Sonodynamic antimicrobial chemotherapy: First steps towards a sound approach for microbe inactivation

Sonodynamic antimicrobial chemotherapy: first steps towards a sound approach for microbe inactivation?

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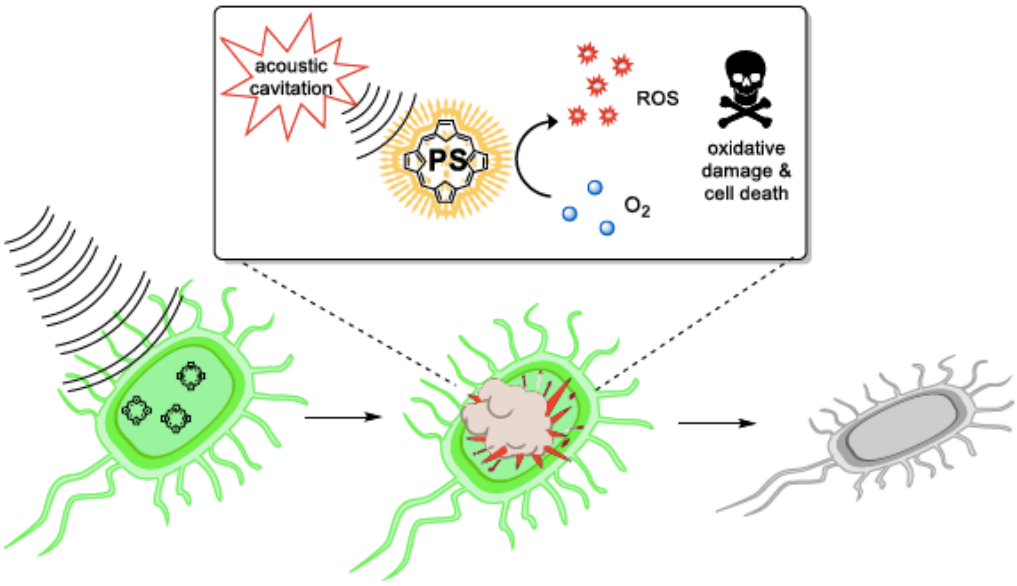
Abstract
Sonodynamic therapy (SDT) relies on the ability of ultrasound to activate sonosensitisers and trigger the generation of reactive oxygen species (ROS) to achieve cell death. SDT was explored as an anticancer approach until 6 years ago, when its potential application as an antimicrobial strategy was pointed out and the term "sonoantimicrobial chemotherapy" (SACT) was coined. The excellent penetration of ultrasound in liquid media make SACT particularly promising approach for the non-invasive treatment of deep-seated infections, and for the reduction of bacterial load in turbid water. In this review we provide an account of the brief history of SACT, from its molecular bases to the current state of the art and perspective applications.

Graphical abstract



Keywords: Sonodynamic therapy, photodynamic therapy, SACT, SDT, PDT, ultrasound, sonosensitisers, microbial inactivation

Introduction

Over the last decades the insurgence of antibiotic-resistant microorganisms has become a pressing concern for public health as it represents the main cause for the failure of antibiotic therapy.[1] The scientific community devoted great efforts to provide alternative solutions not only to eradicate infections in the medical and veterinary setting, but also to reduce bacterial load in the context of environmental and industrial applications, since agricultural/environmental use of antibiotics plays a pivotal role in the spreading of resistance, through food-borne microorganisms[2] and wastewater.[3-5] In the quest for alternative approaches to microbial inactivation, physical methods (*e.g.*, irradiation, heat, high pressure, etc.) gained popularity because they typically have less potential to induce resistance, and are amenable to application on large scale.[6-8] Amongst these methods, ultrasonic waves have been explored as a bactericidal tool has been exploited since Harvey and Loomis provided the first evidence of the lethal effect of ultrasounds on microbes in 1929.[9] Following their work, studies on ultrasound-mediated bacterial inactivation using a plethora of different experimental conditions (*e.g.*, continued/pulsed waves, low/high intensity wave, type of ultrasound generators, etc.) were published, often reporting conflicting results. Nevertheless, some general traits emerged, such as the higher susceptibility of Gram-positive *vs.* Gram-negative bacteria, of rod-shaped bacteria *vs.* cocci, and of larger *vs.* smaller bacteria.[10] It was shown that ultrasounds interfere with the life cycle of both planktonic[11, 12] and biofilm[13] bacteria, but again, depending on the microbe and the irradiation conditions, the outcome can be that of cell replication inhibition, cell inactivation, but also of stimulation of microbes vitality.[14]

Ultrasound-mediated microbial inactivation eventually made its way into applications in medicine and industrial processes. Sonication, both stand-alone and associated with heath or high pressure, has been successfully employed to reduce the bacterial load in foodstuff with no detrimental effect on the quality of the treated food.[15-17] In the medical field, ultrasound-based scalers are employed in dentistry for plaque removal,[18] to promote the microbial uptake and/or the release of antibiotic (*e.g.*, vancomycin, gentamicin) from prosthetic implants.[19, 20]

Until a few years ago, the paths of antimicrobial photodynamic therapy and ultrasound waves hardly crossed. The synergy of ultrasounds and PACT was explored as a way to enhance the efficacy of photodynamic treatment in the disinfection of infected wounds,[21, 22] and in a combined photodynamic/ultrasound treatment to eliminate dental plaque.[23] High-frequency ultrasound was also employed to assess the efficacy of PACT in vitro.[24] While these works were presenting light and ultrasound as the physical triggers of different biological effects (*i.e.*, microbe inactivation and enhanced cell uptake or imaging, respectively), evidences coming from the field of anticancer therapy were showing that in the presence of photosensitisers, ultrasound and light could trigger similar events and elicit similar biological responses.[25] These evidences cast the bases of a new anticancer approach named of sonodynamic therapy (SDT), which few years ago crossed the borders to enter the field of antimicrobial therapy. The aim of this review is to give an account of the state of the art of

antimicrobial SDT, and an overview of the molecular bases of its mechanism of action.

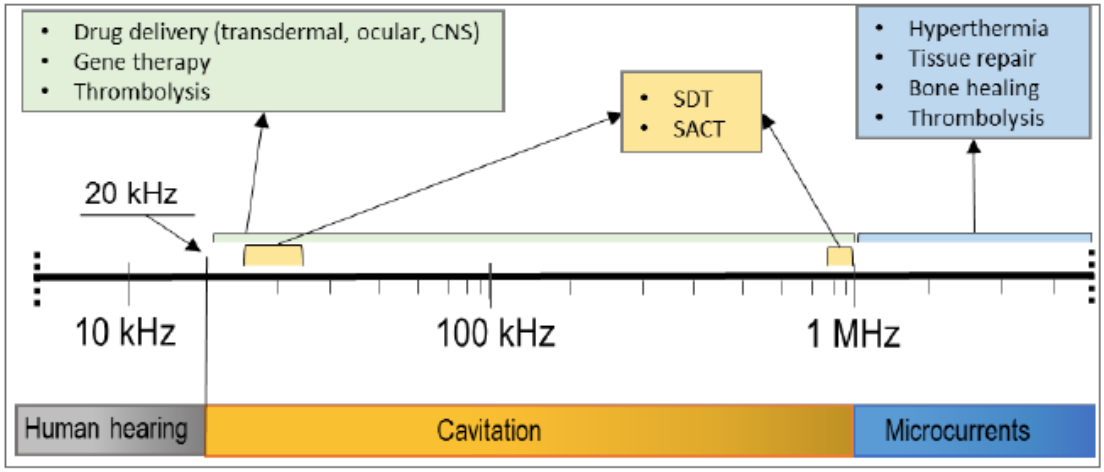


Figure 1: Simplified diagram of the ultrasound frequencies used for therapeutic applications[26]

Ultrasound in living systems

Ultrasound is an acoustic radiation inaudible to humans, with a frequency exceeding 20 kHz. Ultrasound generates a mechanical vibration characterized by repeated cycles of compression and expansion in the surrounding environment.[27] The ability of ultrasound to propagate through tissue and induce transient or permanent changes to biomolecules and cells has been successfully exploited for numerous biomedical applications, including therapeutic intervention (tissue repair, thrombolysis, angioplasty, drug delivery, endodontic disinfection, etc.) and diagnostic techniques.[26] (Figure 1). Modern ultrasound generators for biomedical applications rely on piezoelectric devices to produce a focused ultrasound beam that can be delivered to the target body district through the skin and underlying tissues. The ultrasound frequency range employed for diagnostic purposes is rather broad (2.0-28.0 MHz), whereas therapeutic applications are typically carried out with waves within the 0.5-3.0 MHz frequency range.[27] Ultrasound-based diagnostic techniques rely on low-energy irradiation to avoid damaging cells/tissues, whereas therapeutic intervention require the delivery of higher doses of energy to generate the desired biological outcome.[28]

The interactions of ultrasounds with living systems occur *via* three main pathways, namely, *thermal* (*i.e.*, heat generation), *chemical* (*i.e.*, radical formation) and *mechanical* (*i.e.*, shear stress, liquid jets, shock wave), each of which triggers specific effects. The *thermal* effects are the desired outcome of most ultrasound-based therapeutic applications, as it happens, for example, in the high-intensity focused ultrasound (HIFU) therapy of cancer, where an ultrasound beam focused on the target malignancy delivers a dose of energy that causes a spatially-confined hyperthermic effect and subsequent coagulative necrosis at the focal point.[29] The *mechanical* effects of ultrasounds causes transient alterations of the permeability of cell membrane, a phenomenon that underpins the enhanced cell uptake of both low- and high-molecular weight drugs observed upon exposure to ultrasound.[30] The phospholipidic membrane

is intrinsically able of absorbing the mechanical energy generated by the sonic wave, and of responding with deformations (compressions and expansions) of the intramembrane space: this behaviour has been advantageously exploited in the ultrasound-mediated drug targeting and controlled drug release in several therapeutic fields.[31] The *chemical* effects of ultrasound are associated to the onset of sonochemical reactions (*e.g.*, ionisation, electron-transfer), and/or the sonolytic formation of free radicals: these processes have a great potential for therapeutic applications, but due to the short-life of the species generated, they are very challenging to harness.

It is generally agreed that the effects of ultrasounds in tissues originate from a phenomenon known as acoustic cavitation. The action of ultrasound waves propagating in a liquid promotes the formation of gas- or vapour-filled cavities (microbubbles), which undergo shrinking and growing cycles due to the alternate compression and expansion generated by the different pressure phases of the ultrasound wave. Low-amplitude oscillation of the microbubbles leads to stable cavitation (non-inertial), in which the microbubble "pulsates" generating microstreams in the surrounding medium, causing shear stress to cell membranes and eventually resulting in the transient formation of pores (sonoporation). (Figure 2, A). High-amplitude oscillations of the microbubble give rise to inertial cavitation, in which the alternating shrinking and expansion increase in intensity until the microbubble implodes. Collapsing microbubbles results in the generation of shock waves and/or liquid jet formation, the force of can easily perforate cell membranes. (Figure 2, B and C).

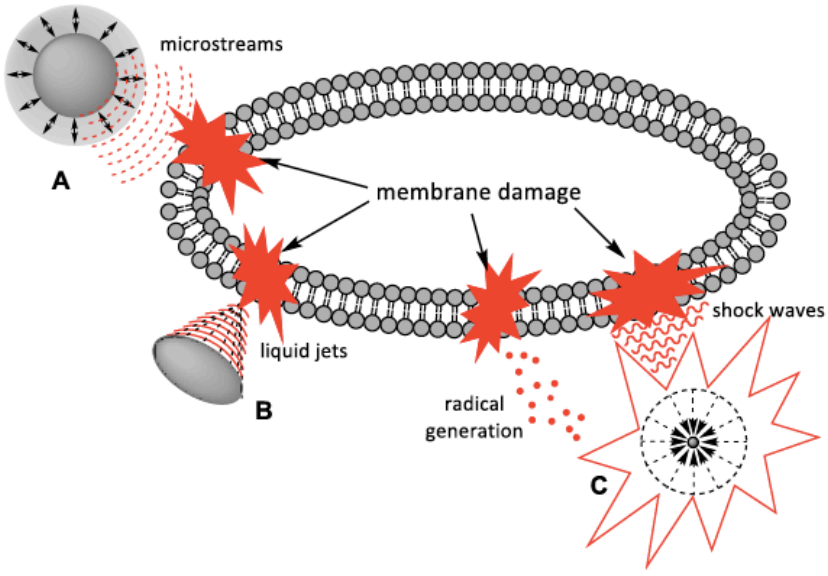


Figure 2: Membrane damages following acoustic cavitation.

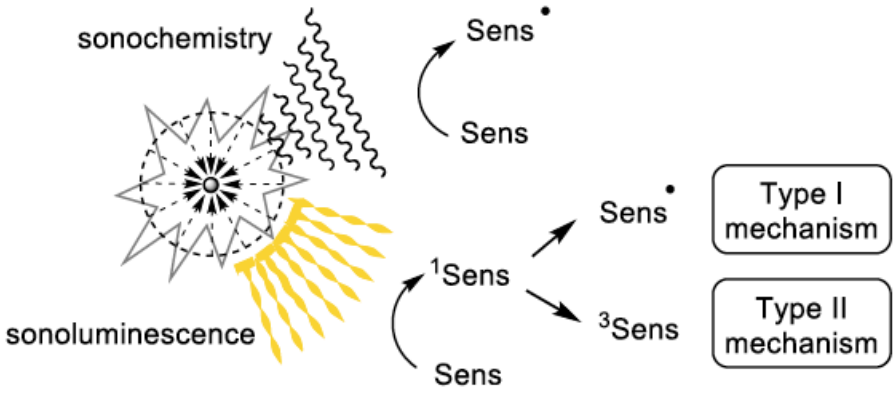
Sensitised living systems to ultrasound: sonodynamic therapy and its molecular bases

A peculiar effect of ultrasound is its ability to induce transient but dramatic changes to the physicochemical behaviour of given molecular species (sensitisers), and trigger localised chain of events culminating in alterations to subcellular structures and

eventually cell damage and death. Following the same rational that led to translate the sensitisation to light into photodynamic therapy, the possibility of sensitising cells to ultrasound received a great attention as a potential warhead for an innovative therapeutic approach that gained the name of sonodynamic therapy (SDT).

As the photodynamic process, sonodynamic treatment relies on two *per se* harmless components, *i.e.*, a sensitiser and ultrasound. Irradiation of the sensitiser with ultrasound initiates a chain of events that culminates with the production of highly reactive cytotoxic species, rapidly leading to cell death via apoptosis and/or necrosis and/or autophagy.[32] The potential of this approach for the reduction of solid tumours has been intensely investigated, and its efficacy has already been demonstrated at the preclinical level on some experimental tumour models. A crucial difference between PDT and STD arises from the different power of penetration of the activating radiation: while light has a relatively limited reach within tissue, ultrasound easily propagates through it, allowing to target more deeply-seated lesions without the need of invasive devices such as implanted fibre-optic.[33]

The binary nature of the treatment is not the only similar trait of SDT and PDT: as for the photodynamic process, ultrasound activation of the sensitiser results in the localised production of ROS. An indirect confirmation of this instance is the fact that the majority of the molecules that are able to sensitise cells to ultrasound are also photosensitisers; porphyrins and related tetrapyrroles, rose Bengal, and ALA, to cite a few examples, displayed sonodynamic efficacy in a number of studies.[34] Although the exact mechanism of SDT has not been entirely elucidated, it is generally accepted that the main effectors of the sonosensitised cell damage are short-lived species, namely ROS and free radicals, generated as a consequence of inertial acoustic cavitation.[35] (Figure 3). When the acoustic pressure amplitude is sufficiently high, the cavitation microbubbles implode violently and a dramatic increase of temperature and pressure occurs. It has been estimated that on a nanosecond time scale the collapse of the microbubble can induce spikes of temperatures reaching 5000 K and pressures of 250 MPa.[36] In these “hot spots” confined to the vicinity of the microbubbles, the high liquid shear-forces, the shock waves, and the localized heating, promote sonochemical reactions (sonolysis), and light emission (sonoluminescence).[37] Two hypotheses were formulated to explain ROS generation from acoustic cavitation; one of them suggests that the high energy released by the collapsing microbubble promotes the sonolysis of water molecules and/or of the sensitiser molecules: the radicals formed can then react with oxygen, triggering the production of reactive oxygen species. The second hypothesis explains the generation of radical on the basis of sonoluminescence, a radiation emitted by excited molecules formed by the recombination of radicals generated from the collapsing microbubble.[38] In the vicinity of the collapsing microbubble, the light emitted can be absorbed by the sensitiser, triggering a purely photodynamic process which can evolve either into a Type I process, again leading to the formation of radicals, or in a Type II process, with singlet oxygen as the main effector.



Evidences supporting the generation of radicals, oxygen radicals and singlet oxygen by either one of these two pathways have been provided in several works.[25, 38, 39] In eukaryotic cells it has been shown that the nature of the damages caused by the sonodynamic process involves alterations to the cytoskeleton,[40] extensive membrane perturbations,[41] and Bcl-2 gene family down-regulation and consequent mitochondria outer membrane permeabilisation, leading to the release of caspase activators (*e.g.,* cytochrome c) or other proapoptotic molecules including apoptosis-inducing factors (AIF).[42, 43]

SACT: sensitizing microbes to ultrasound

In the 90s, Tachibana *et al.*[44] and Umemura *et al.*[39] showed that ultrasound was able to activate photosensitizer to kill cancer cells. Following that work, the research in the field of SDT of the last 20 years has focused primarily on anticancer treatment.[34] Recently, Ma *et al.* suggested that sonodynamic therapy may be exploited for the eradication of microbial infections.[45] In analogy with the concept of PACT, sonodynamic antimicrobial chemotherapy (SACT) was proposed as a therapeutic modality where a sonosensitiser is selectively delivered to target microbial cells and activated by ultrasound to induce the cell death.[46] In the last five years the few groups active in this area achieved ultrasound mediated inactivation of different kind of bacteria with various sensitisers, including fluoroquinolone antibiotics, "classic" photosensitisers, and TiO_2.[47, 48]

Antibiotic-mediated SACT

The observation that ultrasounds enhances the activity of antibiotics was first reported by Liu *et al.*,[49] who studied the sonodynamic antibacterial effect of two fluoroquinolones (ciprofloxacin and levofloxacin) on *E. coli*. Ultrasound irradiation of a 10^4 CFU/mL bacterial population for 45 min achieved a 2-log reduction of the microbial load, although the bacterial inactivation following fluoroquinolone-SACT treatment is less than 10 times higher than the one achieved by the antibiotic alone. (Table 1, Entry 1). The authors show the involvement of ROS by trapping oxygen radicals with a chemical trap.

In another study, the efficacy of gentamycin in association with pulsed ultrasounds in preventing the formation of *E. coli* biofilms in bone cement in vivo (rabbit model) was evaluated.[50] (Table 1, Entry 2) Although the authors do not rationalise the results on the basis of the sonodynamic action, their experiments show that the application of ultrasound (28 to 48 kHz, 0.5 W/cm^2, 48 h) reduces the population of 10^9 CFU/mL of

biofilm bacteria by 2 log on gentamycin-loaded poly-acrylic bone cement implant in rabbit legs.

SACT mediated by photosensitisers

The Nisnevitch group studied the effect of 28 kHz ultrasound irradiation on Gram-negative *E. coli* and Gram-positive *S. aureus* in the presence of Rose Bengal and methylene blue as sonosensitisers.[51] Following on from their previous studies on the photodynamic inactivation of microorganisms exploiting the chemiluminescent oxidation of luminol as the light source,[52] the authors focus on the ultrasound radiation as the initiator of dye-sensitised cell damage. When *E. coli* cells were incubated with rose Bengal (0-15 μM, 15 min) and subsequently exposed to ultrasound irradiation in the dark (to avoid the photodynamic activation of the sensitiser) a reduction of up to 3-log in the bacterial population was observed (initial load: 10^9 CFU/mL). (Table 1, Entry 3). The effect of sonication alone had little effect on the vitality of microbes on a 10^7 CFU/mL cell population, but caused a 2-log reduction in a denser *E. coli* population (10^9 CFU/mL). The highest eradication rate for E. coli was observed when a 106 CFU/mL population was incubated with 15 μM RB (4.2 log). S. aureus proved less susceptible to ultrasound irradiation alone, in agreement with previous evidences, but intriguingly it was more susceptible to the exposure to the sensitiser alone than E. coli. SACT treatment of S. aureus, consisting in the irradiation of a 106 CFU/mL following incubation with 15 μM RB, achieved a 5.5 log reduction in the bacterial load. Surprisingly, this study showed that the efficacy of MB as a SACT sensitiser is negligible. Irradiation of a 10^6 CFU/mL cell population of S. aureus (28 kHz, 0.84 W/cm^2, 1 h) following incubation with 30 μM MB caused a negligible reduction in cell vitality, in sharp contrast with the results obtained following photodynamic activation of the sensitiser (white light, 1.6 mW/cm^2, 0.5 h), which afforded a complete eradication of the microorganisms. The authors explain this unexpected behaviour by considering that although sonoluminescence has a broad emission width, its maximum intensity lies between 250 and 600 nm, offering a minimal overlap with the absorption profile of MB (500-700, $\lambda_{max} > 650$ nm).

Wang *et al.* investigated the efficacy of sonodynamic inactivation of planktonic methicillin-resistant *S. aureus*, using curcumin as the sonosensitiser.[53] 5 min of 1 MHz US irradiation at a fluence rate of 1.6 W/cm^2 achieved a reduction of 5 log in the microbial load. (Table 1, Entry 4). At the dose used in this study (40 μM) curcumin showed no toxicity without irradiation, and similarly exposure to ultrasound did not have intrinsic bactericidal effect. The authors also performed studies to investigate whether curcumin-mediated sonodynamic cell inactivation involved damages to bacterial DNA: pulsed-field electrophoresis showed that the profile of bacterial DNA following SACT treatment was substantially the same as that of the control cells.

Zhou and co-workers studied the effect of hematoporphyrin monomethyl ester (HMME) in the SACT of *S. aureus*.[54] The authors investigated the effect of the sensitiser dose and the ultrasound dose on the cell inactivation efficacy, and they found that under the most effective SACT parameters, which involved the incubation

with 50 µg/L HMME followed by US irradiation (1 MHz, 6 W/cm^2, 30 min), the reduction in microbial load was ca. 2 log. (Table 1, Entry 5).

TiO_2-mediated SACT for wastewater disinfection

The sonodynamic treatment is under study as an alternative approach for water disinfection. While the efficacy of stand-alone ultrasound irradiation for water disinfection has been proven, the feasibility of this approach require the use of high-intensity radiation to achieve high-log reduction of the microbial load. It has been suggested that the sonodynamic treatment could overcome this limitation: the synergy between sonosensitisers and ultrasound radiation could result in a more efficient cell inactivation, allowing to use less intense irradiation and consequently encourage the application of this treatment on a larger scale.*

The sensitiser proposed for the SACT-mediated disinfection of water is titanium dioxide. It has been postulated that under ultrasonic irradiation excited electrons move from the valence band to the conduction band, generating positive holes in the valence band. In the proximity of the surface of TiO_2 electrons are abstracted from water molecules to generate hydroxyl radicals, which are plausible effectors of the cell damage that results in microbial inactivation. Drakopoulou *et al.*[55] showed that 60 minutes irradiation with 24 kHz ultrasound (total ultrasound dose 5400 kJ/L) achieves up to 2-log reduction in the population of Gram-negative bacteria (coliforms and *Pseudomonas* spp.) in wastewater obtained from the outlet of an activated sludge process. (Table 1, Entry 6). The authors studied the microbial reduction of 4 bacteria groups (total coliformis, faecal coliformis, *Pseudomonas* spp., and faecal streptococci) and *C. perfringens*. This study provides yet another evidence that Gram-positive bacteria display a lower susceptibility to the sonodynamic treatment.

In a different study, ultrasound irradiation in the presence of 1.0 g/mL of TiO_2 caused a 3-log load reduction of *Legionella* spp. in wastewater. Variation in the initial bacterial load of the water (from $8.0\text{x}10^2$ to $7.3\text{x}10^7$ CFU/mL) did not seem to affect the efficacy of the treatment.[56] (Table 1, Entry 7). The authors demonstrated that the presence of ROS scavengers (*e.g.,* glutathione, histidine and ascorbic acid) dramatically reduces the efficacy of the bacterial inactivation, indicating the involvement of ROS in the process. The authors argue that SACT is better suited to the disinfection of wastewater thanks to the penetration power of ultrasounds, which, unlike light, is not affected by the turbidity of the medium.

Rahman *et al.* explored the efficacy of a non-woven TiO_2 fabric as a sonosensitiser for the disinfection of water contaminated with *E. coli*.[57] When a colony of 10^8 CFU/mL was exposed to 36 kHz ultrasound radiation (0.28 W, up to 1h) in the presence of non-woven TiO_2, a cell inactivation of ca. 1 log was observed. The authors proved that lipid peroxidation underpins the SACT inactivation of E. coli by performing the experiments in the presence of a fluorescent indicator (DPPP), whose emission intensity increases in the presence of peroxidative chain reactions. Furthermore, the authors demonstrated that in the presence of ROS scavengers (glutathione or *tert*-butanol) the fluorescence emission from the reporter dye was considerably reduced, indicating the involvement of ROS as the cell-damage effectors.

Entry	Sonosensitiser	Dose	Irradiation conditions	Microorganism(s)	Initial load (CFU/mL)	Microbial load reduction (log)	Ref.
1	ciprofloxacin/ levofloxacin	0.01 mg/mL	40 kHz, 1 W/cm^2	*E. coli*	10^4	< 2	[49]
2	gentamycin	-*	28 to 48 kHz, 0.5 W/cm^2, 48 h	*E. coli*	10^9	2	[50]
3	rose Bengal	0-15 μM	28 kHz, 0.84 W/cm^2, 1-2 h	*S. aureus, E. coli*	10^9	2-3	[51]
4	curcumin	40 μM	1 MHz, 1.56 W/cm^2, 5 min	MRSA	10^7	5	[53]
5	HMME	10-50 μg/L	1 MHz, 6 W/cm^2, 30 min	*S. aureus*	10^8	< 2	[54]
6	TiO_2 (21 nm ø nanoparticles)	5 mg/mL	24 kHz, 300W, 15-60 min	*Pseudomonas* spp. faecal coliformis total coliformis faecal streptococci *C. perfringens*	< 10^5	2	[55]
7	TiO_2 (2 mm ø pellets)	1 mg/mL	36 kHz, 300W, 15-60 min	*Legionella* spp.	10^3	< 2	[56]
8	TiO_2 non-woven fabric	-	36 kHz, 0.28W, 0-70 min	*E. coli*	10^8	1	[57]

Conclusions

SACT is a relatively new antimicrobial approach, and unlike in the case of anticancer SDT, the works published in the field are few and far between. The studies surveyed in this review represent, to the best of our knowledge, the complete heritage our knowledge in the field of SACT. The experimental conditions examined differ to such an extent that little margin is left for the comparison and rationalisation of the results.

If SACT is to be translated into an antimicrobial tool for biomedical or environmental applications, much remains to be explored to further our understanding of the field: the large body of work devoted to the establishment of SACT's older sibling (PACT) should encourage the scientific community to work towards the full elucidation of the mechanistic bases of the sonodynamic process and towards the understanding of the pathways that lead to cell death so that they can both be harnessed to enhance the efficacy of the approach. Insofar, SACT has been explored for the inactivation of bacteria: the sonodynamic process possesses the same trait of generality as the photodynamic, and it is reasonable to expect that other microorganisms are sensitive to the treatment, but SACT treatment on viruses, fungi, or yeasts has not been reported yet. Systematic studies on the effect of the various parameters of the sonic wave (continuous or pulsed wave, frequency and intensity, energy delivered, etc.) have not been reported, as, crucially, the selectivity of the sonodynamic treatment for microbes vs. host cells, a factor of paramount importance for any therapeutic application of SDT, remains totally unexplored. Investigations on the possibility of the onset of resistance to SACT should also be undertaken.

While the published data might not suggest that SACT is on the verge of becoming the next panacea for clinical antimicrobial therapy, the studies carried out show that sonosensitisation of microorganisms is well worth investing in, especially for environmental applications: the relatively low prices of ultrasound generators and TiO_2-based sonosensitiser, together with the suitability of the technique for the disinfection of turbid water on a large scale, makes SACT suitable for wastewater treatment. SACT disinfection of implants pre-loaded with sonosensitiser also seems a promising application, because it would offer an attractive alternative to systemic antibiotic administration: in addition, the excellent penetration of ultrasound radiation in tissues would allow the eradication of microbial infections on deep-seated implants without the need of invasive devices to deliver the activating radiation.

Abbreviations

ALA	5-aminolaevulinic acid
CFU	colony-forming units
DPPP	diphenyl-1-pyrenylphosphine
HMME	haematoporphyrin monomethylester
MB	methylene blue
PACT	photodynamic antimicrobial chemistry
PDT	photodynamic chemistry
PpIX	protoporphyrin IX
RB	rose Bengal
ROS	reactive oxygen species
SACT	sonodynamic antimicrobial chemotherapy
SDT	sonodynamic chemistry

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