently, monodispersity in terms of size and acoustic response is regarded as an important condition to unlock the full potential of microbubbles both for imaging and therapy by providing the possibility to finely control the bubble response and efficiently make use of their resonance. Moreover, monodisperse populations allow for a more effective use of bubble nonlinearities, otherwise drowned in the bubble-to-bubble variations in terms of acoustic response. This is particularly important for novel imaging strategies such as super-resolution, three-dimensional and plane wave imaging in which bubble concentrations may be restricted and the need to maximise bubble signal at low ultrasound pressures is critical (Couture et al. 2012; Forsberg et al. 2002; Ghosh et al. 2017; Jones et al. 2018; Lin et al. 2017). Therefore, monodisperse contrast agents, by offering new imaging possibilities (Segers et al. 2018b), could be considered novel agents in their own right. Furthermore, due to their uniform acoustic response (Segers et al. 2016a), monodisperse microbubble populations may potentially solve remaining

fundamental questions as to the optimal acoustic parameters required to induce therapeutic effects such as endocytosis, sonoporation, and cell death (Karshafian et al. 2009)(Roovers et al. 2019; van Rooij et al. 2016) and to precisely measure the role of shell components on the acoustic bubble response (Segers et al. 2018a). Therefore, over the last decades, several techniques have been developed to produce bubble suspensions with a narrow size distribution. The first approach is to enrich polydisperse microbubbles and the second approach is to directly form monodisperse bubbles in a microfluidic flow-focusing device. Regarding the first approach, microbubbles can be enriched through mechanical filtration over a pore filter (Emmer et al. 2009), through decantation (Goertz et al. 2007), and through multiple centrifugation steps (Feshitan et al. 2009). With a higher degree of control, microbubbles can be sorted in microfluidic devices resulting in even narrower size distributions. They can be sorted to size in a pinched microchannel (Kok et al. 2015) and they can be sorted to their resonance behavior using the primary radiation force induced by a traveling acoustic wave (Segers and Versluis 2014). A future challenge for microfluidic sorting methods is to increase robustness and throughput and, therefore, to scale up, e.g. through parallelization. An advantage of sorting methods is the offered high freedom of choice in the lipid mixture used to coat the bubbles, i.e. today, the constraints in terms of the formulations for microfluidic bubble formation at high production rates are rather stringent (Hettiarachchi et al. 2006)(Segers et al. 2017).

Microfluidic flow-focusing

The second approach to produce a monodisperse bubble suspension is through direct bubble formation in a microfluidic flow-focusing device in which a gas thread is focused between two liquid co-flows through a constriction where it destabilizes and pinches off to release monodisperse

bubbles (Figure 5). The bubble size and the bubble formation rate in a flow-focusing device can be controlled through the gas pressure and the liquid flow-rate. Historically, a large gap has separated the classic large-scale microbubble manufacturing techniques presented above and the microfluidic flow-focusing technique, regarded as a low-throughput method. Nevertheless, owing to a better understanding of the microscale fluid dynamics (Anna et al. 2003; Ganán-Calvo and Gordillo 2001; Garstecki et al. 2004) and innovative designs (Castro-Hernández et al. 2011), to date, flow-focusing allows for the formation of several million bubbles per second (Castro-Hernández et al. 2011; Segers et al. 2016c), allowing for the production of a clinically relevant dose (i.e. 10^9 bubbles) in a matter of minutes.

Fluid physics of microfluidic flow-focusing

Much work has been done on the microscale fluid dynamics that governs the production of microbubbles using microfluidics, including the pinch-off process, gas jet formation and break-up, and the effect of the flow-focusing geometry (Anna et al. 2003; Dollet et al. 2008b; Ganán-Calvo and Gordillo 2001; Garstecki et al. 2004; van Hoeve et al. 2011). These investigations, often made in terms of scaling laws, have had a large impact on the capability of microfluidic techniques and led to an improvement of 3 orders-of-magnitude in the production rate of a single microfluidic nozzle. Notwithstanding the importance of these advances, much remains to be done, on the one hand on the fluid dynamics within the chip including the nozzle or constriction to obtain a complete and predictive description of these techniques and, on the other hand, on the impact of the coating material on the production dynamics.

Long-term size stability of microfluidically formed bubbles

Microfluidically formed lipid-coated bubbles are inherently unstable and prone to Ostwald ripening until they have dissolved to their stable size which is typically 2 to 3 times smaller than their initial on-chip bubble size (Segers et al. 2016b; Shih and Lee 2016; Talu et al. 2008). Once the bubbles have reached their final size, they are stable for days in a closed vial (Segers et al. 2019). The size decrease during stabilization has been characterized and it is a function of the lipid mixture, i.e., it increases proportionally with the molar amount of PEGylated lipids and with the PEG chain length (Segers et al. 2017). As a result of the size decrease during stabilization, approx. 90% of the initial gas volume diffuses out of the freshly formed bubbles, which first saturates the surrounding liquid, and then results in large foam bubbles formed through Ostwald ripening (Segers et al. 2016c; Talu et al. 2008). One major challenge is to investigate how foam formation can be mitigated in order to produce readily usable monodisperse microbubble suspensions.

On-chip stability of microfluidically formed bubbles

Microfluidic monodisperse bubble formation requires lipid concentrations approx. 10 times higher than those in classic microbubble production methods to minimize on-chip bubble coalescence in the outlet of the flow-focusing device (Segers et al. 2017; Shih and Lee 2016). Furthermore, a high molar concentration of PEGylated lipids with a long chain length is required to minimize coalescence which to date limits the freedom in the choice of lipid mixture for direct monodisperse bubble formation. It has been shown that the stability against on-chip bubble coalescence can be improved by forming bubbles at elevated temperatures (Segers et al. 2019). However, high lipid concentrations are still required, mainly because of the crucial function of the free liposomes in inhibiting coalescence. A future challenge is therefore to find a cheaper and equally effective

alternative to these free liposomes, allowing to reduce the concentration used and subsequently, the costs.

Microbubble uniformity

Size monodispersity is a critical and necessary aspect to control the acoustic microbubble responses and it can be accurately controlled by the flow-focusing method. It is not, however, sufficient. More precisely, the response of a microbubble depends not only on its size but it is also strongly affected by the viscoelastic properties of the shell (van der Meer et al. 2007). Lipid-coated bubbles, for example, represent the vast majority of ultrasound contrast agents currently under investigation and under clinical use. It has been shown that the response to an ultrasound pulse strongly depends on the lipid packing density, i.e. on the equilibrium surface tension (Overvelde et al. 2010). A future challenge is to measure the acoustic uniformity of mono-sized bubbles formed by flow-focusing. Furthermore, it remains to be investigated how the acoustic response of lipid coated bubbles can be tuned, i.e. how different lipid shell components affect the acoustic microbubble response. Control over the viscoelastic shell properties and thus over the acoustic response would be highly valuable as this would allow for microbubble design dedicated to specific clinical applications such as noninvasive blood pressure measurements, sonothrombolysis, and drug and gene delivery using bubbles and ultrasound.

In vivo performance

It has been shown in an in-vitro setup that the use of a monodisperse bubble population results in a sensitivity increase by 2 to 3 orders of magnitude (Segers et al. 2018c). In a preliminary in-vivo experiment, the measured sensitivity increase was reported to be similar (Jeannot 2018). The *in*

in vivo use of monodisperse bubbles is however still at an early stage, owing to the very recent gain of control over the full production process necessary to enable the testing phase. The expected improvement of therapeutic and molecular imaging applications using monodisperse agents also still remains to be characterized *in vivo*.

Microfluidics for the production of complex agents

The success of microbubbles and ultrasound imaging has led to the creation of the large diversity of contrast agents, as discussed in this review. The same limitations and challenges apply to controlling the response and properties of microfluidic agents in order to maximize their effect, for both contrast enhancement and for therapy. Here again, microfluidic techniques have the potential to address these requirements, and are of special interest for the production of particle-loaded microbubbles, (Peyman et al. 2012; Seo et al. 2010), of hard-shelled agents (Abbaspourrad et al. 2013; Lee et al. 2010), of multi-layered bubbles (Hettiarachchi et al. 2009; Shih et al. 2013) and of droplet precursors (Hsiung et al. 2006; Seo and Matsuura 2012). Although these approaches were met with preliminary success, they often require a modification of the chip and/or of the process driving the chip. Satisfactory microfluidic production of these agents therefore requires revisiting our understanding of bubble production to account for these specificities. Such developments are still at their infancy.

Scale-up challenge

A single flow-focusing device with a single nozzle can deliver a clinical dose in seconds to minutes time which potentially allows for the on-demand production of microbubbles at the bedside. However, in an industrial approach, the production rate achievable with a single microfluidic chip

remains insufficient. A relevant production rate at a reasonable cost requires the parallelization of tens or hundreds of microfluidic nozzles. Some attempt has been made in this direction, with fewer nozzles that led to either a loss of control or a low production rate (Jeong et al. 2017, Hashimoto et al. 2008, Mulligan et al. 2013, Bardin et al. 2013), highlighting the underlying difficulties. Cross-talk between nozzles remains a question since the fluid dynamics in such a system remains yet to be investigated. Scale-up is currently the main challenge for the industrial translation of microfluidic techniques (Holtze et al. 2013). Other techniques that have been explored for microbubble production include electrohydrodynamic atomisation (Farook et al. 2009), electrolytic techniques and hybrid approaches (Parhizkar et al. 2014). All of these, however, also suffer from relatively low production rates in their current format.

APPROVAL OF NEW PRODUCTS

As described in the previous section, multiple methods have been investigated for the production of microbubbles. Each technique exhibits strengths and weaknesses in terms of ease of use, cost effectiveness, production yield, control of the microbubbles size and polydispersity, stability, energy use etc. However, translation of these innovative procedures to the clinic and to the market faces numerous hurdles in terms of scalability, translatability and regulation. In fact, in contrast to conventional small pharmaceutical drugs, microbubbles constructs are rather complex with different ingredients, including typically gas, lipids, excipients, and targeting ligands, making the pharmaceutical development quite challenging. The aim of this section is to outline a possible path to be followed for a successful translation of novel agents, focusing on lipid-shelled gas-filled microbubbles which represent the major part of commercially available ultrasound contrast agents. It should be emphasised that although they are used for diagnostic purposes, gas microbubbles are

currently considered as a medicinal drug and thus have to follow the same development path as therapeutic drugs. Their designation for therapeutic applications remains to be determined.

Chemistry, manufacturing and controls (CMC)

Chemistry, Manufacturing and Controls (CMC) and Regulatory Affairs (RA) are of paramount importance at different stages of pharmaceutical product development (Figure 4). Regulatory agencies such as the US Food and Drug Administration (FDA), European Medicines Agency (EMA) and UK Medicines and Healthcare Products Regulatory Agency (MHRA) provide some guidance and recommendations on the CMC activities to be carried out to guarantee safe, effective and high quality products (FDA (Food and Drug Administration) 2018). The CMC development plan typically involves three main parts that the applicant must submit as part of the quality dossier for any new drug application (NDA), Investigational Medicinal Product Dossier (IMPD) or investigational new drug (IND) filings: 1) Characterization of the manufacturing components and materials, 2) Product manufacturing procedures, and 3) Product testing. These CMC activities are involved at different stages of the microbubble pharmaceutical life cycle.

Characterization of the manufacturing components and materials

Microbubbles are generally available as a liquid suspension or as a freeze-dried powder for reconstitution. The applicant should provide information related to the microbubble components such as gas core, lipid ingredients, excipients and targeting ligand moiety in the case of ultrasound molecular imaging (USMI) applications. The content (mg/vial or mg/g) of each component of the microbubble formulation should be clearly defined and their ranges indicated based on results gathered during the product development stages. Data demonstrating the quality and safety of each

ingredient is compulsory. In particular, the applicant should provide in depth characterization of the microbubble main components such as core gas and lipids (specifications, stability data and microbiological testing, etc.), quality data have to be similar to those for the submission of drug substance (ICH 2003a). In this regard, to ease the regulatory path, the selection of the pharmaceutical gas and lipid components is key for the development of new agents.

Product testing

Physicochemical properties of the gas-filled microbubbles are crucial for quality, efficacy and safety considerations. The applicant should develop and validate dedicated analytical procedures (non-GMP and GMP), in line with current regulations and guidelines, for the comprehensive characterization of both drug substance (gas, phospholipids, etc.) and microbubble drug product, and execution thereof according to the CMC plan. Moreover, the applicant has to design stability studies to determine the shelf life according to ICH guidelines (ICH 2003b) with in-depth characterization of impurities and possible degradation products. In fact, stress and accelerated stability testing data are mandatory to determine the degradation profile, establish stability indicating analytical methods, define appropriate storage conditions ensuring the microbubble quality and performances and ultimately define retest periods. Besides safety and quality considerations, microbubble shelf-life and stability of the reconstituted suspension are important elements from the practical perspective.

The following properties are generally monitored: microbubble size (i.e., mean and distribution profile) and concentration, surface charge or zeta potential, osmolality, viscosity and resistance to hydrostatic pressure, shell lamellarity, and mechanical properties. Moreover, residual solvents

have to be monitored if any organic solvent is used during the manufacturing process. Lipid and gas contents are also systematically determined using dedicated analytical procedures, namely liquid chromatography and gas chromatography. Since microbubbles are sterile products, microbiology activities such as sterilization validations, endotoxin testing, sterility testing, and container closure testing and other USP microbiology tests need to be performed as recommended by regulatory authorities. Critical quality attributes (CQAs) of the contrast agent can thus be defined.

In vitro measurements of the agent's acoustic properties such as attenuation and backscatter coefficients as a function of frequency are also important features to quantify. In addition, mechanical properties (i.e. shell stiffness) are also determined using appropriate methods, e.g. backscatter and attenuation (Gorce et al. 2000), high-speed optical imaging (van der Meer et al. 2007) and atomic force microscopy (AFM) (Sboros et al. 2007). Finally, *in vitro* stability of the agent following incubation in plasma at 37 °C in a time window compatible with clinical examination can also be relevant information for stability assessment.

Manufacturing process

According to the ICH guideline for industry (Q8(R2) Pharmaceutical development), the manufacturing process has to be fully described with a detailed flow diagram (batch size, bulk and finished drug product, purification and sterilization methods, freeze-drying, packaging, etc.) and validated to demonstrate the consistent production of a safe and efficacious agent in line with the specifications set in the filling dossier (NDA). microbubble manufacturing under cGMP conditions for Phases I, II & III clinical trials includes acquisition and identification testing of incoming

materials (gas, lipids, excipients or non-active ingredients, etc.), drafting the master batch record, compounding, filling, labeling and release testing.

All the way through the process scale-up to generate larger batches for commercialization purposes, the process development should yield a product satisfying the CQAs identified during formulation development activities and clinical trials. In order to mitigate the risk and ensure the microbubble performances in terms of safety and efficacy, it is highly recommended to use QbD (Quality by Design) chemometric approach (in contrast to conventional empirical methodology) and to gain better knowledge on the correlation between critical material attributes (CMAs), critical process parameters (CPPs) and the product CQAs. Establishing an extended design space will enable more flexible regulatory approaches (Yu 2008).

Although not new, data integrity is gaining increased attention at different levels of the pharmaceutical product development. In fact, regulatory bodies such FDA and MHRA have released new cGMP guidelines emphasizing the central role of data integrity to ensure that the end-product meets the required quality standards over its entire life cycle. In this respect, computerized systems must be validated to guarantee data accuracy, completeness and consistency. Therefore, pharma professionals including manufacturing and quality management are asked to make certain seamless documentation of all information pertaining to products and processes from collection and storage of the data to its destruction.

Pharmacodynamics, Pharmacokinetics and toxicology

Regulatory approval of new drugs commonly involves preclinical, clinical, and post-marketing phases. The main objective of preclinical study activities is to assess the pharmacokinetic and

safety profile and the pharmacodynamics of the contrast agent. To anticipate clinical trials in human subjects and ensure patient safety, *in vitro* and *in vivo* experiments are performed in compliance with GLP regulations in order to determine maximum tolerated dose of diagnostic agent and identify possible adverse effects. At this stage, the appropriate selection of relevant animal models is fundamental to ensure seamless transition to the clinic. The maximum tolerated dose is determined through dose escalation approach. To demonstrate the effectiveness of the enhancing agent, a diagnostic index can be used to describe the ratio of the dose causing toxicity and the dose eliciting a signal enhancement.

Pharmacokinetic studies are meaningful to establish dosing regimens and develop dose-concentration vs. response relationships. After intravenous injection of the agent at different doses, the pharmacokinetic profile is acquired to assess dose dependency, plasma clearance and elimination pathway. Pharmacokinetics and bioavailability for microbubble studies are also performed within a GLP environment using validated bioanalytical procedures [10-11] (FDA (Food and Drug Administration) 2018; Medicines Agency 2011). The blood kinetic and elimination of the gas can be accomplished by gas monitoring in blood and exhaled air analysis using highly sensitive GC-MS methods (Li et al. 2017; Schneider et al. 2011). In parallel, by radiolabeling key moieties of the microbubbles (e.g. phospholipid), a mass balance can be achieved through the collection and testing of blood, urine and fecal samples.

Preclinical *in vivo* toxicology studies, starting with a single dose followed by a repeated doses approach, enables the identification of a suitable and safe starting dose for clinical trials. Other GLP experiments for the evaluation of chronic toxicity, reproductive and developmental toxicity,

carcinogenicity and genotoxicity, are carried out during the preclinical phase of development, depending on the application purpose. Based on preclinical studies outcome, clinical phases I, II and III are conducted to further demonstrate the safety and efficacy of the imaging agent in healthy subjects and patients.

CONCLUDING REMARKS

In addition to exciting developments in targeted imaging in the diagnostic field, there is a broad wave of advances in applying microbubbles to various therapeutic applications, notably in the delivery of drugs across the blood brain barrier and in the treatment of challenging solid tumours. Other new agents are currently under development such as sub-micrometer liquid droplets, and gas entrapping nanoparticles, monodisperse agents, and phase-change contrast agents with promising therapeutic applications. Bringing these new agents from bench to bedside requires some specific expertise in terms of large-scale cGMP manufacturing, pharmaceutical design, quality and risk management, regulation and preclinical and clinical assessment. The modern pharmaceutical market is however characterized by increased drug development cost, shorter product life cycles and global competition. A new development pathway is therefore a necessity, and close collaboration between ultrasound and pharmaceutical companies is essential. Finally, close interaction with regulatory bodies is key to ensure a seamless translation of these new agents from laboratory bench to patient bedside.

Acknowledgements

The authors gratefully acknowledge funding from the UK Engineering and Physical Sciences Research Council (grants EP/I021795 and EP/LO24012) and US National Institutes of Health (grant R01CA195051).

References:

- Abbaspourrad A, Duncanson WJ, Lebedeva N, Kim SH, Zhushma AP, Datta SS, Dayton PA, Sheiko SS, Rubinstein M, Weitz DA. Microfluidic fabrication of stable gas-filled microcapsules for acoustic contrast enhancement. *Langmuir* 2013;29:12352–12357.
- Anna SL, Bontoux N, Stone HA. Formation of dispersions using “flow focusing” in microchannels. *Appl Phys Lett* 2003;82:364–366.
- Ao M, Wang Z, Ran H, Guo D, Yu J, Li A, Chen W, Wu W, Zheng Y. Gd-DTPA-loaded PLGA microbubbles as both ultrasound contrast agent and MRI contrast agent--a feasibility research. *J Biomed Mater Res B Appl Biomater* 2010;93:551–6.
- Atchley AA, Prosperetti A. The Crevice Model of Bubble Nucleation. *J Acoust Soc Am* 1989;86:1065–1084.
- Beguin E, Shrivastava S, Dezhkunov N V, McHale AP, Callan JF, Stride E. Direct Evidence of Multibubble Sonoluminescence Using Therapeutic Ultrasound and Microbubbles. *ACS Appl Mater Interfaces* 2019;11:19913–19919.
- Bezagu M, Clarhaut J, Renoux B, Monti F, Tanter M, Tabeling P, Cossy J, Couture O, Papot S, Arseniyadis S. In situ targeted activation of an anticancer agent using ultrasound-triggered release of composite droplets. *Eur J Med Chem Elsevier Masson SAS*, 2017;142:2–7.
- Borden MA. Intermolecular Forces Model for Lipid Microbubble Shells. *Langmuir ACS Publications*, 2018;35:10042–10051.
- Borden MA, Kruse DE, Caskey CF, Zhao S, Dayton PA, Ferrara KW. Influence of lipid shell physicochemical properties on ultrasound-induced microbubble destruction. *IEEE Trans Ultrason Ferroelectr Freq Control* 2005;52:1992–2002.

766 Borden MA, Longo ML. Dissolution behavior of lipid monolayer-coated, air-filled
767 microbubbles: Effect of lipid hydrophobic chain length. *Langmuir* 2002;18:9225–9233.

768 Borden MA, Sarantos MR, Stieger SM, Simon SI, Ferrara KW, Dayton PA. Ultrasound radiation
769 force modulates ligand availability on targeted contrast agents. *Mol imaging Off J Soc Mol*
770 *Imaging* 2006;5:139–147.

771 Borden MA, Song K-H. Reverse engineering the ultrasound contrast agent. *Adv Colloid*
772 *Interface Sci Elsevier*, 2018;262:39–49.

773 Borden MA, Streeter JE, Sirsi SR, Dayton PA. In vivo demonstration of cancer molecular
774 imaging with ultrasound radiation force and buried-ligand microbubbles. *Mol Imaging*
775 2013;12:357–63.

776 Borden MA, Zhang H, Gillies RJ, Dayton PA, Ferrara KW. A stimulus-responsive contrast agent
777 for ultrasound molecular imaging. *Biomaterials* 2008;29:597–606.

778 Bourdeau RW, Lee-Gosselin A, Lakshmanan A, Farhadi A, Kumar SR, Nety SP, Shapiro MG.
779 Acoustic reporter genes for noninvasive imaging of microorganisms in mammalian hosts.
780 *Nature Nature Publishing Group*, 2018;553:86–90.

781 Cai W Bin, Yang HL, Zhang J, Yin JK, Yang YL, Yuan LJ, Zhang L, Duan YY. The Optimized
782 Fabrication of Nanobubbles as Ultrasound Contrast Agents for Tumor Imaging. *Sci Rep*
783 *Nature Publishing Group*, 2015;5.

784 Callot V, Canet E, Brochot J, Viallon M, Humblot H, Briguët A, Tournier H, Crémillieux Y. MR
785 perfusion imaging using encapsulated laser-polarized ³He. *Magn Reson Med* 2001;46:535–
786 540.

787 Carpentier A, Canney M, Vignot A, Reina V, Beccaria K, Horodyckid C, Karachi C, Leclercq D,
788 Lafon C, Chapelon JY, Capelle L, Cornu P, Sanson M, Hoang-Xuan K, Delattre JY, Idbaih

789 A. Clinical trial of blood-brain barrier disruption by pulsed ultrasound. *Sci Transl Med*
790 2016;8:343re2.

791 Castro-Hernández E, van Hove W, Lohse D, Gordillo JM. Microbubble generation in a co-flow
792 device operated in a new regime. *Lab Chip* 2011;11:2023–2029.

793 Chen CC, Borden MA. The role of poly(ethylene glycol) brush architecture in complement
794 activation on targeted microbubble surfaces. *Biomaterials* 2011;32:6579–6587.

795 Chen CC, Sirsi SR, Homma S, Borden MA. Effect of surface architecture on in vivo ultrasound
796 contrast persistence of targeted size-selected microbubbles. *Ultrasound Med Biol*
797 2012;38:492–503.

798 Chen H, Konofagou EE. The size of blood-brain barrier opening induced by focused ultrasound
799 is dictated by the acoustic pressure. *J Cereb Blood Flow Metab* 2014;34:1197–1204.

800 Chomas JE, Dayton P, Allen J, Morgan K, Ferrara KW. Mechanisms of contrast agent
801 destruction. *IEEE Trans Ultrason Ferroelec Freq Contr* 2001;48:232–248.

802 Chomas JE, Dayton PA, May D, Allen J, Klivanov A, Ferrara K. Optical observation of contrast
803 agent destruction. 2000;77:1056–1058.

804 Couture O, Fink M, Tanter M. Ultrasound contrast plane wave imaging. *Ultrason Ferroelectr*
805 *Freq Control IEEE Trans* 2012;59:2676–2683.

806 Cox DJ, Thomas JL. Rapid Shrinkage of Lipid-Coated Bubbles in Pulsed Ultrasound. *Ultrasound*
807 *Med Biol* 2013;39:466–474.

808 Crake C, Owen J, Smart S, Coviello C, Coussios CC, Carlisle R, Stride E. Enhancement and
809 Passive Acoustic Mapping of Cavitation from Fluorescently Tagged Magnetic Resonance-
810 Visible Magnetic Microbubbles In Vivo. *Ultrasound Med Biol* 2016a;42:3022–3036.

811 Crake C, Owen J, Smart S, Coviello C, Coussios CC, Carlisle R, Stride E. Enhancement and

812 Passive Acoustic Mapping of Cavitation from Fluorescently Tagged Magnetic Resonance-
813 Visible Magnetic Microbubbles In Vivo. *Ultrasound Med Biol* 2016b;42:3022–3036.

814 Dayton PA, Rychak JJ. Molecular ultrasound imaging using microbubble contrast agents. *Front*
815 *Biosci* 2007;12:5124–42.

816 De Cock I, Zagato E, Braeckmans K, Luan Y, de Jong N, De Smedt SC, Lentacker I. Ultrasound
817 and microbubble mediated drug delivery: Acoustic pressure as determinant for uptake via
818 membrane pores or endocytosis. *J Control Release* 2015;197:20–28.

819 de Saint Victor M, Barnsley LC, Carugo D, Owen J, Coussios CC, Stride E. Sonothrombolysis
820 with Magnetically Targeted Microbubbles. *Ultrasound Med Biol* 2019;45:1151–1163.

821 Delogu LG, Vidili G, Venturelli E, Menard-Moyon C, Zoroddu MA, Pilo G, Nicolussi P, Ligios
822 C, Bedognetti D, Sgarrella F, Manetti R, Bianco A. Functionalized multiwalled carbon
823 nanotubes as ultrasound contrast agents. *Proc Natl Acad Sci U S A* 2012;109:16612–16617.

824 Dixon AJ, Hu S, Klibanov AL, Hossack JA. Oscillatory Dynamics and In Vivo Photoacoustic
825 Imaging Performance of Plasmonic Nanoparticle-Coated Microbubbles. *Small*
826 2015;11:3066–77.

827 Dollet B, Van Der Meer S, Garbin V, De Jong N, Lohse D, Versluis M. Nonspherical
828 oscillations of ultrasound contrast agent microbubbles. *Ultrasound Med Biol*
829 2008a;34:1465–1473.

830 Dollet B, van Hoeve W, Raven JP, Marmottant P, Versluis M. Role of the Channel Geometry on
831 the Bubble Pinch-Off in Flow-Focusing Devices. *Phys Rev Lett* 2008b;100:34504.

832 Dove JD, Borden MA, Murray TW. Optically induced resonance of nanoparticle-loaded
833 microbubbles. *Opt Lett The Optical Society*, 2014a;39:3732.

834 Dove JD, Mountford PA, Murray TW, Borden MA. Engineering optically triggered droplets for

835 photoacoustic imaging and therapy. *Biomed Opt Express* 2014b;5:4417–27.

836 Dove JD, Murray TW, Borden MA. Enhanced photoacoustic response with plasmonic
837 nanoparticle-templated microbubbles. *Soft Matter* 2013;9:7743–7750.

838 Duncan PB, Needham D. Test of the Epstein-Plesset model for gas microparticle dissolution in
839 aqueous media: effect of surface tension and gas undersaturation in solution. *Langmuir*
840 2004;20:2567–78.

841 Emmer M, Vos HJ, Goertz DE, van Wamel A, Versluis M, de Jong N. Pressure-dependent
842 attenuation and scattering of phospholipid-coated microbubbles at low acoustic pressures.
843 *Ultrasound Med Biol* 2009;35:102–111.

844 Farook U, Stride E, Edirisinghe MJ. Preparation of suspensions of phospholipid-coated
845 microbubbles by coaxial electrohydrodynamic atomization. *J R Soc Interface Royal*
846 *Society*, 2009;6:271–277.

847 FDA (Food and Drug Administration). *Bioanalytical Method Validation Guidance for Industry*
848 *Biopharmaceutics Bioanalytical Method Validation Guidance for Industry*
849 *Biopharmaceutics Contains Nonbinding Recommendations*. 2018.

850 Feigenbaum H, Stone JM, Lee DA, Nasser WK, Chang S. Identification of ultrasound echoes
851 from the left ventricle by use of intracardiac injections of indocyanine green. *Circulation*
852 *Am Heart Assoc*, 1970;41:615–621.

853 Feinstein SB, Ten Cate FJ, Zwehl W, Ong K, Maurer G, Tei C, Shah PM, Meerbaum S, Corday
854 E. Two-dimensional contrast echocardiography. I: in vitro development and quantitative
855 analysis of echo contrast agents. *J Am Coll Cardiol* 1984;3:14–20.

856 Feshitan JA, Boss MA, Borden MA. Magnetic Resonance Properties of Gd(III)-Bound Lipid-
857 Coated Microbubbles and their Cavitation Fragments. *Langmuir* 2012a;28:15336–15343.

858 Feshitan JA, Chen CC, Kwan JJ, Borden MA. Microbubble size isolation by differential
859 centrifugation. *J Colloid Interface Sci* 2009;329:316–324.

860 Feshitan JA, Vlachos F, Sirsi SR, Konofagou EE, Borden MA. Theranostic Gd(III)-lipid
861 microbubbles for MRI-guided focused ultrasound surgery. *Biomaterials* 2012b;33:247–255.

862 Forsberg F, Rawool NM, Merton DA, Liu JB, Goldberg BB. Contrast enhanced vascular three-
863 dimensional ultrasound imaging. *Ultrasonics* 2002. pp. 117–122.

864 Ganán-Calvo AM, Gordillo JM. Perfectly monodisperse microbubbling by capillary flow
865 focusing. *Phys Rev Lett APS*, 2001;87:274501.

866 Garstecki P, Gitlin I, DiLuzio W, Whitesides GM. Formation of monodisperse bubbles in a
867 microfluidic flow-focusing device. *Appl Phys Lett* 2004;85:2649–2651.

868 Geers B, Lentacker I, Sanders NN, Demeester J, Meairs S, De Smedt SC. Self-assembled
869 liposome-loaded microbubbles: The missing link for safe and efficient ultrasound triggered
870 drug-delivery. *J Contr Rel* 2011;152:249–256.

871 Ghosh D, Xiong F, Sirsi SR, Shaul PW, Mattrey RF, Hoyt K. Toward optimization of *in vivo*
872 super-resolution ultrasound imaging using size-selected microbubble contrast agents. *Med*
873 *Phys* 2017;44:6304–6313.

874 Goertz DE, de Jong N, van der Steen AFW. Attenuation and size distribution measurements of
875 Definity and manipulated Definity populations. *Ultrasound Med Biol* 2007;33:1376–1388.

876 Goldberg BB, Liu JB, Burns PN, Merton DA, Forsberg F. Galactose-based intravenous
877 sonographic contrast agent: Experimental studies. *J Ultrasound Med* 1993;12:463–470.

878 Gorce JM, Arditi M, Schneider M. Influence of Bubble Size Distribution on the Echogenicity of
879 Ultrasound Contrast Agents. *Invest Radiol* 2000;35:661–671.

880 Gramiak R, Shah PM. Echocardiography of the aortic root. *Invest Radiol LWW*, 1968;3:356–

881 366.

882 Grayburn P. Perflenenpent emulsion (echogen®): A new long-acting phase-shift agent for contrast
883 echocardiography. Clin Cardiol Wiley, 1997;20:12–18.

884 Hernandez C, Nieves L, de Leon AC, Advincula R, Exner AA. Role of Surface Tension in Gas
885 Nanobubble Stability Under Ultrasound. ACS Appl Mater Interfaces 2018;10:9949–9956.

886 Hettiarachchi K, Dayton P, Lee AP. Formulation of Monodisperse Contrast Agents in
887 Microfluidic Systems for Ultrasonic Imaging. 2006;230–232.

888 Hettiarachchi K, Lee AP, Zhang S, Feingold S, Dayton PA. Controllable microfluidic synthesis
889 of multiphase drug-carrying lipospheres for site-targeted therapy. Biotechnol Prog
890 2009;25:938–945.

891 Hitchcock KE, Caudell DN, Sutton JT, Klegerman ME, Vela D, Pyne-Geithman GJ, Abruzzo T,
892 Cyr PEP, Geng YJ, McPherson DD, Holland CK. Ultrasound-enhanced delivery of targeted
893 echogenic liposomes in a novel ex vivo mouse aorta model. J Control Release
894 2010;144:288–295.

895 Hsiung SK, Chen CT, Lee G Bin. Micro-droplet formation utilizing microfluidic flow focusing
896 and controllable moving-wall chopping techniques. J Micromechanics Microengineering
897 2006;16:2403–2410.

898 Hyvelin JM, Gaud E, Costa M, Helbert A, Bussat P, Bettinger T, Frinking P. Characteristics and
899 Echogenicity of Clinical Ultrasound Contrast Agents: An in Vitro and in Vivo Comparison
900 Study. J Ultrasound Med John Wiley and Sons Ltd., 2017;36:941–953.

901 ICH. ICH guideline Q11 on development and manufacture of drug substances (chemical entities
902 and biotechnological/biological entities). 2003a.

903 ICH. ICH Topic Q 1 A (R2) Stability Testing of new Drug Substances and Products Step 5 Note

904 for Guidance on Stability Testing: Stability Testing of New Drug Substances and Products.
 905 2003b.

906 Janssen KGH, Li J, Hoang HT, Tas NR, Linden HJ Van Der, Hankemeier T. Downscaling
 907 Quantitative Isotachophoresis : Limits at the Sub-Picoliter Scale. 2010;1730–1732.

908 Jeannot V. V. Jeannot, A. Helbert, A. Lassus, E. Gaud, T. Segers, C. Botteron, P. Frinking. “In
 909 vivo evaluation of monosize microbubbles: acoustic efficiency and safety”. The 23rd
 910 European Symposium on Ultrasound Contrast Imaging. 23rd Eur Symp Ultrasound Contrast
 911 Imaging <http://www.echocontrast.nl/>, 2018.

912 Jeong HH, Yadavali S, Issadore D, Lee D. Liter-scale production of uniform gas bubbles: Via
 913 parallelization of flow-focusing generators. Lab Chip Royal Society of Chemistry,
 914 2017;17:2667–2673.

915 Jones RM, Deng LL, Leung K, McMahon D, O’Reilly MA, Hynynen K. Three-dimensional
 916 transcranial microbubble imaging for guiding volumetric ultrasound-mediated blood-brain
 917 barrier opening. Theranostics 2018;8:2909–2926.

918 Kabalnov A, Klein D, Pelura T, Schutt E, Weers J. Dissolution of multicomponent microbubbles
 919 in the bloodstream: 1. theory. Ultrasound Med Biol 1998;24:739–749.

920 Kang E, Min HS, Lee J, Han MH, Ahn HJ, Yoon IC, Choi K, Kim K, Park K, Kwon IC.
 921 Nanobubbles from gas-generating polymeric nanoparticles: ultrasound imaging of living
 922 subjects. Angew Chem Int Ed Engl 2010;49:524–528.

923 Karshafian R, Bevan PD, Williams R, Samac S, Burns PN. Sonoporation by Ultrasound-
 924 Activated Microbubble Contrast Agents: Effect of Acoustic Exposure Parameters on Cell
 925 Membrane Permeability and Cell Viability. Ultrasound Med Biol 2009;35:847–860.

926 Ke H, Xing Z, Zhao B, Wang J, Liu J, Guo C, Yue X, Liu S, Tang Z, Dai Z. Quantum-dot-

927 modified microbubbles with bi-mode imaging capabilities. *Nanotechnology*
 928 2009;20:425105.

929 Kim DH, Klibanov AL, Needham D. The influence of tiered layers of surface-grafted
 930 poly(ethylene glycol) on receptor-ligand-mediated adhesion between phospholipid
 931 monolayer-stabilized microbubbles and coated glass beads. *Langmuir* 2000;16:2808–2817.

932 Klibanov. Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging.
 933 *Adv Drug Deliv Rev* 1999;37:139–157.

934 Klibanov AL. Ultrasound Contrast Agents: Development of the Field and Current Status.
 935 2002;73–106.

936 Klibanov AL. Ligand-carrying gas-filled microbubbles: Ultrasound contrast agents for targeted
 937 molecular imaging. *Bioconjug Chem* 2005;16:9–17.

938 Klibanov AL. Ultrasound molecular imaging with targeted microbubble contrast agents. *J Nucl*
 939 *Cardiol* 2007;14:876–884.

940 Kok MP, Segers T, Versluis M. Bubble sorting in pinched microchannels for ultrasound contrast
 941 agent enrichment. *Lab Chip* 2015;15:3716–3722.

942 Kokhuis TJ, Garbin V, Kooiman K, Naaijken BA, Juffermans LJ, Kamp O, van der Steen AF,
 943 Versluis M, de Jong N. Secondary Bjerknes forces deform targeted microbubbles.
 944 *Ultrasound Med Biol* 2013;39:490–506.

945 Kopechek JA, Haworth KJ, Raymond JL, Mast TD, Perrin SR, Klegerman ME, Huang SL,
 946 Porter TM, McPherson DD, Holland CK. Acoustic characterization of echogenic liposomes:
 947 Frequency-dependent attenuation and backscatter. *J Acoust Soc Am* 2011;130:3472–3481.

948 Koshkina O, Lajoie G, Baldelli Bombelli F, Swider E, Cruz LJ, White PB, Schweins R, Dolen
 949 Y, van Dinther EAW, van Riessen NK, Rogers SE, Fokkink R, Voets IK, van Eck ERH,

950 Heerschap A, Versluis M, de Korte CL, Figdor CG, de Vries IJM, Srinivas M. Multicore
 951 Liquid Perfluorocarbon-Loaded Multimodal Nanoparticles for Stable Ultrasound and 19 F
 952 MRI Applied to In Vivo Cell Tracking. *Adv Funct Mater Wiley-VCH Verlag*,
 953 2019;29:1806485.

954 Kremkau FW, Gramiak R, Carstensen EL, Shah PM, Kramer DH. Ultrasonic detection of
 955 cavitation at catheter tips. *Am J Roentgenol Radium Ther Nucl Med* 1970;110:177–183.

956 Kripfgans OD, Fowlkes JB, Miller DL, Eldevik OP, Carson PL. Acoustic droplet vaporization
 957 for therapeutic and diagnostic applications. *Ultrasound Med Biol* 2000;26:1177–1189.

958 Kwan JJ, Borden MA. Microbubble Dissolution in a Multigas Environment. 2010;26:6542–
 959 6548.

960 Kwan JJ, Borden MA. Lipid monolayer mechanics during microbubble gas exchange. *Soft*
 961 *Matter* 2012;8:4756–4766.

962 Kwan JJ, Myers R, Coviello CM, Graham SM, Shah AR, Stride E, Carlisle RC, Coussios CC.
 963 Ultrasound-Propelled Nanocups for Drug Delivery. *Small* 2015;11:5305–5314.

964 Lajoinie G, Lee J-Y, Owen J, Kruizinga P, de Jong N, van Soest G, Stride E, Versluis M. Laser-
 965 driven resonance of dye-doped oil-coated microbubbles: Experimental study. *J Acoust Soc*
 966 *Am Acoustical Society of America (ASA)*, 2017;141:4832–4846.

967 Lee SS, Park JW, Pelet S, Hegemann B, Jeon NL, Peter M. Microfluidic-Based Assay Platform
 968 for Studying Polarization Mechanism of Budding Yeast Under Gradient of Mating
 969 Pheromone. *14th Int Conf Miniaturised Syst Chem Life Sci* 2010. pp. 309–311.

970 Lee TM, Oldenburg AL, Sitafalwalla S, Marks DL, Luo W, Toublan FJ-J, Suslick KS, Boppart
 971 SA. Engineered microsphere contrast agents for optical coherence tomography. *Opt Lett*
 972 2003;28:1546–8.

973 Leighton TG. The Acoustic Bubble. Academic Press, 1994.

974 Li MK, Fogler HS. Acoustic emulsification. Part 2. Breakup of the large primary oil droplets in a
 975 water medium. J Fluid Mech 1978a;88:513–528.

976 Li MK, Fogler HS. Acoustic emulsification. Part 1. The instability of the oil-water interface to
 977 form the initial droplets. J Fluid Mech 1978b;88:499–511.

978 Li P, Hoppmann S, Du P, Li H, Evans PM, Moestue SA, Yu W, Dong F, Liu H, Liu L.
 979 Pharmacokinetics of Perfluorobutane after Intra-Venous Bolus Injection of Sonazoid in
 980 Healthy Chinese Volunteers. Ultrasound Med Biol Elsevier USA, 2017;43:1031–1039.

981 Lin F, Tsuruta JK, Rojas JD, Dayton PA. Optimizing Sensitivity of Ultrasound Contrast-
 982 Enhanced Super-Resolution Imaging by Tailoring Size Distribution of Microbubble
 983 Contrast Agent. Ultrasound Med Biol Elsevier USA, 2017;43:2488–2493.

984 Liu Y, Feshitan JA, Wei M-Y, Borden MA, Yuan B. Ultrasound-modulated fluorescence based
 985 on fluorescent microbubbles. J Biomed Opt 2014;19:085005.

986 Mannaris C, Bau L, Grundy M, Gray M, Lea-Banks H, Seth A, Teo B, Carlisle R, Stride E,
 987 Coussios CC. Microbubbles, Nanodroplets and Gas-Stabilizing Solid Particles for
 988 Ultrasound-Mediated Extravasation of Unencapsulated Drugs: An Exposure Parameter
 989 Optimization Study. Ultrasound Med Biol 2019;45:954–967.

990 Mannaris C, Teo BM, Seth A, Bau L, Coussios C, Stride E. Gas-Stabilizing Gold Nanocones for
 991 Acoustically Mediated Drug Delivery. Adv Healthc Mater 2018;7:1800184.

992 Matsunaga TO, Sheeran PS, Luo S, Streeter JE, Mullin LB, Banerjee B, Dayton PA. Phase-
 993 Change nanoparticles using highly volatile perfluorocarbons: Toward a platform for
 994 extravascular ultrasound imaging. Theranostics. 2012. pp. 1185–1198.

995 McEwan C, Owen J, Stride E, Fowley C, Nesbitt H, Cochrane D, Coussios CC, Borden M,

996 Nomikou N, McHale AP, Callan JF. Oxygen carrying microbubbles for enhanced
 997 sonodynamic therapy of hypoxic tumours. *J Control Release* 2015;203:51–56.
 998 Medicines Agency E. 2** Committee for Medicinal Products for Human Use (CHMP) Guideline
 999 on bioanalytical method validation. 2011.
 1000 Meng Z, Zhou X, She J, Zhang Y, Feng L, Liu Z. Ultrasound-Responsive Conversion of
 1001 Microbubbles to Nanoparticles to Enable Background-Free in Vivo Photoacoustic Imaging.
 1002 *Nano Lett* 2019;19:8109–8117.
 1003 Millard TP, Endrizzi M, Everdell N, Rigon L, Arfelli F, Menk RH, Stride E, Olivo A. Evaluation
 1004 of microbubble contrast agents for dynamic imaging with x-ray phase contrast. *Sci Rep*
 1005 Nature Publishing Group, 2015;5.
 1006 Millard TP, Endrizzi M, Rigon L, Arfelli F, Menk RH, Owen J, Stride E, Olivo A. Quantification
 1007 of microbubble concentration through x-ray phase contrast imaging. *Appl Phys Lett*
 1008 2013;103.
 1009 Minnaert M. On musical air-bubbles and the sound of running water. *Philos Mag* 1933;16:235–
 1010 248.
 1011 Mullin L, Gessner R, Kwan J, Kaya M, Borden MA, Dayton PA. Effect of anesthesia carrier gas
 1012 on in vivo circulation times of ultrasound microbubble contrast agents in rats. *Contrast*
 1013 *Media Mol Imaging* 2011;6:126–131.
 1014 Mulvana H, Browning RJ, Luan Y, De Jong N, Tang MX, Eckersley RJ, Stride E.
 1015 Characterization of contrast agent microbubbles for ultrasound imaging and therapy
 1016 research. *IEEE Trans Ultrason Ferroelectr Freq Control* 2017;64:232–251.
 1017 O’Brien J-P, Ovenden N, Stride E. Accounting for the stability of microbubbles to multi-pulse
 1018 excitation using a lipid-shedding model. *J Acoust Soc Am* 2011;130:EL180–EL185.

1019 Overvelde M, Garbin V, Sijl J, Dollet B, de Jong N, Lohse D, Versluis M. Nonlinear shell
 1020 behavior of phospholipid-coated microbubbles. *Ultrasound Med Biol* 2010;36:2080–2092.
 1021 Owen J, Crake C, Lee JY, Carugo D, Beguin E, Khrapitchev AA, Browning RJ, Sibson N, Stride
 1022 E. A versatile method for the preparation of particle-loaded microbubbles for multimodality
 1023 imaging and targeted drug delivery. *Drug Deliv Transl Res* 2018;8:342–356.
 1024 Parhizkar M, Stride E, Edirisinghe M. Preparation of monodisperse microbubbles using an
 1025 integrated embedded capillary T-junction with electrohydrodynamic focusing. *Lab Chip*
 1026 *Royal Society of Chemistry*, 2014;14:2437–2446.
 1027 Pene F, Courtine E, Cariou A, Mira J-P. Toward theragnostics. *Crit Care Med* 2009;37:S50-8.
 1028 Peyman SA, Abou-Saleh RH, McLaughlan JR, Ingram N, Johnson BRG, Critchley K, Freear S,
 1029 Evans JA, Markham AF, Coletta PL, Evans SD. Expanding 3D geometry for enhanced on-
 1030 chip microbubble production and single step formation of liposome modified microbubbles.
 1031 *Lab Chip The Royal Society of Chemistry*, 2012;12:4544–4552.
 1032 Postema M, Marmottant P, Lancée CT, Hilgenfeldt S, Jong N De. Ultrasound-induced
 1033 microbubble coalescence. *Ultrasound Med Biol* 2004a;30:1337–1344.
 1034 Postema M, Van Wamel A, Lancee CT, De Jong N. Ultrasound-induced encapsulated
 1035 microbubble phenomena. *Ultrasound Med Biol* 2004b;30:827–840.
 1036 Rapoport NY, Gao Z, Kennedy A. Multifunctional nanoparticles for combining ultrasonic tumor
 1037 imaging and targeted chemotherapy. *J Natl Cancer Inst* 2007;99:1095–1106.
 1038 Raymond JL, Luan Y, Peng T, Huang SL, McPherson DD, Versluis M, de Jong N, Holland CK.
 1039 Loss of gas from echogenic liposomes exposed to pulsed ultrasound. *Phys Med Biol*
 1040 2016;61:8321–8339.
 1041 Roovers S, Segers T, Lajoinie G, Deprez J, Versluis M, De Smedt SC, Lentacker I. The Role of

1042 Ultrasound-Driven Microbubble Dynamics in Drug Delivery: From Microbubble
1043 Fundamentals to Clinical Translation. *Langmuir* American Chemical Society,
1044 2019;35:10173–10191.

1045 Sboros V, McDicken WN, Koutsos V. Nanomechanical probing of microbubbles using the
1046 atomic force microscope. 2007;46:349–354.

1047 Schneider M. Characteristics of SonoVue. *Echocardiography* 1999;16:743–746.

1048 Schneider M, Anantharam B, Arditi M, Bokor D, Broillet A, Bussat P, Fouillet X, Frinking P,
1049 Tardy I, Terrettaz J, Senior R, Tranquart F. BR38, a new ultrasound blood pool agent.
1050 *Invest Radiol* 2011;46:486–94.

1051 Segers T, de Jong N, Versluis M. Uniform scattering and attenuation of acoustically sorted
1052 ultrasound contrast agents: Modeling and experiments. *J Acoust Soc Am* 2016a;140:2506–
1053 2517.

1054 Segers T, de Rond L, de Jong N, Borden M, Versluis M. Stability of Monodisperse
1055 Phospholipid-Coated Microbubbles Formed by Flow-Focusing at High Production Rates.
1056 *Langmuir* 2016b;32:3937–44.

1057 Segers T, de Rond L, de Jong N, Borden M, Versluis M. Stability of monodisperse phospholipid-
1058 coated microbubbles formed by flow-focusing at high production rates. *Langmuir*
1059 2016c;32:3937–3944.

1060 Segers T, Gaud E, Versluis M, Frinking P. High-precision acoustic measurements of the non-
1061 linear dilatational elasticity of phospholipid coated monodisperse microbubbles. *Soft Matter*
1062 Royal Society of Chemistry, 2018a;14:9550–9561.

1063 Segers T, Kruizinga P, Kok MP, Lajoinie G, de Jong N, Versluis M. Monodisperse Versus
1064 Polydisperse Ultrasound Contrast Agents: Non-Linear Response, Sensitivity, and Deep

1065 Tissue Imaging Potential. *Ultrasound Med Biol* Elsevier USA, 2018b;44:1482–1492.

1066 Segers T, Kruizinga P, Kok MP, Lajoinie G, de Jong N, Versluis M. Monodisperse versus
 1067 polydisperse ultrasound contrast agents: non-linear response, sensitivity, and deep tissue
 1068 imaging potential. *Ultrasound Med Biol* 2018c;44:1482–1492.

1069 Segers T, Lassus A, Bussat P, Gaud E, Frinking P. Improved coalescence stability of
 1070 monodisperse phospholipid-coated microbubbles formed by flow-focusing at elevated
 1071 temperatures. *Lab Chip* Royal Society of Chemistry, 2019;19:158–167.

1072 Segers T, Lohse D, Versluis M, Frinking P. Universal equations for the coalescence probability
 1073 and long-term size stability of phospholipid-coated monodisperse microbubbles formed by
 1074 flow-focusing. *Langmuir* 2017;33:10329–10339.

1075 Segers T, Versluis M. Acoustic bubble sorting for ultrasound contrast agent enrichment. *Lab*
 1076 *Chip* 2014;14:1705–1714.

1077 Seo HK, Kim HO, Kim YJ. Hydrodynamics and Magnetophoresis Based Hybrid Blood.
 1078 2010;223–225.

1079 Seo M, Matsuura N. Monodisperse, submicrometer droplets via condensation of microfluidic-
 1080 generated gas bubbles. *Small* 2012;8:2704–2714.

1081 Shapiro MG, Goodwill PW, Neogy A, Yin M, Foster FS, Schaffer D V., Conolly SM. Biogenic
 1082 gas nanostructures as ultrasonic molecular reporters. *Nat Nanotechnol* Nature Publishing
 1083 Group, 2014;9:311–316.

1084 Sheeran P, Luo S, Dayton PA, Matsunaga T. Formulation and acoustic studies of new phase-
 1085 shift agent for diagnostic and therapeutic ultrasound. *Langmuir* 2011;27:10412–10420.

1086 Sheeran PS, Streeter JE, Mullin L, Matsunaga TO, Dayton PA. Ultrasound molecular imaging
 1087 with customizable nanoscale phase-change contrast agents: An in-vitro feasibility study.

1088 IEEE Int Ultrason Symp IUS 2012. pp. 2309–2312.

1089 Sheeran PS, Yoo K, Williams R, Yin M, Foster FS, Burns PN. More Than Bubbles: Creating
 1090 Phase-Shift Droplets from Commercially Available Ultrasound Contrast Agents. *Ultrasound*
 1091 *Med Biol* 2017;43:531–540.

1092 Shih CP, Chen HC, Chen HK, Chiang MC, Sytwu HK, Lin YC, Li SL, Shih YF, Liao AH, Wang
 1093 CH. Ultrasound-aided microbubbles facilitate the delivery of drugs to the inner ear via the
 1094 round window membrane. *J Control Release* 2013;167:167–174.

1095 Shih R, Lee AP. Post-formation shrinkage and stabilization of microfluidic bubbles in lipid
 1096 solution. *Langmuir*, 2016;32:1939–1946.

1097 Slagle CJ, Thamm DH, Randall EK, Borden MA. Click Conjugation of Cloaked Peptide Ligands
 1098 to Microbubbles. *Bioconjug Chem* 2018;29:1534–1543.

1099 Smeenge M, Tranquart F, Mannaerts CK, de Reijke TM, van de Vijver MJ, Laguna MP, Pochon
 1100 S, de la Rosette JJMCH, Wijkstra H. First-in-Human Ultrasound Molecular Imaging With a
 1101 VEGFR2-Specific Ultrasound Molecular Contrast Agent (BR55) in Prostate Cancer: A
 1102 Safety and Feasibility Pilot Study. *Invest Radiol* 2017;52:419–427.

1103 Sontum P, Kvåle S, Healey AJ, Skurtveit R, Watanabe R, Matsumura M, Østensen J. Acoustic
 1104 Cluster Therapy (ACT) - A novel concept for ultrasound mediated, targeted drug delivery.
 1105 *Int J Pharm Elsevier*, 2015;495:1019–1027.

1106 Stieger SM, Dayton PA, Borden MA, Caskey CF, Griffey SM, Wisner ER, Ferrara KW. Imaging
 1107 of angiogenesis using Cadence™ contrast pulse sequencing and targeted contrast agents.
 1108 *Contrast Media Mol Imaging* 2008;3:9–18.

1109 Straub JA, Chickering DE, Church CC, Shah B, Hanlon T, Bernstein H. Porous PLGA
 1110 microparticles: AI-700, an intravenously administered ultrasound contrast agent for use in

1111 echocardiography. *J Control Release* 2005;108:21–32.

1112 Stride E, Porter C, Prieto AG, Pankhurst Q. Enhancement of Microbubble Mediated Gene
 1113 Delivery by Simultaneous Exposure to Ultrasonic and Magnetic Fields. *Ultrasound Med*
 1114 *Biol* 2009;35:861–868.

1115 Stride E, Saffari N. Microbubble ultrasound contrast agents: A review. *Proc Inst Mech Eng Part*
 1116 *H J Eng Med* 2003;217:429–447.

1117 Sutton JT, Raymond JL, Verleye MC, Pyne-Geithman GJ, Holland CK. Pulsed ultrasound
 1118 enhances the delivery of nitric oxide from bubble liposomes to ex vivo porcine carotid
 1119 tissue. *Int J Nanomedicine* 2014;9:4671–4683.

1120 Szebeni J. Complement activation-related pseudoallergy: A stress reaction in blood triggered by
 1121 nanomedicines and biologicals. *Mol. Immunol.* Elsevier Ltd, 2014. pp. 163–173.

1122 Talu E, Hettiarachchi K, Powell RJ, Lee AP, Dayton PA, Longo ML. Maintaining
 1123 Monodispersity in a Microbubble Population Formed by Flow-Focusing. *Langmuir*
 1124 2008;24:1745–1749.

1125 Tartis MS, Kruse DE, Zheng H, Zhang H, Kheirrolomoom A, Marik J, Ferrara KW. Dynamic
 1126 microPET imaging of ultrasound contrast agents and lipid delivery. *J Control Release NIH*
 1127 *Public Access*, 2008;131:160–6.

1128 Thomas DH, Butler M, Pelekasis N, Anderson T, Stride E, Sboros V. The acoustic signature of
 1129 decaying resonant phospholipid microbubbles. *Phys Med Biol* 2013;58:589–599.

1130 Ueguchi T, Tanaka Y, Hamada S, Kawamoto R, Ogata Y, Matsumoto M, NAKAMURA H,
 1131 JOHKO T. Air Microbubbles as MR Susceptibility Contrast Agent at 1.5 Tesla. *Magn*
 1132 *Reson Med Sci* 2006;5:147–150.

1133 Unger E, Fritz T, Shen DK, Lund P, Sahn D, Ramaswami R, Matsunaga T, Yellowhair D, Kulik

1134 B. Gas filled lipid bilayers as imaging contrast agents. *J Liposome Res Informa Healthcare*,
1135 1994;4:861–874.

1136 Unger E, Porter T, Lindner J, Grayburn P. Cardiovascular drug delivery with ultrasound and
1137 microbubbles. *Adv Drug Deliv Rev* 2014;72:110–126.

1138 Upadhyay A, Dalvi S V., Gupta G, Khanna N. Effect of PEGylation on performance of protein
1139 microbubbles and its comparison with lipid microbubbles. *Mater Sci Eng C Elsevier Ltd*,
1140 2017;71:425–430.

1141 van der Meer S, Dollet B, Voormolen M, Chin CT, Bouakaz A, de Jong N, Versluis M, Lohse D.
1142 Microbubble spectroscopy of ultrasound contrast agents. *J Acoust Soc Am* 2007;121:648–
1143 656.

1144 van Hoeve W, Dollet B, Versluis M, Lohse D. Microbubble formation and pinch-off scaling
1145 exponent in flow-focusing devices. *Phys Fluids* 2011;23:92001.

1146 van Rooij T, Skachkov I, Beekers I, Lattwein KR, Voorneveld JD, Kokhuis TJA, Bera D, Luan
1147 Y, van der Steen AFW, de Jong N, Kooiman K. Viability of endothelial cells after
1148 ultrasound-mediated sonoporation: Influence of targeting, oscillation, and displacement of
1149 microbubbles. *J Control Release* 2016;238:197–211.

1150 Viti J, Mori R, Guidi F, Versluis M, de Jong N, Tortoli P. Nonlinear oscillations of deflating
1151 bubbles. *IEEE Trans Ultrason Ferroelec Freq Contr* 2012;59:2818–2824.

1152 Vlaskou D, Mykhaylyk O, Pradhan P, Bergemann C, Klivanov AL, Hensel K, Schmitz G, Plank
1153 C. Magnetic Microbubbles: Magnetically Targeted and Ultrasound-Triggered Vectors for
1154 Gene Delivery In Vitro. *Hum Gene Ther* 2010;21:1191.

1155 Willmann JK, Bonomo L, Testa AC, Rinaldi P, Rindi G, Valluru KS, Petrone G, Martini M, Lutz
1156 AM, Gambhir SS. Ultrasound molecular imaging with BR55 in patients with breast &

1157 ovarian lesions: First-in-human results. *J Clin Oncol American Society of Clinical*
 1158 *Oncology*, 2017;35:2133–2140.

1159 Wilson K, Homan K, Emelianov S. Biomedical photoacoustics beyond thermal expansion using
 1160 triggered nanodroplet vaporization for contrast-enhanced imaging. *Nat Commun* 2012;3.

1161 Yang F, Li Y, Chen Z, Zhang Y, Wu J, Gu N. Superparamagnetic iron oxide nanoparticle-
 1162 embedded encapsulated microbubbles as dual contrast agents of magnetic resonance and
 1163 ultrasound imaging. *Biomaterials* 2009;30:3882–3890.

1164 Yu LX. Pharmaceutical quality by design: Product and process development, understanding, and
 1165 control. *Pharm Res* 2008;25:781–791.

1166 Yumita N, Nishigaki R, Umemura K, Umemura S -i. Hematoporphyrin as a Sensitizer of Cell-
 1167 damaging Effect of Ultrasound. *Japanese J Cancer Res* 1989;80:219–222.

1168 Zhang G, Harput S, Lin S, Christensen-Jeffries K, Leow CH, Brown J, Dunsby C, Eckersley RJ,
 1169 Tang MX. Acoustic wave sparsely activated localization microscopy (AWSALM): Super-
 1170 resolution ultrasound imaging using acoustic activation and deactivation of nanodroplets.
 1171 *Appl Phys Lett American Institute of Physics Inc.*, 2018;113.

1172 Zhang J, Chen Y, Deng C, Zhang L, Sun Z, Wang J, Yang Y, Lv Q, Han W, Xie M. The
 1173 Optimized Fabrication of a Novel Nanobubble for Tumor Imaging. *Front Pharmacol*
 1174 2019;10.

1175 Zhao Y, Zhu YC. Synergistic cytotoxicity of low-energy ultrasound and innovative mesoporous
 1176 silica-based sensitive nanoagents. *J Mater Sci* 2014;49:3665–3673.

1177 Ziskin MC, Bonakdarpour A, Weinstein DP, Lynch PR. Contrast agents for diagnostic
 1178 ultrasound. *Invest Radiol* 1972;7:500–505.

1179
 1180

Figure Legends

Figure 1: Overview of the different classes of agents for ultrasound imaging and therapy. The white scale bar represents 1 μm . (Image of echogenic liposomes reproduced with permission from (Hitchcock et al. 2010) © 2016, Elsevier); image of nanobubbles reproduced with permission from (Zhang et al. 2019) © 2019 Zhang, Chen, Deng, Zhang, Sun, Wang, Yang, Lv, Han and Xie; image of solid nuclei reproduced with permission from (Kwan et al. 2015)© Wiley 2015).

Figure 2: Overlays of contrast-specific CPS (green) and B-mode (grey) ultrasound scans of HUVEC cells incubated with targeted PCNs: Prior to activation, no contrast-specific echogenicity is detected, suggesting no microbubbles have formed. After exposure to a mechanical index of 1.1 at 8 MHz, targeted droplets vaporize to the highly-echogenic microbubble state and remain in the plane of the HUVEC cells. Reprinted with permission from (Sheeran et al. 2012) © 2012 IEEE.

Figure 3: Conjugation strategies for microbubbles agents used for molecular imaging and/or therapeutic delivery applications. Reproduced with permission from (Mulvana et al. 2017) © 2017 IEEE.

Figure 4: Examples of multi-modality imaging of microbubbles. a. Photoacoustic image of gold nanoparticles conjugated to lipid microbubbles within a murine tumour (reproduced with permission from (Meng et al. 2019) © 2019 American Chemical Society). b. MR image of murine tumour following injection of lipid microbubbles containing iron oxide nanoparticles

(reproduced with permission from (Crake et al. 2016b) © 2016, Elsevier). c. Time series of phase contrast X-ray images of tube containing a suspension of polymeric microbubbles with diminishing concentration (reproduced with permission from (Millard et al. 2015) © 2015, Springer Nature. d. Maximum intensity projection PET image of a mouse following injection of radiolabelled microbubbles (reproduced with permission from Tartis et al. 2008 Copyright © 2008 Elsevier B.V).

Figure 5: a. Flow-focusing microfluidic chip used to produce monodisperse phospholipid-coated microbubbles (reproduced with permission from Segers et al. 2017 © 2017, American Chemical Society). b. Microfluidic chip designed for the production of multi-layer bubbles (reproduced with permission from Hettiarachchi et al. 2009 © 2019 American Institute of Chemical Engineers) c. parallelized microfluidic chip (reproduced with permission from (Jeong et al. 2017) © Royal Society of Chemistry) d. SEM image of a silica particle-coated microbubble (reproduced with permission from (Lee et al. 2010)© 2013 Royal Society of Chemistry) e. Hard shell microbubbles (reproduced with permission from (Shih et al. 2013)© 2013 Elsevier B.V.) f. lipid-coated microbubble (reproduced with permission from (Seo and Matsuura 2012) © 2013 Wiley) g. droplet as microbubbles precursors produced using microfluidics (reproduced with permission from (Abbaspourrad et al. 2013) © 2013 American Chemical Society).