Ultrasound Mediated Therapies for the Treatment of Biofilms in Chronic Wounds: A Review of Present Knowledge

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Abstract

Bacterial biofilms are an ever-growing concern for public health; featuring both inherited genetic resistance and a conferred innate tolerance to traditional antibiotic therapies. Consequently, there is a growing interest in novel methods of drug delivery, in order to increase the efficacy of antimicrobial agents. One such method is the use of acoustically activated microbubbles, which undergo volumetric oscillations and collapse upon exposure to an ultrasound field. This facilitates physical perturbation of the biofilm, and provides the means to control drug delivery both temporally and spatially. In line with current literature in this area, this review offers a rounded argument for why ultrasound-responsive agents could be an integral part of advancing wound care. To achieve this, we will outline the development and clinical significance of biofilms in the context of chronic infections. We will then discuss current practices used in combating biofilms in chronic wounds, and then critically evaluate the use of acoustically activated gas microbubbles as an emerging treatment modality. Moreover, we will introduce the novel concept of microbubbles carrying biologically active gases that may facilitate biofilm dispersal.

The bacterial biofilm: development and aetiology

Though the microbial world is vastly diverse, the development of a biofilm remains perhaps the most ubiquitous means by which microbial cells can thrive within their given

environment (Wu *et al.*, 2015; Flemming *et al.*, 2016). A biofilm can be described as a localised aggregation of microorganisms in a heterogeneous, sessile community, embedded in a dynamic matrix of extracellular polymeric substances (EPS) (Singh *et al.*, 2017). From biogeochemical cycling in the ecosystem and the human microbiome, to biofouling and disease; biofilms are simultaneously an essential part of life and a prominent concern for industry and public health (Donlan, 2002; Flemming *et al.*, 2016; Kuliasha *et al.*, 2017). The gross architecture of the biofilm is complex; proteomic investigation has shown that at least in *Pseudomonas aeruginosa* biofilm development is regimented and sequential *(Fig. 1)* (Hall-Stoodley *et al.*, 2004). Though the specific stages of biofilm development have not been characterised for each prokaryotic organism individually, it should be noted that the development archetype is markedly conserved *in vitro* (Figueiredo *et al.*, 2017; Lohse *et al.*, 2017; Bartell *et al.*, 2018).

(FIGURE 1)

The initial stages of biofilm formation are characterised by an impermanent association of planktonic cells with a surface, which is superseded by a robust adhesion and the production of EPS (Hall-Stoodley *et al.*, 2004; Holt *et al.*, 2017). Although proteins, carbohydrates, lipids, DNA, RNA and water are staple constituents of the EPS (Taglialegna *et al.*, 2016), there is a notable variability in the quantity of each macromolecule present between biofilms (Flemming *et al.*, 2007). The mucoid phenotype of *P. aeruginosa* is found extensively in the cystic fibrosis lung, and usually arises from the overproduction of the exopolysaccharide alginate (Schurr, 2013). This serves as a good example of the individual differences seen in the EPS of biofilms, exemplifying that variation can be both species specific and influenced by the immediate environment (Maleki *et al.*, 2016). Adherent cells will then begin to propagate and assemble into micro-colonies; the initiation of this process is attributed to monomeric adhesins (Jamal *et al.*, 2015). Aggregation and micro-colony formation in *P. aeruginosa* is attributed to the Type IV pili surface adhesin, whereas in *Staphylococcus aureus* it is thought to be induced by polysaccharide intercellular adhesin (Persat *et al.*, 2015; Maleki *et al.*, 2016).

Maturation of a biofilm into its distinctive three dimensional structure, is achieved through sophisticated cell-cell communication termed quorum sensing (Laganenka and Sourjik, 2017). Quorum sensing is facilitated by the production of auto-inducer signalling molecules, which allow microbial cells to determine cell density and collectively adjust gene expression in response (Rutherford and Bassler, 2012). This ensures the formation of an encapsulating extracellular matrix, with water-filled channels for the transport and exchange of nutrients and waste products within the biofilm (Parsek and Singh, 2003). Programmed dispersal of microbial cells is the final stage of biofilm development, which is achieved by either the release of newly formed cells from biofilm aggregates, or detachment of constituent peripheral cells by species specific saccharolytic enzymes (Marsh and Zaura, 2017). Erosion and sloughing may also occur due to mechanical (i.e. shear) stress upon the biofilm, which causes peripheral cells to disengage indiscriminately from the biofilm and enter the local environment (Rmaile *et al.*, 2014; Jamal *et al.*, 2018). Though dispersed cells regain motility they remain physiologically unique from cells in the planktonic and biofilm phase; these dispersed cells are highly virulent in nature towards macrophages, which is a useful attribute given their main purpose is the colonisation of new sites (Chua *et al.*, 2014). Dispersion represents one of the most virulent stages in the biofilm lifecycle, but perhaps also one of the easiest to target and thus potentiate killing of microbial cells (Hall and Mah, 2017).

Significance of biofilms in chronic wound infections

The causality between pathogenic microorganisms and infection has been understood for over a century; yet most research into the pathogenesis of microorganisms has focused solely on acute infection by planktonic cells. Over the last decade this focus has shifted; greater emphasis is now placed on the role of multi-drug resistant (MDR) organisms and biofilms, which mediate over 90% of chronic wound infections (Attinger and Wolcott, 2012; Bjarnsholt, 2013). The augmented persistence of biofilms can be attributed in part to inherited genetic antibiotic resistance traits, which actively reduce the efficacy of an administered antimicrobial agent. This commonly includes the use of membrane associated efflux pumps,

which prevent antimicrobial agents reaching lethal intracellular concentrations; and antibiotic degradation enzymes such as beta-lactamase, which alter the pharmacokinetic properties of beta-lactam antibiotics (Høiby *et al.*, 2011; Geisinger and Isberg, 2017). However, of particular concern is that when compared to their planktonic counterparts, it has been conclusively shown that the biofilm phenotype confers an innate physical tolerance to antimicrobial agents (Hengzhuang *et al.*, 2012; Algburi *et al.*, 2017). It is also noted that the extracellular polysaccharides within the dynamic EPS matrix potentiate this effect by acting as a shield, compromising the ability of the host immune system to detect the biofilm infection (Limoli *et al.*, 2015; Kumar *et al.*, 2017). Moreover, the production of extracellular toxins and lytic enzymes facilitates destruction of local immune cells, which provides a source of cellular components that can be utilised by microbial cells (Cooper *et al.*, 2014).

It is evident that current treatment options available for biofilms are both limited in availability and effectiveness; thus, seeking to resolve a chronic infection by eradicating a competent biofilm formed in a wound bed is a multifactorial challenge. In response to a wound derived from acute trauma, tissue will undergo a sequential process of reparation that results in the reinstitution of anatomical integrity (Clark, 1993). Under normal physiological conditions, the progression of wound healing can be broadly categorised into four phases: haemostasis, inflammation, proliferation and remodelling (Guo and DiPietro, 2010). The phases within the process of wound repair are not mutually exclusive, but involve dynamic integration of cellular processes that overlap temporally (Robson, 2004). Diabetic foot ulcers (DFU) are a prevalent example of a chronic wound; commonly arising from comparatively minor trauma to the foot, they have been strongly associated with substantial morbidity and mortality (Walsh *et al.*, 2016). The key pathophysiological differences between a common acute wound and a chronic wound is that the latter is typically associated with recalcitrant infection, ischaemia of the tissue, and a prolonged or arrested inflammatory phase (Wolcott *et al.*, 2008). One of the hallmarks of a chronic wound is high microbial burden and diversity, which is routinely attributed to the formation of poly-microbial drug-resistant biofilms in the wound bed (Banu *et*

al., 2015). Given the pathogenicity and associated virulence factors of biofilms, there is credible evidence that they are implicit in preventing normal mechanisms of wound healing (Malone *et al.*, 2017). It is reported that every 30 seconds worldwide, there is a lower-limb amputation as a direct result of DFU (Yazdanpanah *et al.*, 2015). This is undoubtedly accompanied by significant physical and emotional stress, as well as an increase in mortality rate (Costa *et al.*, 2017). In addition, there is an undeniable economic burden associated with the cost of health care, from disease management to major intervention (Walsh *et al.*, 2016).

Current treatment strategies for biofilms in chronic wounds

The most established treatment for the removal of necrotic tissue and biofilms from chronic wounds is sharp debridement, but this mechanical method of biofilm disruption lacks both efficiency and effectiveness (Cooper *et al.*, 2014; Yazdanpanah *et al.*, 2015). Although the debridement of chronic wounds in clinical trials is largely concurrent with a reduction in the surface area of a wound, the period over which intervention is required is typically in the order of weeks to months and does not significantly correlate with complete wound closure (Williams *et al.*, 2005; Rhoads *et al.*, 2008; Wolcott *et al.*, 2009; Yazdanpanah *et al.*, 2015). Studies have shown that debridement can expedite wound healing by stimulating re-epithelisation of the tissue, however complete healing is typically observed in less than 20% of patients (Cardinal *et al.*, 2009; Kim *et al.*, 2018). The presence of persister cells allows the regeneration of the biofilm within the wound bed, which means that debridement is by no means a complete or permanent solution (Lebeaux *et al.*, 2014). The efficacy of debridement can be improved by chemical and biological adjuvants, such as hydrogen peroxide and enzymes respectively (Watters *et al.*, 2016). By causing the EPS matrix of the biofilm to degrade, and thus removing its principal means of protection and nutrition, the rate of wound healing is significantly increased (Kim *et al.*, 2018). The physical perturbation of the biofilm caused by debriding has also been shown to temporarily restore antibiotic sensitivity; as the biofilm begins to regenerate, key antibiotic targets such as cell-wall synthesis (glycopeptides) and protein synthesis (aminoglycosides) become viable (Wolcott *et al.*, 2009; Hall and Mah, 2017).

Quorum sensing is an important regulator of biofilm development; it is the principal means by which microbial cells communicate within a given environment (Miller and Bassler, 2001; Rutherford and Bassler, 2012). The ability of microbial cells to carry out such sophisticated communication is a potent advantage, therefore quorum sensing can also be considered as a valuable therapeutic target (Singh *et al.*, 2017). By incapacitating this signalling mechanism, the regulation of gene expression, essential metabolic processes and virulence can be irreparably altered (Khmel, 2006). Natural and synthetic inhibitors of quorum sensing such as furanones and Manuka honey, work by downregulating four major quorum sensing genes, which in turn has downstream consequences for genes associated with the biofilm phenotype (Wang *et al.*, 2012; Jakobsen *et al.*, 2018). However, it is important to note this is principally a method to render a biofilm more inert; it does not offer an immediate solution to detachment or physical eradication (LaSarre and Federle, 2013).

An additional means of controlling biofilms in chronic wounds is impeding their attachment to surfaces; one way in which this has been achieved is with the iron chelating glycoprotein, lactoferrin (García-Montoya *et al.*, 2012). Lactoferrin is an important endogenous antimicrobial component of the innate immune system; it is principally found in tears, saliva, mucous secretions, and breast milk of mammals (Cooper *et al.*, 2014). Its most potent properties include sequestering iron essential for bacterial motility, and direct interaction with Gram-negative bacterial cell walls to induce lysis at the site of infection (Valenti *et al.*, 2015). This consequently means that its bacteriostatic effect can prevent biofilm development, but also disrupt cells which have already become adherent. Lactoferrin has the additional benefit of exhibiting anti-inflammatory properties, which may play an important role in mitigating chronic inflammation associated with delayed wound healing (Valenti *et al.*, 2017). Allison *et al.* (2015) showed that as a component of breast milk, lactoferrin at a concentration of 3 mg/mL significantly decreased S*treptococcus mutans* biofilm formation *in vitro.* The artificial sweetener xylitol has been shown to bind to Gram-positive organisms, preventing the organism from adhering to other cells or surfaces (Ferreira *et al.*, 2015). The co-administration

of lactoferrin and xylitol has shown great efficacy in eliminating *Pseudomonas aeruginosa* biofilms *in vitro* (Ammons *et al.*, 2011). The acquisition of iron by lactoferrin causes membrane disruption, while xylitol prevents *P. aeruginosa* biofilms successfully responding to the environmental change (Rhoads *et al.*, 2008). This treatment modality has been successfully implemented for the treatment of chronic wounds; a lactoferrin and xylitol hydrogel, in conjunction with a silver wound dressing, were shown to achieve a significant log reduction in *Staphylococcus aureus* and *P. aeruginosa* biofilm viability of 6·5 ± 2·4 and 4·9 ± 0·9, respectively (Ammons *et al.*, 2011). However, this method is comparably only as efficacious as sharp debridement and still requires treatment over a number of weeks to months, but may provide a means to prevent recalcitrant and exacerbated infection (Kim *et al.*, 2018). By avoiding mechanisms of microbial resistance, quorum sensing inhibition, bacteriostasis and adhesion prevention, are pertinent alternatives to traditional antimicrobial therapies. To meet the multi-factorial challenge presented by biofilms in chronic wounds, this review proposes that gas-filled microbubbles (MBs) can be the versatile biomedical-tool required.

Gas microbubbles: A method of controlled drug delivery

The architecture of gas-filled microbubbles is variable according to their intended application, though they commonly consist of a surfactant, polymer, protein or phospholipid shell, which encapsulates a gaseous-core *(Fig. 2)* (Sirsi and Borden, 2009; Owen *et al.*, 2018). The composition of the MB shell is integral to conferring mechanical stability, preventing coalescence, and determining its acoustic response to stimulation by ultrasound (US) (Borden *et al.*, 2005; Stride, 2008; Carugo *et al.*, 2017). Characterising MB size is an important step not only for determining its acoustic response and drug-loading capacity, but also its longevity in circulation and thereby its safety for *in vivo* applications (M. Lee *et al.*, 2015). MBs are typically manufactured with a diameter distribution in the range between 1 – 10 µm, and the mean MB diameter during storage increases over time (Ferrara *et al.*, 2007). Notably, there exists a pressure difference between the inside of a MB and the outer environment (known as Laplace pressure), which is caused by the surface tension of the curved gas-liquid interface.

For a given MB shell formulation, the Laplace pressure is inversely proportional to MB radius. Therefore, gas diffuses from the smaller bubbles to the larger ones, leading to dissolution and disappearance of the smaller bubbles. It is well documented that factors such as shell composition, fabrication method used, the relative chemical environment, and temperature can substantially influence MB size and its temporal evolution (Mulvana *et al.*, 2010; Sun *et al.*, 2014; M. Lee *et al.*, 2015; Taylor *et al.*, 2017).

Phospholipids are perhaps the most common excipients of the MB shell; key examples include distearoyl phosphatidylcholine (DSPC) and dipalmitoyl phosphatidylcholine (DPPC) (Swanson *et al.*, 2010). The lipid molecules assemble in a monolayer at the hydrophilic-hydrophobic interface to stabilise the gas core, exposing their hydrophilic polar head to the aqueous environment and their hydrophobic tails towards the gaseous core *(Fig. 2)*. The acyl chain length of a lipid dictates the permeability of the MB shell to gases (Borden, 2016); longer hydrophobic acyl chains provide greater cohesion (or packing density) between adjacent lipids (Hosny *et al.*, 2013), consequently reducing MB shell permeability to gases and increasing its stability during storage and upon administration (Zhuang *et al.*, 2016). Surfactants can be integrated into the MB shell, which is particularly important for biomedical applications of MBs such as drug delivery (Abou-Saleh *et al.*, 2014). The addition of the surfactant polyethylene glycol (PEG) to a MB shell, simultaneously provides the means to functionalise the MB shell with biological components or assemblies such as targeting ligands, antibodies and liposomes (Abou-Saleh *et al.*, 2014), whilst mitigating immunogenicity to lipid antigens and preventing phagocytosis of the MB by macrophages (Paolino *et al.*, 2017). The content of the MB gaseous core, is arguably just as important as the composition of the encapsulating shell, as it dictates some of the MB properties (Vohra and Jasuja, 2016). High molecular weight perfluorocarbon compounds and sulphur hexafluoride are commonly used as the gaseous core for MBs *in vivo*; the poor water solubility and low diffusion rate of fluorinated gases prolongs MB longevity by enhancing stability (Casini *et al.*, 2016; Carugo *et al.*, 2017). The drug loading capacity of a MB is strongly linked to the efficacy of the treatment, as it directly impacts the amount of a

given therapeutic agent that can be delivered to a target site (Tzu-Yin *et al.*, 2013). The use of electrostatic force to bond drugs to the MB surface (Rychak and Klibanov, 2014), insertion into the MB shell (Lentacker *et al.*, 2009), loading the drug into the gaseous void and placing a layer of oil at the interface between the gaseous core and MB shell (Tinkov *et al.*, 2009), are all considered low-capacity methods of drug loading (Sirsi and Borden, 2009). To yield a greater MB drug loading capacity, it is common for nanoparticles or liposomes to be conjugated to MBs covalently or with the use of biotin-avidin bridges (Lentacker *et al.*, 2010; Liang *et al.*, 2018). However, methods of achieving a greater drug loading capacity also directly impact upon the stability and acoustic properties of the MB, due to their altered shell thickness and composition (Tzu-Yin *et al.*, 2013; Kooiman *et al.*, 2014). Drug loading modalities, methods of microbubble fabrication, and their biophysical effects have been extensively reviewed elsewhere (Unger *et al.*, 2002; Stride and Edirisinghe, 2008; Fix *et al.*, 2015; M. Lee *et al.*, 2015).

(FIGURE 2)

Ultrasound mediated physical effects of gas microbubbles

Manipulation of the MB fabrication method and shell composition dictates their physico-chemical properties and size, allowing them to elicit different modes of action in response to acoustic stimulation. These ultrasound-mediated behaviours of the MBs can be further regulated by adjusting the parameters of the US delivered (e.g. frequency, acoustic pressure, duration, pulse repetition frequency); this makes MBs perhaps one of the most versatile tools available in biomedicine. In biomedical applications of MBs their efficacy for a given task is correlated to their acoustic response (Datta *et al.*, 2006; Choi *et al.*, 2014), which is typically categorised into either stable or inertial cavitation (Lentacker *et al.*, 2014). In response to the pressure changes of ultrasound waves, MBs experience alternating volumetric compression and expansion (or rarefaction) (De Jong *et al.*, 2002). Upon exposure to low-intensity US, the nature of these oscillations is typically repetitive over several US cycles; this behaviour is referred to as stable cavitation (Stride and Coussios, 2009). Above a

critical US intensity, the periodicity of this oscillation is lost; MBs expand rapidly, and the inertia of the surrounding fluid during contraction causes them to collapse violently (Wu and Nyborg, 2008; Stride and Coussios, 2009). This process is known as inertial or transient cavitation, and often leads to MB fragmentation into smaller bubbles. It should be noted that while there is a simple relationship between US intensity and pressure for a plane travelling wave, it is more complex in 2D fields and standing wave fields. The propensity for fragmentation of lipid-shelled MBs is inversely correlated to the phospholipid alkyl chain length, and also depends upon the type of emulsifier used (Borden *et al.*, 2005). Notably, the MB resonant frequency and amplitude of oscillation, the transition pressure from stable to inertial cavitation, and MB fragmentation diameter all strongly depend upon the initial MB size (for a given shell formulation and suspension medium) (Povey and Mason, 1998; Borden *et al.*, 2005). Therefore, the selection of a specific US frequency to elicit a desired acoustic response should take into account the typically broad size distribution of a MB suspension, and variations in MB size during sample storage and/or handling.

Concerning the bio-physical effects of MBs, it has been postulated that the systematic expansion and compression of MBs in stable cavitation creates localised pushing and pulling forces, which in turn cause disruption to the integrity of cell membranes located in their vicinity (*Fig*. 3B) (J. Y. Lee *et al.*, 2015). It is also possible for MB oscillation to drive a steady flow of the surrounding fluid (also known as cavitation micro-streaming). The volumetric oscillation of MBs generates flows that are purely divergent (i.e., radial), while interaction with a dissimilar surface (e.g., a target tissue) can generate a circulatory flow that enhances shear stress over nearby cells, potentially causing transmembrane pores to form (*Fig*. 3A) (Ferrara *et al.*, 2007). Furthermore, the streaming flow field can drive shedding of shell constituents - such as therapeutic compounds - away from a MB (Luan *et al.*, 2014), which in turn can be exploited as a mechanism to deposit (or ‘print’) therapeutic material over the membrane of target cells (De Cock *et al.*, 2016). Micro-jet formation and shockwaves are more transient physical effects attributed to the collapse of MBs in inertial cavitation, which respectively puncture proximal

membranes and increase membrane permeability through mechanical stress (*Fig.* 3C and 3D) (Collis *et al.*, 2010). Although inertial cavitation can release energy in the form of heat, this is rapidly dissipated in the surrounding fluid, which has a significantly greater total volume than the volume occupied by MBs (Ye et al., 2013). In comparison to the mechanical stresses imparted by inertial cavitation, it could be inferred that stable cavitation is a comparatively gentle means of facilitating drug uptake.

(FIGURE 3)

The use of ultrasound alone as an anti-biofilm treatment

The ability of low frequency US to improve the efficacy of antibiotics was shown as early as 1994; the minimum inhibitory concentration (MIC) of aminoglycoside and macrolide antibiotics was consistently reduced by up to 50%, in planktonic cultures of *P. aeruginosa* and *S. aureus* exposed to continuous US at 67 kHz and 0.3 W/cm^2 intensity (Pitt *et al.*, 1994). This was later supported in a study by Runyan *et al.* (2006), who demonstrated that US both potentiates antibiotics against planktonic cultures and biofilms by facilitating the transport of antibiotics into biofilms. The prevailing theory is that US increases the permeability of cell membranes to systemically available antibiotics, without physically disrupting or dispersing the biofilm (Mohammad *et al.*, 2015). The efficacy of US as an adjuvant to antibiotics can be supported by a study conducted by Pitt and Ross (2003), which showed that in the absence of antibiotic low frequency US (70 kHz) of < 2 W/cm^2 acoustic intensity increased the growth rate of planktonic *Escherichia coli* and *P. aeruginosa*. It can be reliably deduced that by the same means US potentiates antibiotics by promoting transport across cell membranes, in the absence of antibiotics US may increase the rate at which waste products are removed and metabolites are transported to cells, consequently enhancing their growth.

Investigations conducted *in vivo* on the effect of adjuvant US on biofilms implanted subcutaneously in rabbits, showed that after 24 hours of continuous US exposure (28.48-kHz, 100- and 300-mW/cm^2) recovered *E. coli* colony forming units (CFU) were significantly

reduced, whereas there was no observable effect on *P. aeruginosa* (Rediske *et al.*, 1999). The literature in this area is clear that US alone has no effect on cell viability, though they do lack congruency in the US frequency, intensity, and pulse length or repetition frequency used (Jiang *et al.*, 2016; Cai *et al.*, 2017). The principal limitation of this approach is that it currently lacks translation to a viable clinical application; the most efficacious low-frequency US (28.48-kHz) and intensity (100- and 300-mW/cm^2) used *in vitro,* was shown to induce tissue damage when applied *in vivo* (Rediske *et al.*, 1999; Jiang *et al.*, 2016). It should also be noted that the majority of studies have continuous treatment times of 24-48 hours, which is unlikely to be considered economically or socially practical. Moreover, this method of utilising the synergistic relationship between US and antibiotic efficacy does not allow for targeted or controlled delivery of the antibiotic to a localised region, since it relies on the traditional oral or intravenous administration of antibiotics of sufficient dose to ensure an effective serum concentration. The limitation of this method is that any antibiotic administered in this manner would still have a non-specific impact on unintended systemic targets, which would include dysbiosis of the host commensal microbiota (Carding *et al.*, 2015). Due to the localised release of antibiotics conjugated with microbubbles, the dosage required to achieve an effective local antibiotic concentration is significantly lower than orally or intravenously administered antibiotic (Horsley *et al.*, 2019). In quantitative terms the typical recommended dosage for systemic administration of aminoglycoside and β-lactam antibiotics ranges from 2 – 16 g/day (Taccone *et al.*, 2011), whilst the concentration associated with microbubble administration is typically of MIC (i.e. µg/mL) (Pitt *et al.*, 2004; Zhu *et al.*, 2014). Therefore, the residual serum concentration of antibiotics delivered by microbubbles is much lower, likely reducing or removing non-specific systemic targets.

The application of low-frequency (20-60 kHz) ultrasound for chronic wound debridement has shown great potential, with recent advancements extensively reviewed elsewhere (Chang *et al.*, 2017; Liu *et al.*, 2017). To summarise, ultrasonic debridement has been shown to potentiate antibiotics against bacteria within the chronic wound, emulsify dead

cells within a localised area, and stimulate peripheral healthy cells to promote the healing process. The efficacy of this treatment modality has been assessed in a number of clinical trials, which generally concur that ultrasonic debridement is a valuable wound care adjuvant (Amini *et al.*, 2013; Murphy *et al.*, 2018). It is important to note that although these trials have demonstrated that ultrasonic debridement improves short-term treatment outcomes, the frequency and total duration of the treatment is comparable to non-surgical sharp debridement with no significant difference in healing rate between the modalities after 6 months of treatment (Amini *et al.*, 2013; Michailidis *et al.*, 2018).

Acoustically activated gas microbubbles for the treatment of bacterial biofilms

The success of low frequency US (20–100 kHz) in facilitating the uptake of systemic antibiotics by biofilms has been variable, therefore the use of US responsive MBs in light of their controllable physical response may be viewed as an evolution in antimicrobial drug delivery. Alteration in the permeability of biofilms to macromolecules such as antimicrobial compounds, has been routinely demonstrated with the use of the red-fluorescent nucleic acid stain, propidium iodide (PI). Dong *et al.* (2017) demonstrated that acoustically activated MBs were capable of enhancing the permeability of *Staphylococcus epidermidis* biofilms grown in OptiCell™ chambers *in vitro.* The fluorescent signal emitted increases proportionally with the quantity of PI that has intercalated with DNA, therefore it can be utilised as a direct means of assessing how MBs facilitate uptake of macromolecules (Stiefel *et al.*, 2015). It could be therefore hypothesised that US activated MBs facilitate the uptake of antibiotics by biofilms; principally through increasing membrane permeability of bacterial cells and *via* heterogeneous alterations to the biofilm architecture, which can include the development of pores in the EPS matrix (Ying Dong *et al.*, 2013; Jang *et al.*, 2017; Hu *et al.*, 2018). Fluid shear stress has also been shown to significantly affect biofilm morphology; at shear stresses under 1 Pa biofilms are shown to grow in a lab-typical mushroom-like shape with interstitial channels and voids (Salta *et al.*, 2013). However, when the shear stress overcomes the biofilm adhesion strength (> 2 Pa) erosion and sloughing of the biofilm is more pronounced, which leads to low density,

biofilm aggregates with large interspaces (Rmaile *et al.*, 2014). Shear stress in terms of human physiology is variable according to location; arteries are typically subject to average wall shear stress of 1-3 Pa, whilst in arterioles and capillaries it is commonly of 2-6 Pa (Sheikh *et al.*, 2003; Shaik *et al.*, 2009). The effect of fluid shear stress on the detachment of biofilms has shown to be compounded in the presence of microbubbles, clearing up to 70% of biofilm in a species dependent manner *in vitro* (Sharma *et al.*, 2005). It has been shown that the *in vitro* application of 0.08 MHz, 1.0 W/cm^2, 50% duty cycle, and 10-min duration US, MBs and vancomycin can significantly decrease the number of viable cells (7.17 $\log_{10}$ CFU/mL) from *S. epidermidis* biofilms compared to an untreated control (10.51 $\log_{10}$ CFU/mL) (He *et al.*, 2011). It is important to note however, that this study could not demonstrate a significant difference between these groups using an *in vivo* rabbit model, in which *S. epidermidis* biofilms grown on polyethylene disks were subcutaneously implanted bilateral to the vertebral column. The work carried out by He *et al.* (2011), demonstrated that US activated sulphur hexafluoride MBs with a mean diameter of 2.5 µm and vancomycin create micro-pores within the biofilm architecture, which does support the hypothesis of facilitated uptake mediated by membrane/EPS disruption. An interesting point is that not only does the membrane disruption facilitate antibiotic uptake, but the influx of nutrients may induce a phase of active growing in deeper layers of the biofilm, potentiating the efficacy of the antibiotic (Y. Dong *et al.*, 2013). In addition, studies have consistently shown that the combination of US and MB is capable of halving the MIC of the administered antibiotic (Kasimanickam *et al.*, 2013). This has been supported in recent work by Horsley *et al.* (2019) in which gentamicin loaded liposomes bound to ultrasound-responsive microbubbles, were utilised to significantly enhance direct antibiotic delivery to intracellular uropathogenic bacteria. The ultrasound-mediated delivery of gentamicin in concentrations of 0.53-1.32 µg/mL with a 20 second exposure time, showed an equivalent efficacy in bacterial killing to a two hour exposure to free gentamicin at the significantly higher clinically approved dosage of 200 µg/mL (Horsley *et al.*, 2019). Moreover, the ultrasound-mediated therapy achieved a 75% greater reduction in bacterial bioburden than free gentamicin alone, with no evidence of cellular damage (Horsley *et al.*, 2019). This effect

is perhaps indicative of the mechanical action of the oscillating microbubble, aiding the physical detachment of bacteria from proximal surfaces. This work has exemplified the utility of ultrasound mediated intracellular-delivery of antimicrobial agents, as a viable alternative to the use of orally administered antibiotics. It should not be overlooked that to date, research on enhancing efficacy of US activated microbubbles has only been performed on naive single-species biofilm models. Furthermore, although the biophysical effect of acoustically activated MBs is evident, the underlying mechanisms of interaction between the bubbles and the biofilm have not been elucidated yet.

The applications of nitric oxide for the treatment of bacterial biofilms

Nitric oxide (NO) has been utilised to facilitate healing of chronic wounds such as DFU for a number of years, this is motivated by the role of NO as an important biological signalling molecule (Witte and Barbul, 2002). Cellular proliferation, angiogenesis and remodelling are key biological and physiological processes mediated by NO, which have been principally applied to wounds in the form of inducible NO synthase (Dhall *et al.*, 2014). It is important to note that traditionally NO has only been applied to chronic wounds in the context of tissue repair, and not with the specific intention to treat the underlying biofilm (Saidkhani *et al.*, 2016; Han and Ceilley, 2017). The administration of NO in a therapeutic capacity has historically been difficult; at high concentrations NO is bactericidal and cytotoxic, which significantly impairs the progression of the normal healing process (Schulz and Stechmiller, 2006). Studies of the biofilm life cycle have elucidated endogenous mechanisms which can be exploited as therapeutic targets, these principally involve the use of NO in the sub-micromolar range to induce the biofilms dispersal phase (Barraud *et al.*, 2015). By inducing dispersal of the biofilm with NO, the physical barrier imposed by the EPS matrix can be negated entirely. Research has shown that the dispersed cells are considerably more susceptible to antimicrobial treatments; it can therefore be inferred that adjuvant NO potentiates antibiotics against biofilms (Howlin *et al.*, 2017). Utilising the spontaneous NO donor sodium nitroprusside (SNP), Howlin *et al.* (2017) successfully showed that NO disrupted *P. aeruginosa* biofilms from cystic fibrosis

sputum samples *in vitro*. The same study also highlighted the importance of dispersal as a means of therapy, since the administration of the antibiotic tobramycin alone caused a significant increase in biomass and biofilm thickness compared to untreated controls. Xu *et al.* (2017) have proposed and tested the implementation of NO releasing agents for indwelling medical device surfaces, and they were able to successfully prevent biofilm formation on a functionalised polyurethane surface. Utilising NO to prevent bacterial growth and adhesion on implanted surfaces in conjunction with antimicrobial therapy, may result in better clinical outcomes for patients and significant cost savings for health service providers. There is relatively little published data either *in vivo* or *in vitro,* on the successful implementation of exogenous NO in a gaseous form for biofilm dispersal. The inhalation of NO has been an FDA approved therapeutic agent for nearly two decades, and clinical trials have shown that NO gas at 5-10 ppm can achieve a 3.5 log reduction in respiratory *P. aeruginosa* biofilm aggregates (Howlin *et al.*, 2017). The major problem with the delivery of NO in a gaseous form is that it is highly reactive, which translates to a half-life of only seconds (Thomas *et al.*, 2001). A solution to this challenge is to utilise an US responsive agent such as the MB, which could provide the means to successfully control the delivery and release of NO.

The mechanism of action for nitric oxide induced dispersal of biofilms

The control of dispersal events in the biofilm life cycle are linked to the intracellular second messenger molecule cyclic-di-guanosine monophosphate (c-di-GMP), which is regulated by cellular phosphodiesterase (Reinders *et al.*, 2016). Recent studies have shown that the activity of cellular phosphodiesterase is increased in the presence of NO at concentrations in the pico- and nano-molar range, which results in the degradation of c-di-GMP and subsequent dispersal of the biofilm (Algburi *et al.*, 2017; Howlin *et al.*, 2017). The dispersed cells return to a motile state and become susceptible to antimicrobial treatment, this effect is shown to be conserved across species such as *P. aeruginosa and Staphylococcus aureus* and in both single and complex multispecies biofilms (Barraud *et al.*, 2009). Dispersal

has been achieved with NO concentrations as low as 450 pM, reaching peak efficacy at 450 nM (Howlin *et al.*, 2017).

Biologically active nitric oxide gas microbubbles and their applications

Though high molecular weight gases are most commonly used as the MB core constituent, it is also possible to generate MBs that possess a biologically active gaseous core. Recent research has already highlighted the advantages of using MBs with an oxygen core, to increase the therapeutic efficacy of chemo-sonodynamic therapy (SDT) in the targeted treatment of solid malignant tumours (McEwan *et al.*, 2015; Nesbitt *et al.*, 2018). Bioactive gases such as NO have significant therapeutic potential, as they mediate a number of important biological signal pathways (Basudhar *et al.*, 2016). However, the molecule is highly reactive with both exogenous molecules such as oxygen, as well as endogenous scavengers like haemoglobin (Azarov *et al.*, 2005). This can be mitigated by utilising the gas as the core of acoustically active MBs; protected by the MB shell, the release of NO can then be both spatially and temporally controlled by US *(Fig. 4)* (Fix *et al.*, 2015). The stable expansion and contraction of the MB is an important attribute of its associated biophysical effects, however the effects of cavitation-enhanced gas exchange have been less investigated. During MB compression there is an efflux of core-gas into the local environment, followed by a net influx of gas upon expansion (Crum, 1984; Lentacker *et al.*, 2014; Yusof *et al.*, 2016). This is particularly important in regard to the use of nitric oxide microbubbles (NOMBs), since it shows that the MB has the capacity to deliver a locally concentrated therapeutic NO payload.

(FIGURE 4)

There have been few studies to date, which investigated the use of microbubbles for the delivery of NO. Tong *et al.* (2013) and Wang *et al.* (2013), generated nitric oxide microbubbles (NOMBs) with a mean diameter of 3.85 µm by continuous sonication of a lipid suspension at 100 W. The organophosphorus compound 1,2-Bis(diphenylphosphino)ethane (DPPE), PEG_{2000} and phosphatidylcholine were used to encapsulate NO, which was

administered at a constant stream of 4 mL/min for five minutes under anoxic conditions. In contrast, Grishenkov *et al.* (2015) and Cavalieri *et al.* (2008), used biocompatible polyvinyl alcohol (PVA) and high shear stirring to fabricate air-filled MBs, which were then freeze-dried with liquid nitrogen. The subsequent 4 µm diameter PVA capsules were enclosed in a pressure chamber purged with nitrogen, before loading with NO and re-suspend in water. All four studies focused on the intravascular application of NOMBs, thus their research is linked by some common themes.

The work by Cavalieri *et al.* (2008) was the first *in vitro* study of its kind, utilising NOMBs for localised delivery of NO for the prevention of clot formation. Similarly, the NOMBs developed by Grishenkov *et al.* were implemented in a theranostic capacity for myocardial ischemia, showing that they are a highly effective tool for the localised and targeted delivery of NO. Unlike the work by Tong *et al.* (2013), this study sought to use NOMBs as a prophylaxis for patients at risk of thromboembolism. In a rodent model of myocardial infarction, 60 seconds of continuous US (1 MHz, 1 W/cm^2) applied to NOMBs in conjunction with mesenchymal stem cells, was successfully used to promote angiogenesis (Tong *et al.*, 2013). The applications of this as a successful therapy are promising, with the scope to prevent heart failure by restoring adequate blood flow to damaged cardiac tissue (Cochain *et al.*, 2013). The use of NOMBs without ultrasound for the resolution of deep vein thrombosis was investigated by Wang *et al.* (2013), who successfully showed a reduction in thrombus size by 40% and mitigated development of chronic inflammation.

The research conducted by Grishenkov *et al.* (2015), was the only study to assess the final NOMB gas content and dissolution rate in solution, with and without the application of US (1-15 MHz, <100 kPa). High-performance liquid chromatography (HPLC) was used to measure nitrite and nitrate in both aerated and degassed saline, which are oxidised products of NO. Passive release of NO from degassed saline occurred in 17 minutes and this is increased to 55 minutes in aerated saline; the application of US reduced the exponential time constant to 10 minutes and 4 minutes, respectively (Grishenkov *et al.*, 2015). The assessment

of nitrate and nitrite in solution is concordant with expected parameters of diffusion; NO enters the degassed solution at a faster rate than an aerated solution.

Multifunctional agents for the delivery of nitric oxide to biofilms: present and future perspectives

To the best of the authors' knowledge, only one previous study has investigated the use of a NO releasing particle for the treatment of biofilms. The research carried out by Hetrick *et al.* (2009) investigated the use of NO-releasing silica nanoparticles, however it focused only on the bactericidal efficacy of the NO and not on biofilm dispersal. Consequently, there are a number of unexplored and novel aspects in this area, pertaining specifically to the application of NOMBs for the treatment of bacterial biofilms. There are currently no acoustically stimulated NOMBs used for the treatment of biofilms *in vitro* or *in vivo*, consequently there is currently no evidence on the efficacy of NOMBs or nanoparticle induced dispersal of biofilms. Furthermore, though its effects have been observed, the specific interaction between MBs and the biofilm has not been fully elucidated. Previous research has shown that lipid transfer occurs between MBs and biological membranes, which undoubtedly impacts upon cellular integrity, permeability and signalling (Carugo *et al.*, 2017). This effect could be successfully employed to exert a priming effect upon biofilms, to stimulate disruption of the biofilm prior to the administration of US.

Bacterial infection and subsequent development of biofilms in open wounds, poses a significant risk to human health. Due to the increased tolerance of biofilms to traditional antimicrobial therapies and non-specific drug delivery, interdisciplinary techniques are being explored as novel treatment methods. Ultrasound responsive drug delivery agents provide a dynamic means of delivering therapeutic compounds, with high temporal and spatial specificity. Ongoing research has shown that ultrasound responsive agents can facilitate drug delivery, utilising both bioactive components and mechanical stimulation to eliminate biofilms. There is no clinically viable translation of this treatment modality for chronic wounds at present, perhaps due to the complexity of ensuring consistent and efficacious implementation with

minimal training or expertise. Essential parameters such as the consistent production and administration of MBs, controlled transmission of US, and handling of biohazardous residuum, would require a robust system with clearly defined instructions for use in lay terms. Moreover, due to the lack of congruity in size, shape and depth of wounds, it is likely a successful clinical translation would be primarily targeted at early stages of DFU development for patients presenting with small lesions. This would then have the potential to either prevent or limit biofilm development, in addition to dispersing and treating any adherent cells already present in the wound bed. The transmission of US *via* a fluid stream to biotic and abiotc surfaces for the purpose of biological decontamination, has recently demonstrated that clinical translation and utility in this field is achievable (Birkin *et al.*, 2015, 2016). In order to achieve fundamental change in healthcare practices such as the treatment of chronic wounds, we believe this review exemplifies the need for collaborative and interdisciplinary research to potentiate existing therapies and develop novel treatment modalities.

Conflict of interest

None declared

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Figure Legends

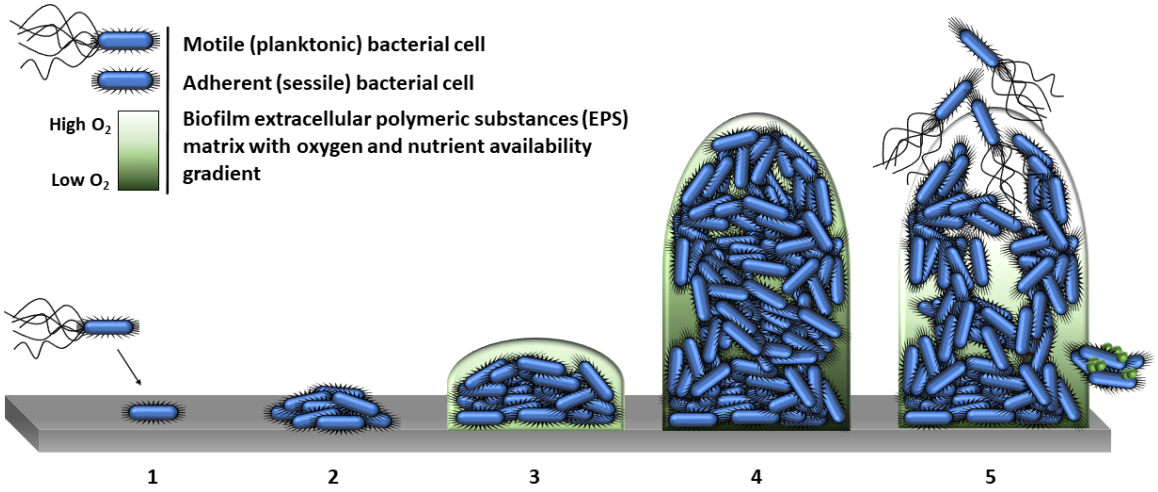

Figure 1 – A simplified schematic representation of the sequential biofilm formation described for *P. aeruginosa*. (1) Initial attachment can be transient, but this association can become robust and lead to the aggregation of cells (2). This stabilised attachment leads to the production of extracellular polymeric substances (EPS), which encapsulate aggregated cells forming micro-colonies (3). The maturation of the biofilm structure is achieved through inter-cellular signalling (4), a mature biofilm commonly features a concentration gradient of oxygen and nutrient availability. Oxygen is consumed by biofilm cells at a faster rate than it can diffuse in, which coincides with the gradient of nutrient availability. Consequently, the majority of metabolically active cells are located at the periphery of the biofilm, whilst persister, dormant or dead cells are found at the biofilm-substratum interface. In its final stages the biofilm undergoes programmed dispersal of cells, which includes cellular mass and EPS sloughing off (5).

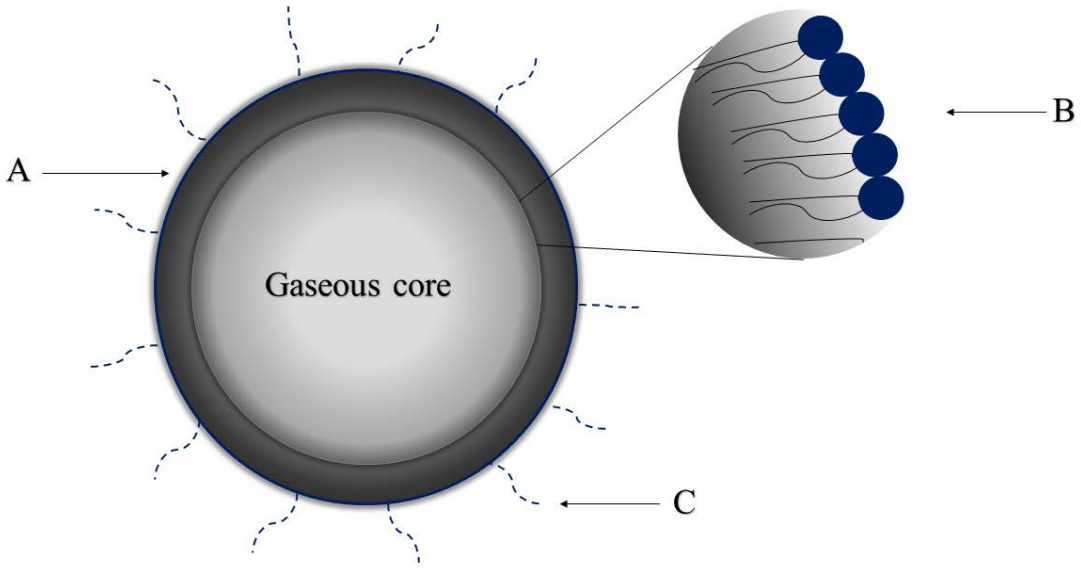

Figure 2 – A schematic representation of a gas microbubble depicting a gaseous core encapsulated by (A) stabilising lipid-shell. An expanded view (B) shows the orientation of the phospholipid monolayer at the gas-liquid interface, with polar heads in contact with the aqueous phase and polar tails internalised towards the gaseous core. The addition of polyethylene glycol surfactant chains is represented by (C).

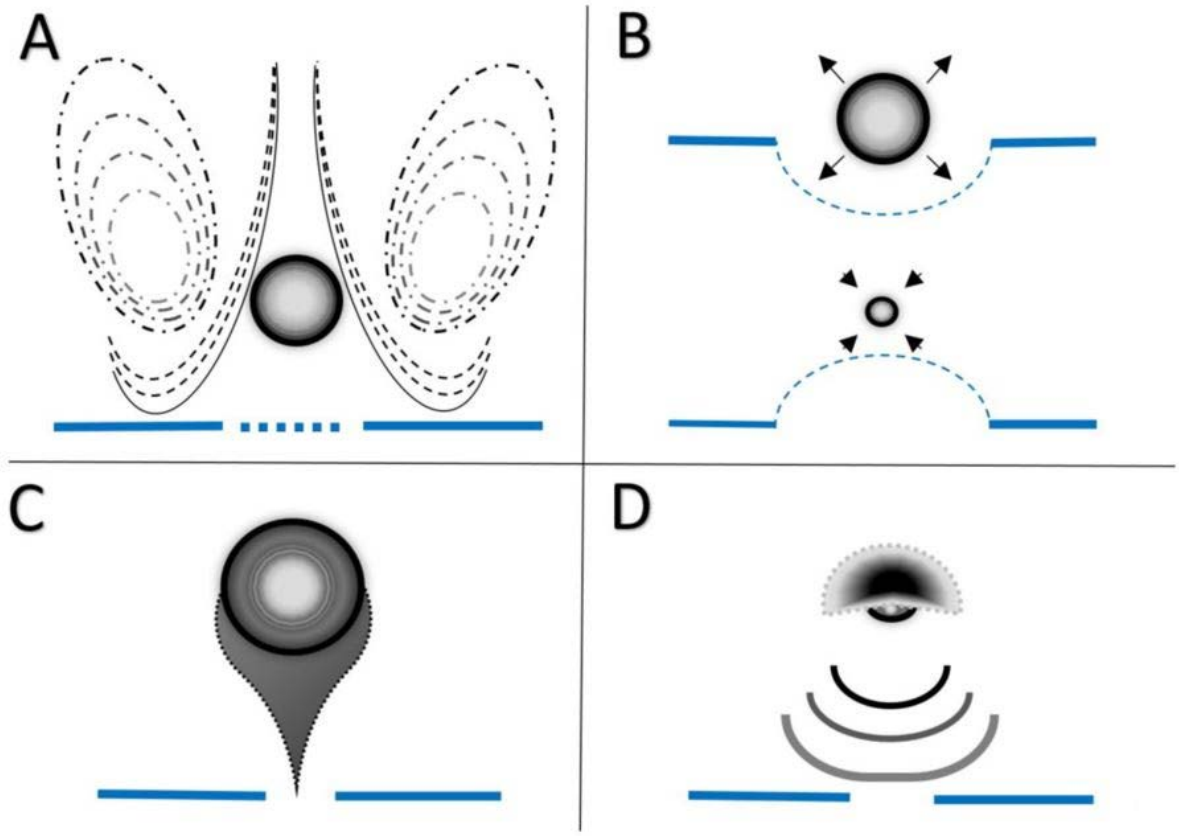

Figure 3 – Examples of the biophysical effects of microbubbles on cell membranes (▬), when exposed to varied ultrasound parameters: (A) Micro-streaming of fluid is generated upon temporally sustained oscillation of the microbubble; the mechanical stress imparted on proximal cell membranes can lead to pore formation. (B) Microbubble pushing upon expansion and pulling upon compression are characteristic effects of stable cavitation; this can increase cell membrane permeability and/or compromise membrane integrity. (C) If a microbubble undergoes inertial cavitation near a surface, the resulting collapse of the microbubble is asymmetrical and leads to the generation of a liquid microjet directed towards the surface. Fluid jetting can cause membrane perforation and thus enhance intracellular transport of therapeutic compounds. (D) In addition to liquid jetting, shock waves can be produced by microbubbles that collapse forcefully. The stress imparted on a cell membrane can equally cause membrane perforation.

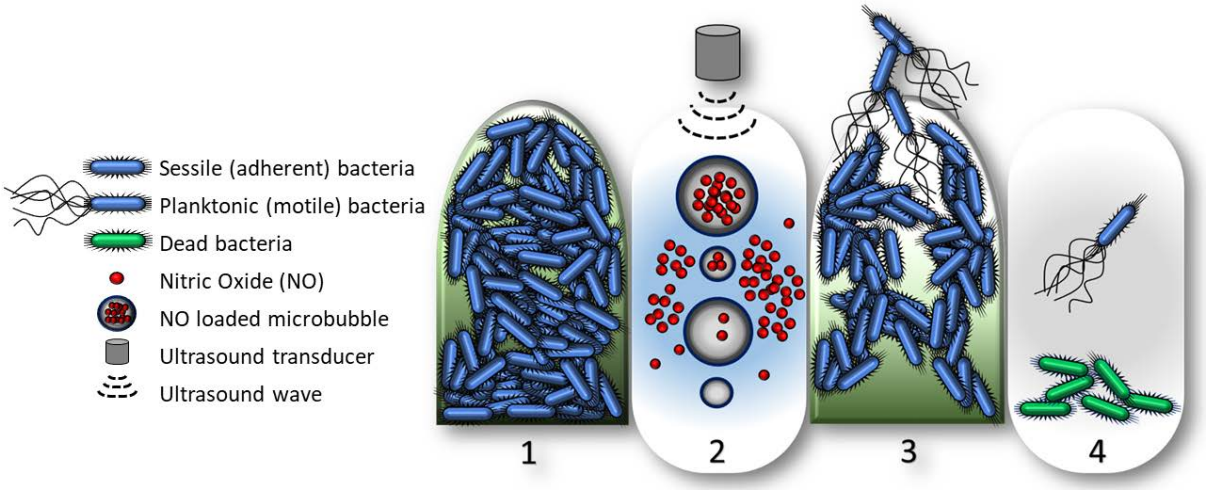

Figure 4 – Gas microbubbles undergoing stable cavitation in response to ultrasound have been shown to enhance membrane permeability; this can increase the local intracellular concentration of a target drug. By encapsulating biologically active nitric oxide as the gaseous core of a shelled microbubble, the combined mechanical action of the oscillating microbubble and biological effect of nitric oxide may induce targeted dispersal and elimination of biofilms from a surface. The co-administration of antibiotic and microbubbles, may provide a novel means of combatting biofilm-associated antibiotic tolerance