**Using Ultrasound to Combat Microorganisms**

Eight decades after the discovery of the first antibiotic, penicillin, bacterial infections have reemerged as a serious threat to human health (Cai 2017). The main reason for antibiotic therapy failure is increased resistance of microorganisms to antibiotics (Serpe 2015; Nisnevitch 2016). Combining different antimicrobial therapeutic strategies is not always effective, even when combinations cover a broad range of activities and exert a synergistic effect. Such combinations can sometimes lead to increased antibiotic use and may accelerate the emergence of drug resistance (lvan 2011; Cai 2017). In addition, bacterial biofilms exhibit heightened antibiotic resistance hundreds and even thousands of times higher than that of planktonic bacteria (Yu 2012). There is increasing attention focusing on alternative large-scale microbial inactivation methods that do not induce microbial resistance, such as irradiation, heat, high pressure, magnetic and electrical fields, and ultrasound (US), (Yu 2012; Serpe 2015). Low-frequency ultrasound (LFU) is a promising method that is both safe and exhibits good tissue penetration without significant energy attenuating (Rosenthal 2004; Cai 2017). The processes during the cavitation bubbles collapse that lead to the damage or destruction of microbial cells, as described by Joyce (2003), include:

1. Gas bubbles exert increased pressure on the bacterial cell wall, leading to its collapse and cell destruction due to mechanical fatigue.

2. Microstreaming induces the action of shear forces within bacterial cells.

3. Free radicals (H˙ and OH˙), formed during cavitation in an aqueous medium, cause the weakening and destruction of the cell wall.

4. Hydrogen peroxide (H2O2), an end product of sonochemical water decomposition, has an antibacterial effect.

In addition to the direct damaging effects on microorganisms, US can synergistically amplify the effectiveness of other antibacterial agents, for example, by increasing cell penetration. Applying US by sonication can also be used to control the release of antimicrobial agents delivered by special means, such as liposomes, into affected tissues. Finally, US can activate sonosensitizer molecules that can form compounds that are toxic to microbes. This review presents the current knowledge concerning the use of applying ultrasound to combat microorganisms.

**1. Direct effect of ultrasound on microbial cells**

**1.a. Effects of ultrasound on planktonic bacterial cells and viruses**

The antimicrobial effect of ultrasound was first demonstrated almost a century ago when algae *Spirogyra* cells were ruptured using high frequency and high-intensive sound waves (Wood 1927). Subsequently, the cell-damaging effect of sonication was shown to extend to many other microorganisms (Harris 2014). Scherba et al. (1991) showed the damaging impact of low-frequency (26 kHz) ultrasound on various types of bacteria, fungi, and viruses in aqueous suspensions. Their study demonstrated that the planktonic bacteria *E. coli, S. aureus, B. subtilis*, and *P. aeruginosa* are destroyed by sonication, dependent upon US exposure duration and intensity. A significant reduction in the growth of the fungus *Trichophyton mentagrophytes* was also demonstrated, also directly correlated with US intensity (Scherba et al. 1991).

Since this research, other studies have reported on the bactericidal effect of US on various bacteria types. A recent review by Lattwein et al. (2020) provides a detailed summary of these studies’ main findings.

In addition to the above-mentioned US-induced effects on bacterial cells (acoustic cavitation, mechanical forces, including shock waves, shear forces, and induced microstreams [Cai 2017]), specific outcomes in different cell types must be taken into account. For this reason, comparing separate studies is problematic, as the tested microorganisms may differ in morphology, metabolism, and defense mechanisms. Differences in cell shape, such as between spherical-shaped versus rod-shaped or kidney-shaped cells, can affect sonication sensitivity, as cell shape is related to the total surface area, cell tension, and rigidity. In addition, the presence of fimbriae or filamentous on the surface of bacteria can protect cells by preventing the contact of cavitation nuclei with the cell wall (Lattwein 2020). Liao (2018) also found that the Gram-positive *S. aureus* is more resistant to US than is the Gram-negative bacteria *E. coli*, indicating that the structural differences in cell envelopes also affect US sensitivity. This result was attributed to the increased thickness, toughness, and strength of Gram-positive bacteria cell envelopes compared to Gram-negative. The spherical *S. aureus* are less sensitive to US treatment than are the rod-shaped *E. coli* (X. Liao 2018). Bacterial cell size, the cell division stage, growth phases, and cell state (planktonic or adherent) can also contribute to US treatment efficacy (Lattwein 2020). The wide variety of reported results on the effect of US treatment on microorganisms can also be due to specific device types (high or low frequency, probe or bath), mode of use (pulse duration, total treatment duration, temperature conditions, etc.), and intensities of US processing.

The Ding research group has recently studied the mechanisms of ultrasonic damage to *E. coli* and *S. aureus* microbial cells using a variety of methods (Li 2016; Liao 2018). They found several target sites within the microbial cell for the destructive action of US, including the outer membrane, the cell wall, the cytoplasmic membrane, and inner structures. They suggest that the primary cellular target depends on the bacteria type: in Gram-negative bacteria, the outer membrane is the primary target, and in Gram-positive bacteria, it is the inner (cytoplasmic) membrane (Li 2016). The type of cell damage can also differ, depending on the location of the cell relative to the zone of acoustic cavitation. Bacteria situated directly within the field of ultrasonic waves are destroyed quickly and entirely due to high-power mechanical forces. The microbial cells that are not within the ​​ultrasonic cavitation area can endure internal injuries, including DNA destruction and enzyme inactivation, without any damage to the cell membranes and walls. These effects may be mediated by free radicals generated during sonication and introduced into the cells via cavitation microjets (Liao 2018).

In yeast, in contrast to bacterial cells, ultrasonic treatment primarily damages the cell wall leading to cellular disruption (Tao 2015).

It is also worth noting the stimulating effect of low-intensity US irradiation on the bacterial growth rate, as discovered and studied by Pitt et al. (1994). They showed that low-frequency, low-intensity US (70 kHz, <2 W/cm2) did not decrease *Staphylococcus epidermidis, Pseudomonas aeruginosa,* and *Escherichia coli* viability, but in fact, increased their growth rate in comparison to untreated control cells. The authors attribute this effect to increased oxygen and nutrients transport to the cells, increased waste removal from the cells, and stress-induced cellular growth following sonication (Pitt 1994; Pitt 2003; Erriu 2014).

Although this review focuses on microorganisms, the antiviral properties of US should also be noted. The target of ultrasonic damage in viruses is the viral envelope, consisting of a membrane with virus-specific glycoproteins. These glycoproteins mediate virus entry by acting as ligands for cell surface receptors so that envelope destruction inactivates the virus. However, viruses with a viral capsid, an outer protein coat, are insensitive to sonication, in contrast to enveloped viruses (Scherba 1991). Indeed, in feline herpesvirus type 1, lesions appeared in the viral envelope following US, with their severity a function of sonication intensity. However, feline calicivirus was not susceptible to US. The physical mechanism of viral inactivation was suggested to be transient (or “collapse”) cavitation (Scherba 1991; Erriu 2014).

**1.b. Ultrasound effect on microbial biofilms**

The topic of the ultrasonic effect on microbial biofilms deserves special attention. Biofilms are formed when communities of microorganisms attach to a solid substrate. They can appear on any moist non-sterile surface (such as teeth and dental implants, wounds, medical instruments, etc.) and cause detrimental health effects like pathogenesis and persistence of nosocomial infections (Costerton 1999). Therefore, it is critical to find a way to remove the biofilms without damaging the surrounding surfaces. Since US can both inactivate planktonic bacteria and deagglomerate bacterial flocs via physical, mechanical, and chemical action of acoustic cavitation (Joyce 2003), it may provide an effective treatment against bacterial biofilms (Erriu 2014, Vyas 2019).

However, the effect of sonication on biofilms is not entirely clear. US's ability to remove bacterial cells from the surface is widely recognized and used in many areas. This effect is associated with the mechanical destruction of the extracellular biofilm matrix, as well as with the destruction of the cells themselves, under low-frequency (40-100 kHz) and high intensity (>10 W/cm2) ultrasonic cavitation conditions (Bigelow 2009; Kirzhner 2009; Erriu 2014). However, low-frequency, low-intensity ultrasonic radiation (≤2 W/cm2) stimulates bacterial metabolism. In this scenario, sonication causes increased transport of oxygen and nutrients into deeper biofilm layers, leading to further biofilm formation that becomes more stable and more firmly attached to the surface (Pitt 2003; Erriu 2014). A detailed review of the effect of US on bacterial biofilm is presented in Erriu (2014), and Vyas (Vyas 2019), where the US mechanism of action on the biofilm is analyzed, and the parameters affecting biofilm removal by US are considered.

**2. Augmenting antibacterial drug effect on bacteria by ultrasound.**

**2.a. Synergistic effect of ultrasound and antibiotics on planktonic bacteria**

In addition to the direct bactericidal effect of US on cells, US treatment enhances the action of antibacterial drugs. Pitt et al. (1994) first demonstrated the synergistic effect of low-intensity US and antibiotics. Low-frequency US (67 kHz), used at subinhibitory levels (0.3 W/cm2), increased gentamicin bactericidal activity on planktonic cultures of Gram-negative *P. aeruginosa* and *E. coli* (Pitt et al. 1994). This effect was later confirmed by Rediske et al. (1998), who studied the combination of a low-frequency (70 kHz) sonication at a non-toxic intensity rate (3 W/cm2) with antibiotics belonging to the aminoglycoside (gentamicin, streptomycin, and kanamycin), tetracycline (tetracycline), and penicillin (ampicillin) families. Their study shows that the combination of antibiotics and ultrasound can significantly increase antibiotic efficacy against both Gram-negative (*Enterobacter aerogenes, Serratia marcescens*, and *Salmonella derby*) and Gram-positive (*Streptococcus mitis* and *Staphylococcus epidermidis*) microorganisms. Several research groups have successfully reproduced this synergistic effect using various antibiotics against different bacteria (Williams 1997; Rediske 2002; Runyan 2006; Si-Feng Shi 2013; Zhu 2014; Selan 2019). The review by Cai summarizes the effect of antibiotics in combination with LFU on planktonic bacteria (Yu 2012; Cai 2017;).

The mechanism underlying the synergistic bactericidal action of US and antibiotics is complex. Rapoport (1997), Rediske (1999), and Runyan (2006) have all shown that low-frequency US increases outer membrane permeability of *P. aeruginosa* cells. At high sonication intensities (>150 mW/cm2), cavitation causes microbubble implosion. However, at low sonication intensities, stable cavitation of microbubbles occurs when the latter oscillates symmetrically in the medium. Such stable oscillations create liquid microstreams around the oscillating bubbles. If this phenomenon occurs close to a cell, shear stress can stimulate pore formation in the cell membrane without impairing cell viability (Runyan 2006; Zhu 2014). This temporary change in cell membrane permeability following low-frequency low-intensity ultrasound is termed “sonoporation” (Ward 1999). Sonoporation manifests as temporary defects in a phospholipid bilayer, thus allowing enhanced penetration of lipophilic substances. Sonoporation can also facilitate hydrophobic antibiotics entrance (e.g., erythromycin) through the microbial cell membrane (Rapoport 1997; Rediske 1999), since the pores formed following US are large enough to allow more antibiotic to enter into the bacterial cell, but are not big enough to cause cell destruction (Zhu 2014). This effect is not permanent, and after the termination of sonication, initial membrane permeability is restored (Cai 2017).

Unlike hydrophobic compounds that penetrate bacterial cells through phospholipid bilayers, hydrophilic antibiotics (e.g., gentamicin) penetrate cells through porin channels, which are not sensitive to ultrasonic processing. Therefore, the synergistic effect between hydrophilic antibiotics and low-frequency US (LFU) in killing Gram-negative bacteria occurs via a different mechanism than that of hydrophobic antibiotics (Rapoport 1997; Cai 2017).

Using electron microscopy, Zhu et al. (2014) demonstrated structural changes in the cell walls of *E. coli* following low-intensity US treatment (average 100 mW/cm2), which led to an increase in cell wall permeability and antibiotic entry. *E. Coli* cell walls exposed to US in the presence of gentamicin were wrinkled and thicker than control samples (Zhu 2014). *S. aureus* cells exhibit a similar phenomenon of bacterial cell wall following low-intensity US treatment (Ayan 2008). At the same time, these researchers noted that US did not affect the sensitivity of bacteria to antibiotics and caused no discernable genetic differences.

The bactericidal activity of fluoroquinolones against *E. coli* is also enhanced by US (Liu 2011). This study showed that 40 kHz sonication-activated fluoroquinolones produced reactive oxygen species (ROS), such as superoxide radical anion (·O2-) and hydroxyl radical (·OH), which then likely enhance the overall bactericidal effect (Liu 2011).

**2.b. Synergistic effect of ultrasound and antibiotics on biofilms**

Combining antimicrobial drugs with US to combat bacterial biofilms can help overcome several factors interfering with antibiotic efficiency on bacterial biofilms. First, the biofilm structure is difficult for antibacterial drugs to penetrate. The internal biofilm environment (oxygen gradient, waste accumulation, etc.) can also negatively affect antibacterial agent activity. Biofilms are encapsulated in a self-produced extracellular polysaccharide matrix, which acts as a physical obstacle for the inflow of antibiotics. In addition, due to decreased nutrients, oxygen, and energy availability, the cells located at the base of a mature biofilm remain dormant, and their metabolic activity is reduced, additionally reducing the rate of antibiotic entry. Together, these factors provide bacterial cells in biofilms with significant resistance to traditional antimicrobial drugs, withstanding antibiotic concentrations 100-1000 times higher than in a planktonic state (Brown 1988; Pitt 1994; Costerton 1999; Carmen 2005; Malone 2016; Cai 2017). However, if the cells in the upper biofilm layers are destroyed by sonication, nutrients and oxygen availability in the underlying bacteria increase, making these bacteria more active, and therefore more susceptible to antibiotics (Pitt 1994).

Pitt's group studied in detail the bactericidal effect of antibiotics on microbial biofilms in combination with sonication. They showed that LFU of an intensity of 10 mW/cm2 enhanced the gentamicin bactericidal impact on *P. aeruginosa* biofilms, while US itself did not affect biofilm viability (Qian 1996; Qian 1997). By varying the US frequency (70 kHz-10 MHz), they also found that when combined with gentamicin, low-frequency US was more effective in destroying *P. aeruginosa* and *E. coli* biofilms than was high-frequency US (Qian 1997; Johnson 1998). The authors concluded that the main factor underlying this bioacoustic effect was stable cavitation accompanied by microstreaming (Qian 1999).

Successful combinations of various antibiotics with the US using different treatments were deployed against several bacterial biofilms *in vitro* and *in vivo*. Rediske et al. described a synergistic bactericidal effect of gentamicin and low-frequency low-power ultrasound on *E. coli* biofilms implanted subcutaneously in rabbits (Rediske 1999; Rediske 2000). Later, Carmen et al. showed a positive effect of US on gentamicin transport through *E. coli* and *P. aeruginosa* biofilms *in vitro* and *in vivo* (Carmen 2005; Carmen 2006).

Recently, the Cai group have studied several aspects of US and antibiotic combination. In 2016, they presented an effective bactericidal combination of sonication, colistin, and vancomycin on a pan-resistant biofilm of *Acinetobacter baumannii* (Liu 2016). Each of these agents alone, or in combination with only one additional agent, did not significantly affect the number of bacteria in the biofilms. In a follow-up study, this group searched for conditions (antibiotics type and concentrations, US intensity, and sonication mode) that enhance antimicrobial agents' activity against *S. aureus* biofilms. Three drugs, vancomycin, linezolid, and levofloxacin, combined with LFU, showed synergistic effects against methicillin-susceptible *Staphylococcus aureus* (MSSA). However, only vancomycin showed a synergistic effect with LFU against a methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm (Wang 2018). A recent work published by this group addresses combating the highly resistant *Klebsiella pneumoniae* biofilm (Liu 2020). When combined with LFU, the antibiotics meropenem, tigecycline, and fosfomycin significantly reduced the number of bacteria in these biofilms (Liu 2020). Cai et al. (2017) present and summarize the *in vitro* and *in vivo* studies on the effect of antibiotics in combination with sonication on microbial biofilms, including reaching three important conclusions. First, it was confirmed that the impact of LFU is more potent in reducing bacteria viability in the biofilm than high-frequency ultrasound. Second, when using pulsed US, the antimicrobial synergism between US and antibiotics depends mostly on the temporal peak intensity rather than average intensity. Third, unlike the US and antibiotics synergism, skin damage is associated with the average US intensity. Therefore, it is possible to achieve a maximal bactericidal effect with minimal tissue damage by regulating the mode of the pulsed LFU (Cai 2017).

**3. Using ultrasound for drug delivery**

Another area of US ​​application for microorganism eradication is drug delivery and release in target tissues. Focused US irradiation can be used to increase local cell membrane permeability to allow for drug entry. Additionally, drug transport in the blood or other extracellular fluids can be enhanced by a local US-induced increase in fluid oscillatory motion, thus increasing drug transfer rates. Cavitation can also increase the permeability of the skin, capillary walls, and other tissue systems (Pitt 2003; Pitt 2004; Ueda 2009; Polat 2011; Azagury 2014). Since US treatment is highly effective and safe, this method has garnered high interest for drug delivery. In a pilot study published by Silberg et al. (2013), cefazolin was injected subcutaneously and dispersed by US with a power density of 3 W/cm for several minutes. This method allowed the direct delivery of the antibiotic to a localized soft tissue infection without subjecting the entire body to antibiotic treatment, as in a traditional intravenous administration. The concentrations of antibiotics achieved using ultrasonic delivery to tissues were up to 1000 times higher than those obtained using systemic delivery (Silberg 2013). The possibility of using US to improve drug penetration through the skin into circulation is also of great importance. The mechanisms, main achievements, and new trends in using ultrasonic irradiation for transdermal drug delivery are presented in reviews by Polat (2011) and Azagury (2014).

Liposomes, microbubbles, or micelles introduced into the blood can be induced for the local release of encapsulated drugs by US. The mechanism of this release is based on the effect of pressure on bubble size, since pressure waves passing through a liquid cause gas bubble contraction or expansion at high or low pressure, respectively. In the case of sufficiently stable fluctuations in bubble size repeated over multiple cycles, a stable or non-inertial cavitation phenomenon can occur. Such stable vibrations create microstreaming, with fluid flowing around microbubbles, and is characterized by speeds and shear rates proportional to oscillation amplitudes. Vesicles denser than the surrounding fluid are convected toward the microstreaming field around the oscillating bubble. If the shear stress exceeds the vesicle's strength, the vesicle is ruptured, releasing the drug within the vesicle (Canaparo 2019). Another possible mechanism for vesicle rupture is a shock wave resulting from inertial or collapse cavitation. In addition, a jet of fluid created by the collapse cavitation can also result in high shear stress, thus piercing or shear opening the surrounding bubbles. Moreover, focused US can lead to local heating of tissues, which can be enhanced by bubble cavitation, and can also be used for drug release from heat-sensitive carriers (Pitt 2004; Lentacker 2014; Shashank 2014; Canaparo 2019; Kooiman 2020). Thus, cavitation can exert two different effects that can be manipulated for targeted drug delivery: cavitation can cause both carrier vesicle opening and drug release, as well as increase cell membrane and capillary permeability for efficient drug transfer (Pitt 2004; Canaparo 2019).

Spherical vesicles consisting of one or more phospholipid layers, liposomes, and micelles can contain drugs within their membranes or in the internal space, preventing premature drug release (Goyal 2005; Drulis-Kawa 2010; Shashank 2014). Since vesicles are gas-free, they are not acoustically active, but can be sheared open by other cavitating bubbles. However, liposomes are assumed to contain some gas, and, therefore, react to sonication by releasing the drug during the cavitation-induced liposome disintegration (Pitt 2004).

Successful use of sonication for enhancing liposome-encapsulated antibiotics release efficacy was recently demonstrated by Ma et al. in *Ralstonia insidiosa* bacteria. Bacteria were efficiently eradicated using gentamicin containing liposomes following US treatment (Ma 2015; Ma 2016). The authors also noted that non-focused US increases liposome penetration into the biofilm (Ma 2015), and that focused US efficiently bursts the liposomes, thus releasing the contained drugs (Ma 2016).

Unlike liposomes, microbubbles contain gas in their internal cavity and, therefore, respond to US pressure waves. Microbubbles are gas bubbles encapsulated in a shell of surfactants, polymers, proteins, or phospholipids, with a diameter distribution between 1 and 10 µm. High molecular weight perfluorocarbon compounds and sulfur hexafluoride are usually used as a gas core of microbubbles. Poor water solubility and a low diffusion rate of fluorinated gases increase microbubble stability (LuTheryn 2020). The size and composition of the microbubble membrane determine stability and lifespan, as well as the acoustic response to US stimulation (Carugo et al. 2017; LuTheryn 2020). The microbubbles’ acoustic response can be regulated by the US's parameters, such as frequency, power, duration of treatment, and pulse mode (Pitt 2004; LuTheryn 2020). When stable cavitation occurs at lower US intensities, cavitation bubbles pulsate in the course of many acoustic pressure cycles without collapsing. Simultaneously, applying higher US intensities leads to inertial cavitation, which can ultimately cause the microbubbles to burst, thus amplifying the biophysical effects (Lentacker 2014).

Zhu et al. used microbubbles of gaseous sulfur hexafluoride encapsulated in a phospholipid membrane combined with low-intensity US to increase the antimicrobial efficacy of gentamicin against planktonic *E. coli* (Zhu 2014). Using electron microscopy, they found the appearance of sonoporation in *E. coli* and suggested that, when treated by US, microbubbles in the medium served as cavitation nuclei, which affected cell membranes, promoted shear stress, and increased microstreams, causing the formation of additional pores in cell membranes.

It is worth noting that using microbubbles responding to US increases the penetration of systemic antibiotics into biofilms. During the last decade, two different research groups demonstrated a significant increase in both vancomycin uptake by *S. epidermidis* biofilms following ultrasonic treatment and in biofilm permeability in the presence of microbubbles (He 2011; Dong 2013). This effect was later studied in more detail both *in vitro* and *in vivo* (Dong 2017; Dong 2018; Hu 2018). Ronan et al. (2016) applied a combined treatment against *P. aeruginosa* biofilm, including the aminoglycoside antibiotics gentamicin sulfate and streptomycin sulfate, and microbubbles under ultrasonic processing, to increase antibiotic efficacy. They found that the most significant damage to biofilms is achieved by simultaneous cell exposure to antibiotics and microbubbles during ultrasonic treatment (Ronan 2016). It was concluded that US, alone or in combination with microbubbles, negatively affects biofilms by increasing bacterial cell membranes permeability, followed by changes in biofilm structure, such as the appearance of pores in the extracellular polymer matrix, as well as by interfering with the expression of genes controlling the biofilm (Dong 2017; Hu 2018; LuTheryn 2020). In addition, fluid shear stress can cause erosion and flaking of the biofilm and decrease biofilm aggregate density (Rmaile et al. 2014; LuTheryn 2019).

Gas-carrying microbubbles can be used not only as activators of ultrasonic drug delivery but also as drug carriers themselves. Drugs can be electrostatically bound to the surface of microbubbles or injected into the microbubble membrane or gas cavity. However, using microbubbles appears to be more efficient for drug delivery when used in combination with nanoparticles or liposomes loaded with an active substance (LuTheryn 2019). For example, Horsley et al. (2019) used gas-filled lipid microbubbles coated with liposomes containing the antibiotic gentamicin to deliver the drug into an apical cell cytoplasm. The US-activated intracellular delivery of gentamicin with liposome-coated microbubbles was over 16 times higher than in the control group and twice as high as with free liposomes. When applied under sonication at low doses, these liposome-coated vesicles quickly and efficiently removed and destroyed the intracellular uropathogenic bacteria *E. faecalis* in a human urothelial organoid model (Horsley 2019).

Interestingly, unbound microbubbles and liposomes also exhibit synergistic antibacterial properties under the influence of ultrasound. As shown by Fu et al. (2018), polymyxin B encapsulated in chitosan-modified liposomes in the presence of phosphatidylcholine microbubbles carrying perfluoropropane showed significant bactericidal activity under ultrasonic activation, which led to substantial damage to drug-resistant biofilms of *Acannbacter baumannii* (Fu 2018).

Another promising use of US for local drug application is the release of antibiotics from implanted materials or artificial organs. Removing bacterial biofilms from medical implants is very difficult and usually requires invasive interventions. Pre-loading antibiotics on the implanted materials that can be locally released may prevent biofilm formation altogether. An example of this method is developing acrylic bone cement with antibiotics.

In addition, loading antibiotics on implanted materials is very effective for preventing and treating orthopedic infections (Anagnostakos 2009). However, in this case, antibiotics are released from the surface of the implanted materials at low efficiency, and local antibiotic concentrations drop rapidly (Yu 2012). US can improve the release of antibiotics from acrylic bone cement or other polymers. Recently, Shi et al. (2018) demonstrated that sonication enhanced and prolonged the release of high concentrations of vancomycin and gentamicin from calcium phosphate cement, which serves as a basis for bone cement used to fill bone defects (Shi 2018a, Shi 2018b). It was suggested that the improved release of antibiotics from bone cement is caused by microstreams arising from stable cavitation and US thermal effects. The US-induced antibiotic release from a bone implant is accompanied by other US treatment effects, including the direct destruction of bacteria, an increase in antibacterial drug activity, and an increase in tissue permeability for antibiotics. Thus, US can enhance the bactericidal effect of immobilized antibiotics in two ways: by increasing their antibacterial activity and by enhancing and prolonging their release from the implants (Anagnostakos 2009; Shi 2018a; Shi 2018b).

However, some adverse effects of US should be taken into account. Cavitation phenomena, which are necessary to stimulate drug release from the carrier or drug transport into cells, can be harmful to the tissues. Therefore, it is essential to select a cavitation rate that will be sufficient to increase cell membrane permeability and release drugs from the carrier but will not damage the cells themselves (Pitt 2004).

**4. Sonodynamic antimicrobial chemotherapy**

Another critical application of sonication for combating microorganisms is sonodynamic therapy (SDT). SDT is based on the ultrasonic activation of molecular species susceptible to US (sonosensitizers) and is accompanied by reactive oxygen species (ROS) formation and damaging cells. Sonodynamic antimicrobial chemotherapy (SACT) refers to the application of SDT to kill microorganisms (Ma 2009; Nakonechny 2013; Harris 2015; Serpe 2015; Nakonechny 2019). As in photodynamic treatment, sonodynamic treatment is based on the combined action of three components: US, sensitizer, and molecular oxygen. Some molecules with photodynamic activity produce free radicals when irradiated with US, as do sonosensitizers. Some PSs, including porphyrins and related tetrapyrroles, protoporphyrin IX, Rose Bengal, ALA (5-aminolaevulinic acid), are also sonosensitizers.

The ability of US to penetrate deep into liquids and tissues makes SACT a promising approach both for the disinfection of turbid liquids and for non-invasive treatment of deep infections. Treatment efficiency is further enhanced due to increased tissue permeability under the US influence (Rosenthal 2004, Serpe 2015, Pang 2016).

The mechanism of ROS generation under acoustic cavitation is not fully understood. According to one hypothesis, collapsing microbubbles release energy, which leads to the sonolysis of water molecules and/or sensitizer molecules. The resulting radicals react with oxygen to form ROS.

Another hypothesis suggests that radicals are formed by radiation emitted by excited molecules as a result of sonoluminescence. Sonoluminescent radiation is generated during the recombination of radicals formed due to collapsing microbubbles. Sonoluminescent light, emitted in the proximity of the collapsing microbubbles, can be absorbed by sensitizer molecules, leading to their excitation. This effect is accompanied by cytotoxic ROS formation, such as singlet oxygen and free radicals. As in classic photodynamic therapy (PDT), sonoluminescence may cause either a type I process, leading to the formation of secondary radicals, or a type II process, in which mainly singlet oxygen eradicates the pathogenic cells (Macdonald 2001; Nakonechny 2013; Serpe 2015).

The idea of SACT as a new and promising antimicrobial strategy was proposed by Ma et al., who suggested exploiting the unique capabilities of US in generating free radicals in the dark, thus enabling its use for treating infectious diseases (Ma 2009). In 2013, our group demonstrated the efficient eradication of Gram-positive *S. aureus* and Gram-negative *E. coli* bacteria by the xanthene dye Rose Bengal excited in the dark by a 28 kHz ultrasound (Nak 2013), confirming SACT applicability. Since Rose Bengal was initially characterized as a photosensitizer (Silva 2018), its high sonodynamic activity is likely due to the excitation of its molecules by sonoluminescent light. In our later studies, Rose Bengal was immobilized onto silicone, which also showed significant antibacterial activity against *S. aureus* in the dark under ultrasonic activation, probably due to Rose Bengal leaching from the polymer during ultrasonic treatment (Nak 2019). Since Rose Bengal also possesses photo- and sound-sensitive properties, it has been the subject of scientific attention (Vanerio 2019). Costley et al., (2019) demonstrated the sonodynamic antibacterial activity of Rose Bengal when conjugated to an antimicrobial peptide klaklack against planktonic cultures of *S. aureus* and *P. aeruginosa*, as well as *P. aeruginosa* biofilms, and *in vitro* and *in vivo* mouse models (Costley 2019). Activation of Rose Bengal by low-frequency ultrasound increased its antimicrobial activity (Costley 2019). In another study, Alves (2018) described the sonodynamic activity of Rose Bengal against planktonic forms and biofilms of *Candida albicans*, which are highly resistant to antifungal drugs. Also, a synergistic effect of PDT with SDT was found, and their combination was proposed as an alternative approach to fungal biofilm eradication (Alves 2018).

Another well-known representative of photosensitizers, chlorin e6, is very active under ultrasonic activation. Recent studies have demonstrated its sonodynamic activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria (Xu 2016), and fungi (*C. albicans*) (Alves 2018). The photosensitizer hematoporphyrin monomethyl ether also exhibits sonodynamic properties and was successfully used in *in vitro* eradication of *S. aureus* (Zhuang 2014) and *Porphyromonas gingivalis* (Zhang 2020), as well as in the treatment of periodontal diseases in rats (Zhuang 2016). Xu's group studied the sonodynamic activity of another photosensitizer, Hypocrellin B, and showed its significant antibacterial efficacy in killing *Staphylococcus epidermidis* (Wang 2015b) and methicillin-resistant *S. aureus* (Wang 2016).

Curcumin, a natural photosensitizer, demonstrates a broad spectrum of pharmacological properties, including antioxidant, anti-inflammatory, antiviral, antimicrobial, and antitumor activities, while at the same time inducing very low or no internal toxicity. Curcumin is effective in killing methicillin-resistant *S. aureus* (Wang 2014), *Bacillus cereus*, and *E. coli* (Wang 2015a) and is active under combined sono- and photo-activation. A new approach, called sono-photodynamic therapy (SPDT), is based on the simultaneous use of ultrasound and light to activate photosensitizers. Under the influence of ultrasound and blue light, curcumin successfully destroyed *S. aureus* biofilms (Alves 2019) and inactivated *E. coli* *and S. aureus* in orange juice (Bhavya 2019). SPDT was also successfully employed against polymicrobial periopathogenic biofilms using indocyanine green (Pourhajibagher 2020) and against *E. coli* using ZnO nanoparticles (Zhang 2017) as sensitizers.

**Conclusions**

Data drawn from the literature suggest multiple uses for LFU to combat various microorganisms. However, there are still many unknowns concerning the interactions of US with microorganisms.

US can damage not only Gram-positive and Gram-negative bacteria, but also yeast, fungi, algae, and even viruses. Comparing the effectiveness of various methods is impeded by the significant variations in sonication frequency, intensity, and pulse cycle between studies. The observed synergistic effect of US and antibiotics against multiple microorganisms opens up new prospects in the treatment and eradication of pathogenic cells. To date, only a few antibiotics have been tested against a limited number of bacteria under ultrasonic excitation. Further in-depth studies are required to investigate the effects of additional antibiotic types against an expanded list of bacterial cells.

Using sonication for targeted delivery and release of drugs, using micelles, liposomes, microbubbles, together with the release of antibacterial agents from implants, has significant advantages over traditional methods. Since this method is still under development, there is a wide field for future studies into new formulations and conditions for enhancing antibacterial compound effectiveness under ultrasonic activation.

**Table 1.** Overview of the Effects of Ultrasonic US on Planktonic Microorganisms and Viruses

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| **Effect on Cells** |
| Effective destruction of bacteria under the influence of sonication was observed. The amount of cells killed was directly dependent on exposure duration and intensity. |
| US treatment caused cell death by compromising membrane integrity, inactivating intracellular esterases, and inhibiting metabolic performance. The primary target is the cytoplasmic membrane.  |
| Lethal effect as a result of synchronous membrane damage with esterase inhibition during sonication exposure was observed. |
| US exerted an irreversible effect on bacteria without sublethal injury. A proportion of bacteria subpopulation suffered from severe damage to intracellular components (e.g., DNA and enzymes) but retained intact cell envelopes. |
| Effective destruction of bacteria under the influence of sonication was observed. The amount of cells killed was directly dependent on exposure duration and intensity. |
| The effective destruction of bacteria was directly dependent on the duration of the US’s exposure and intensity in the low-frequency range (20 and 38 kHz) and deagglomerating of bacterial clusters at higher frequencies (512 and 850 kHz). |
| Effective destruction of bacteria under the influence of sonication was observed. The number of cells killed was directly dependent on exposure duration and intensity. |
| Bacterial lysis correlated with the duration of exposure to US. Cells were destroyed in less than 0.4 seconds. |
| US treatment caused cell death by compromising membrane integrity, inactivating intracellular esterases, and inhibiting metabolic performance. The primary target was the outer membrane.  |
| US exerted an irreversible effect on bacteria without sublethal injury. A proportion of bacteria subpopulation suffered from severe damage to intracellular components (e.g., DNA and enzymes) but retained cell envelopes. |
| Effective destruction of bacteria under the influence of sonication was observed. The number of cells killed was directly dependent on exposure duration and intensity.  |
| An immediate lethal effect due to membrane damage caused by cavitation during US exposure. |
| US first destroyed the cell wall and then the cell membrane in yeast cells. At low acoustic intensity (10 W/cm2), polysaccharides were released before proteins. At higher acoustic intensities (24 and 39 W/cm2), the release of proteins was faster. |
| The destruction of yeast cells correlated with the duration of US exposure. Complete lysis is achieved in about 1.0 second. |
| Reduction in fungal growth under the influence of sonication decreased growth was correlated with increased US intensity. |
| Twenty minutes of liquid shear ultrasonication disrupted ~68% of algae cells. |
| Significant reduction in virus with US intensity was observed. |
| No apparent effect of US on the virus was observed. |

**Table 2.** Overview of the Manifestations of the Ultrasonic Effect on Microbial Biofilms

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| **Effect on Cells** |
| In vitro experiments: 280 kHz US was more effective at removing biofilm than was 1 and 2 MHz. More than 80% of adhered biofilm was removed after three minutes of US exposure. *In vivo,* moderate biofilm removal was achieved using 280 kHz for five minutes. |
| The largest reduction was achieved during 10-cycle tone burst exposures. |
| Most of the biofilms treated at the higher exposures of 6.2 and 7.6 MPa biofilm were destroyed. Cells in some biofilms were still viable  |
| Most of the biofilms treated at the higher exposures of 6.2 and 7.6 MPa had no detectable live cells (the treatment killed bacteria in the biofilm, or the treatment disrupted the biofilm and released bacteria from the surface). |
| Thirty-second exposure and 6 ms pulse period were sufficient to destroy the biofilms. 15-second exposure and 3 ms pulse period were optimal when considering exposure time, efficacy, and safety. |
| Up to 98% of microorganisms were removed from the roots of *Eichhornia crassipes* by US exposure for five minutes at an intensity of 64.5 W/cm2. The efficiency of removing microorganisms from the roots improved with increasing exposure time and power input. |

**Table 3**. Overview of the Manifestations of the Synergistic Effects between Ultrasound and Antibiotics on Planktonic Microorganisms

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| **Research Model** |
| No synergistic antibacterial effect of gentamicin and US was observed. |
| There was a significant reduction in viable bacteria count in the test group due to LFU treatment: 5 log10 CFU/ml to 3.66 log10 CFU/ml. Antimicrobial susceptibility tests did not detect any resistance. Partial cell wall destruction or disintegration and increased bacterial cell wall thickness were observed in test samples compared to the control group. |
| Low-frequency US increased the antibiotic efficacy against *S. aureus* internalized in osteoblasts. There was a slight reversible effect of US on osteoblast viability. |
| The mechanism of LFU-mediated bacterial cell death was damage to the bacterial cell membrane. MRSA cultures treated with LFU showed altered colony characteristics and antibiotic resistance, possibly due to changes in gene expression resulting in a less virulent strain of MRSA. |
| US reduced the effective dosage of ampicillin required to impair bacterial viability. |
| No synergistic antibacterial effect of gentamicin and US was observed. |
| A combination of ampicillin and US produced a killing efficiency of over 1.5 logs higher than ampicillin alone. Tetracycline killing was not enhanced by US. |
| The antibiotic's bactericidal effect was not significantly different from the effect of the combined antibiotic and US after three hours. A slight synergistic effect with US was noted at shorter exposure times. |
| A synergistic effect of gentamicin and US was observed. Bacterial viability was reduced several orders of magnitude by gentamicin concentrations and ultrasonic levels, which by themselves did not reduce viability. |
| The synergistic antibacterial effect of gentamicin and US was observed as a function of ultrasonic intensity. The most significant bactericidal effect, with a 5-log reduction in viable population, was achieved at 4.5 W/cm2. Reductions in power density reduced bactericidal activity, and at 0.01 mW/cm2, no significant US enhanced bactericidal effect was observed. |
| A synergistic effect was observed between LFU and gentamicin |
| Simultaneous application of LFU with levofloxacin and ciprofloxacin enhanced antibacterial effectiveness. The synergistic effect of fluoroquinolones and US was observed, and the bacterial viability was reduced when antibiotics and US were combined. Antibacterial activity was enhanced with increasing antibiotic concentrations, US irradiation time, and solution temperature. LFU activated fluoroquinolones to produce reactive oxygen species, mainly the superoxide radical anion and hydroxyl radical. |
| A synergistic effect of gentamicin and US was observed. Bacterial viability was reduced several orders of magnitude by gentamicin concentrations and ultrasonic levels, which alone did not reduce viability. |
| The simultaneous application of an antibiotic and US reduced the number of living bacteria by over one order of magnitude. At the same time, US itself did not kill cells but led to transient destabilization of the outer membrane. |
| US increased antibiotic uptake by disturbing or stressing the membrane. Simultaneous US and antibiotic application reduced *P. aeruginosa* viability by one to two orders of magnitude compared with an antibiotic alone, even at concentrations below MIC. |
| A synergistic effect was observed between LFU and gentamicin |
| The US induced large holes in the bacterial outer membrane. The ultrasonically enhanced nitrocefin increased via US-perturbed porins or holes in the outer membrane. The rate of nitrocefin hydrolysis in cell suspensions rose with increased US intensity, reflecting the rate of antibiotic penetration. |
| Gentamicin at the minimum inhibitory concentration (MIC), in combination with US, decreased bacteria viability 3 logs greater than antibiotic alone. Kanamycin at the MIC did not reduce cell viability, but, in combination with US, decreased viability by 2 logs. In combination with US, streptomycin at the MIC decreased bacteria viability 1 log greater than the antibiotic alone. The combination of US and tetracycline (at a concentration above the MIC) produced greater than 2 logs more killing than the antibiotic alone. |
| Gentamicin at the MIC, combined with US, decreased bacteria viability 3 logs greater than the antibiotic alone. No statistically significant killing by tetracycline alone or by tetracycline with ultrasound was seen at MIC concentration. |
| US and gentamicin at the MIC killed 1 to 2 logs greater than gentamicin alone, and at over double, the MIC killed 3 to 4 logs greater than gentamicin alone. Experiments with tetracycline at and above the MIC (up to x 4) showed no bioacoustic effect.  |

**Table 4.** Overview of the Manifestations of the Synergistic Effect between Ultrasound and Antibiotics on Microbial Biofilms

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| **Effect on Cells** |
| Immediate following US, synergistic effects were observed in groups of S-LFU combined with three antimicrobial drugs. At 24 h, treatment with M-LFU plus vancomycin had a concentration-dependent synergistic bactericidal effect against biofilm. No synergistic effects were observed in any of the S-LFU combinations. |
| Immediate following US, only S-LFU plus vancomycin had a synergistic effect. At 24 h, treatment with M-LFU plus Vancomycin had a concentration-dependent synergistic bactericidal effect against biofilm. No synergistic effects were observed in any of the S-LFU combinations. |
| Combinations of US and vancomycin successfully reduced the number of viable bacteria in the biofilm of *S. epidermidis*, but this Gram-positive organism required a longer treatment time than previously tested Gram-negative species. The insonation of implanted biofilms for 48 h with vancomycin significantly reduced the average number of viable bacteria. |
| The lower frequencies of US produced higher levels of bacteria killing. Sterilization of a 14 h biofilm was achieved after 6 h of exposure. |
| Low-frequency and low-power-density US, when combined with gentamicin, reduced the number of viable bacteria in the biofilm. Exposure to US alone did not affect bacterial viability. |
| LFU significantly enhanced the killing of biofilm *E. coli* by gentamicin, which was increased with higher ultrasonic intensity and decreased with increasing frequency. |
| US enhanced the bactericidal action of gentamicin against *E. coli* with a pulse intensity ≧300 mW/cm2. Bacteria responded to peak US intensity and not to average intensity or the total amount of energy delivered. Skin damage correlated with average ultrasonic power and not maximum pulse power. The average intensity of 100 mW/cm2 produced no skin damage, but an average intensity of 300 mW/cm2 produced significant damage. |
| US significantly increased gentamicin transport across the biofilm. A 45-minute US treatment more than doubled the amount of penetrated gentamicin compared to analogs without sonication. The 2.9 W/cm2 intensity was more effective in enhancing gentamicin transport than 1.9 W/cm2. |
| The combination of gentamicin and ultrasonic exposure for 48 h reduced *E. coli* bacteria viability in implanted biofilms to nearly undetectable levels. |
| Low-power US alone did not disrupt the biofilm, but, when combined with gentamicin, increased cell killing by nearly two orders of magnitude. The bioacoustic effect was due to the enhancement of antibiotic transport through the cell membrane or interference with bacterial internal metabolic activity. |
| Simultaneous application of LFU enhanced the antibacterial effectiveness of gentamicin. Lower frequency sonication was significantly more effective than higher frequency in reducing bacterial viability within the biofilm. US by itself did not reduce biofilm viability. |
| US increased the antibiotic efficacy of gentamicin. The synergistic bactericidal effect decreased with frequency increase from 44 kHz to 10 MHz. The bioacoustic effect was associated with the US temporal peak intensity. The 10 mW/cm2 power density was more effective in enhancing the antibiotic action than 1 mW/cm2. |
| The colony biofilms effectively blocked gentamicin transport within 45 minutes. US application significantly increased gentamicin transport of gentamicin through the same biofilm. Higher sonication intensity was associated with increased gentamicin transport. |
| 24- and 48-hour ultrasonic treatment did not increase gentamicin's bactericidal efficacy in killing *P. aeruginosa* biofilms implanted into rabbits. |
| LFU application on biofilm-infected wounds reduced viable bacteria amounts and improved wound healing. |
| LFU enhanced the bactericidal activity of antimicrobial agents against *K. pneumoniae* biofilm. Synergistic effects were observed with US combined with meropenem, tigecycline, and fosfomycin, but not with amikacin and colistin. The synergistic antibiofilm effect of multiple US lasted longer than a single US when combined with antimicrobial agents. |
| LFU alone or combined with a single antibacterial agent did not significantly reduce bacteria counts in biofilms but enhanced the antibacterial effectiveness of colistin and vancomycin combinations. Higher concentrations of colistin in the combined treatments resulted in a better US-enhanced antibacterial effect. |

**Table 5**. Overview of the Effects of Ultrasound-Mediated Drug Delivery to Planktonic Microorganisms

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| **Effect on Cells** |
| *In vitro*: elution from [Vancomycin + US + MBs] cement was higher than from either the [Vancomycin] or [Vancomycin + US] specimens. The activity of the eluted vancomycin from the [Vancomycin + US + MBs] cement against *S. aureus* was higher than from either the [Vancomycin] or [Vancomycin + US] specimens. In rabbits, eluted Vancomycin activity from the [Vancomycin + US + MBs] cement against *S. aureus* was higher than from either the [Vancomycin] or [Vancomycin + US] specimens. |
| US-activated liposome-coated bubbles efficiently delivered high concentrations of gentamicin into the intracellular compartment of human urothelial cells. This delivery was over 16 times greater than that of the control group and twice that achieved by liposomes not associated with MBs. |
| As a result of the simultaneous application of LFU with levofloxacin-loaded nanoparticles, the bacterial cell wall was ruptured, the cell structure was incomplete, and the bacteria was severely damaged. US, combined with levofloxacin-loaded nanoparticles, exhibited a tenfold higher antibacterial activity against *M. smegmatis* residing inside macrophages compared to the free drug. |
| *In vitro*: *P. acnes* growth was inhibited by 86.08 ± 2.99% in the Lysozyme-shelled MB group and 57.74 ± 3.09% in the Lysozyme solution group. For US power densities of 1, 2, and 3 W/cm2 in the Lysozyme-shelled MB group, *P. acnes* growth was inhibited by 95.79 ± 3.30%, 97.99 ± 1.16%, and 98.69 ± 1.13%, respectively. *In vivo*: the recovery rate on day 13 was higher in the US group with Lysozyme-shelled MBs (97.8 ± 19.8%) than in the Lysozyme-shelled MBs group (90.3 ± 23.3%). |
| Short pulses of 1 MHz US induced stress responses in bacteria and were able to cause bacterial death. |
| The viable counts of *E. coli* in [Gentamicin + MBs + US] were decreased by 1.01 to 1.42 log 10 CFU/mL compared to [Gentamicin + US]. The minimal inhibitory concentration of gentamicin against *E. coli* was 1 μg/mL in the [Gentamicin + MBs + US] and [Gentamicin + US] groups, lower than that in the [Gentamicin] and [Gentamicin + MBs] groups (2 μg/mL).  |
| Reparable sonoporation (transient membrane permeabilization by US), during which the induction of temporary pores on the cell membrane was followed by pore resealing, and did not affect cell survival.  |
| Reparable sonoporation  |

**Table 6**. Overview of the Effects of Ultrasound Mediated Drug Delivery to Microbial Biofilms.

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| **Effect on Cells** |
| Phase-shift acoustic nanodroplets coupled with vancomycin exhibited significant bactericidal efficacy against MRSA biofilms through sequential stimulation by low-intensity pulsed US and heating to 37°C, and were more effective than microbubbles in damaging the biofilm. |
| A model of infective endocarditis biofilm exposed to a combination of oxacillin, recombinant tissue plasminogen activator, US, and Definity US contrast agent, achieved a 99.3% fractional infected clot loss, greater than any other treatment approach. |
| US-targeted microbubble destruction further enhanced antibiotic activity against methicillin-resistant bacterial biofilms. Biofilms treated with a combination of human β-defensin 3 and US-targeted microbubbles contained a lower percentage of live bacteria and lower biofilm density compared to the other mouse groups. |
| UTMD further enhanced antibiotic activity against bacterial biofilms, compared with antibiotics combined with US alone. Biofilms treated with vancomycin combined with US and MBs contained the largest percentage of dead bacteria compared to the other groups. Biofilm density was significantly lowered, and larger micropores were observed after the treatment. |
| US-mediated MBs exerted a bactericidal effect on biofilms and enhanced biofilm susceptibility to vancomycin, with a dose-dependent bioeffect. Vancomycin transport *S. epidermidis* biofilms was significantly enhanced by US, and MBs further increased biofilm permeability to vancomycin. |
| Simultaneous application of US and MBs increased vancomycin bactericidal action against *S. epidermidis* biofilms *in vivo* without exerting obvious harm to the animals. The antibacterial effect of US in combination with MBs and vancomycin was more pronounced than that of US with vancomycin only. |
| Direct damage to bacterial cells and increased metabolic activities enhanced the bactericidal capacity of antibiotics to biofilm under UTMD treatment. The energy of the US waves or UTMD had no direct lethal effect on bacteria, but enhanced the bactericidal activity of vancomycin. Inhibition of cell wall synthesis in cell-wall-damaged cells caused cell death. |
| The addition of sSonazoid MBs enhanced the US biofilm removal effect. After Sonazoid addition, approximately 80% of the biofilm was removed in one minute of ultrasonic exposure at a frequency of 280 kHz (approximately equal to three minutes exposure using the same probe and duty cycle without Sonazoid). |
| Bacterial biofilms were effectively destroyed due to inertial (collapse) cavitation, the effectiveness of which depended on the initial distance between the microbubble and the bacterial biofilm and on US parameters. |
| Concurrent treatments of USMB with gentamicin and USMB with streptomycin caused the greatest reduction in biofilm biomass and the highest proportion of cell death. |
| The liposome-capture density by the alginate film linearly increased with ultrasound intensity up to ISATA = 6.2 W/cm2, threefold higher than without US. US-driven penetration of antibiotic-loaded liposomes into the biofilm was more effective at killing bacteria compared to a variety of controls. |
| High-intensity focused US alone, gentamicin in solution alone, and gentamicin in solution with high intensity focused US were ineffective at killing bacteria in alginate-based biofilms. Focused US and gentamicin-encapsulated liposomes reduced the number of viable bacteria in biofilms by 72%. |
| USMB and chitosan-modified polymyxin B-loaded liposomes had a more significant antibacterial effect on biofilm-forming bacteria than did polymyxin B alone. The synergistic antibacterial effect of USMB with chitosan-modified liposomes containing 2 µg/mL polymyxin B was sufficient to almost eliminate drug-resistant biofilm-producing bacteria. |

**Table 7**. Overview of the Effects of Sonosensitizes on Planktonic Microbial Cells

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| **Effect on Cells** |
| Bacteria incubation with HMME without US had no effect. HMME combined with US effectively destroyed over 95% of the bacteria, while the same US level without HMME only reduced CFU by 38%. Higher HMME concentrations and higher US intensities caused more bacteria death. |
| Curcumin had a significantly sonodynamic killing effect on MRSA in a dose-dependent manner, up to a 5-log reduction in CFU. |
| Sonodynamic action of chlorin e6 had significant antibacterial activity and induced a 7-log reduction in *S*. *aureus* CFU. |
| Sonodynamic action of hypocrellin B had a significant bactericidal effect on MRSA and caused a 5-log reduction in CFU. |
| Under the simultaneous action/influence of conjugate and US, 5 log reductions in bacterial numbers were observed. The conjugate also improved bacterial uptake compared to a mammalian cell line. |
| US or PS + blue light treatments resulted in 0.18 ± 0.14 and 2.34 ± 0.13 log reductions of *S. aureus*, respectively, while combining of PS  with US and  blue light treatment at optimized conditions resulted in a 2.35 ± 0.16 log reduction. |
| Sonodynamic action of hypocrellin B had significant antibacterial activity on *S. epidermidis,* probably through increased intracellular ROS level that caused damage to bacterial membrane integrity. |
| Curcumin had sonodynamic bactericidal activity in a dose-dependent manner up to a 5.6-log CFU reduction. |
| Curcumin had sonodynamic bactericidal activity in a dose-dependent manner, up to a 2-log CFU reduction. |
| Sonodynamic action of chlorin e6 had significant antibacterial activity and induced a 2-log reduction in CFU of *E*. *coli* cells. |
| Sonophotocatalysis significantly increased the outer membrane and inner membrane permeability of *E. coli* cells mainly due to ROS induced from oxidative stress.  |
| US or PS + blue light treatments resulted in 3.02 ± 0.52 and 1.06 ± 0.13 log reduction of *E. coli,* respectively. Combined PS with US and blue light treatment resulted in 4.26 ± 0.32 log reductions. |
| Under the simultaneous action/influence of conjugate and ultrasound, 7 log reductions in bacterial numbers were observed. The conjugate also displayed improved uptake by bacterial cells compared with a mammalian cell line. A preliminary *in vivo* experiment involving SACT treatment of *P. aeruginosa*-infected wounds in mice demonstrated that US irradiation of conjugate-treated wounds substantially reduced bacterial burden. |
| *P. gingivalis* CFU decreased with increased HMME concentration in SACT. For the same HMME concentration, the antimicrobial effect of SACT depends on the ultrasonic time. |
| Treatment with SACT mediated by PDZ eradicated the microorganism, while treatment with US or PDZ separately did not significantly impact *C. albicans* viability. Treatment with RB alone significantly decreased planktonic cultures viability, however combined with SACT resulted in microorganism eradication. |

**Table 8**. Overview of the Manifestations of the Effect of Sonosensitizes on Biofilms

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| **Effect on Cells** |
| Curcumin, LED light or US alone did not affect *S. aureus* biofilms, while US in combination with PDT showed a higher and more significant bacteria reduction compared to SACT and PACT applications. |
| The antibacterial photodynamic effect enhanced by US led to a maximum effect of a 99.99998% reduction in viable bacteria. The combination of PACT with US resulted in a synergistic effect reducing viable counts by 6.8 log 10 reduction. |
| Pretreatment of a *P. aeruginosa* biofilm with low-intensity US for five minutes before RB addition resulted in a 2.6-fold increase in sensitizer diffusion through the biofilm. |
| Simultaneous use of PACT and SACT with Chitosan Nanoparticles-Indocyanine green significantly increased the periopathogens’ biofilm elimination compared to other groups. |
| Individual PACT or SACT had little impact on biofilms. Combined PACT and SACT significantly reduced the viability and total biomass of biofilms. Biofilms treated with a combination of PACT and SACT were thinner and were comprised mainly of dead cells. |