Can microbial cells develop resistance to oxidative stress in antimicrobial photodynamic inactivation?

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Abstract

Infections have been a major cause of disease throughout the history of humans on earth. With the introduction of antibiotics, it was thought that infections had been conquered. However, bacteria have been able to develop resistance to antibiotics at an exponentially increasing rate. The growing threat from multi-drug resistant organisms calls for intensive action to prevent the emergence of totally resistant and untreatable infections. Novel, non-invasive, non-antibiotic strategies are needed that act more efficiently and faster than current antibiotics. One promising alternative is antimicrobial photodynamic inactivation (APDI), an approach that produces reactive oxygen species when dyes and light are combined. So far, it has been questionable if bacteria can develop resistance against APDI. This review paper gives an overview of recent studies concerning the susceptibility of bacteria towards oxidative stress, and suggests possible mechanisms of the development of APDI-resistance that should at least be addressed. Some ways to potentiate APDI and also to overcome future resistance are suggested.

1. Introduction

Microorganisms have existed on the earth for more than 3.8 billion years and exhibit the greatest genetic and metabolic diversity of any known life form. They are an important component of the biosphere and serve a vital role in the maintenance of ecosystems. In order to survive, they have evolved mechanisms that enable them to respond to selective pressure

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exerted by a range of different environments and competitive challenges. Humans have continuously exposed pathogenic microbial populations to antibiotics, antiseptics, and other antimicrobial agents, in attempts to control infectious disease. These microorganisms have then responded by developing a variety of resistance mechanisms to escape this offensive against their survival. Currently, antimicrobial resistance among bacteria, viruses, parasites, and other disease-causing organisms is a serious worldwide threat to management of infectious disease.

Antibiotics were discovered in the middle of the 20th century, and almost immediately reduced the threat of infectious bacterial diseases, which had devastated the human population for centuries. But, surprisingly soon after the discovery of penicillin in 1940, a number of treatment failures and isolation of some bacterial strains such as staphylococci which were no longer sensitive to penicillin started to occur. This marked the beginning of the era of antibiotic resistance [1].

Resistance can be categorized into two mechanisms.

> **A.**   Intrinsic or natural resistance whereby microorganisms naturally either do not possess the appropriate target sites for the specific drug (and therefore the drug does not affect them), or they naturally have only low permeability to those agents, because of the differences in the chemical nature of the drug and the microbial membrane structures. This is particularly relevant for those antibiotics that require entry into the microbial cell in order to effect their action.

> **B.**   Acquired resistance whereby a naturally susceptible microorganism develops mechanisms that prevent it from being affected by the drug.

Acquired resistance mechanisms can occur through various ways (summarized in Figure 1) [2].

> -   the presence of an enzyme that inactivates the antimicrobial agent

> -   a mutation in the antimicrobial target of the agent, which reduces the binding of the antimicrobial agent

> -   post-transcriptional or post-translational modification of the antimicrobial target of the agent, which reduces binding of the antimicrobial agent

> -   reduced uptake of the antimicrobial agent into the cell

> -   active efflux of the antimicrobial agent out of the cell

Resistance elements can be acquired by transmission of free (naked) DNA from one bacterial species to another bacterial species (horizontal gene transfer). Genes responsible for antibiotic resistance in one species of bacteria can be transferred to another species of bacteria through various mechanisms such as F-pilus generation as well as via bacteriophages [3].

Although antimicrobial resistance is a naturally occurring biological phenomenon, it is often enhanced as a consequence of the adaptation forced upon microbes by continuous or repeated exposure to antimicrobial agents used to prevent or treat infections in humans or

livestock, and the widespread routine use of disinfectants in farms, hospitals, and households [1]. It is now accepted that excessive antimicrobial use is the single most important factor responsible for increased antimicrobial resistance [4, 5]

In 2015 the O'Neill report garnered much international attention when it delivered the alarming forecast that by 2050 (if nothing were done to stem the growth of multi-drug-resistant bacteria) there would have been 300 million premature deaths that would have cost the world economy $100 trillion [6].

The current worldwide increase in drug-resistant bacteria and the simultaneous decline in efforts by both academic laboratories and pharmaceutical companies directed towards the discovery of new antibacterial agents to combat resistant strains now poses a serious threat to the treatment of life-threatening infections. Therefore, it is necessary to develop novel noninvasive and non-toxic antimicrobial strategies that act more efficiently and faster than the current antibiotics, and to which pathogens will not easily develop resistance [7]. One promising alternative to current antibiotics is antimicrobial photodynamic inactivation (APDI).

2 Antimicrobial photodynamic inactivation

APDI is defined as the application of a non-toxic dye known as a photosensitizer (PS), which can be photo-activated with light of the appropriate wavelength in the presence of oxygen to generate cytotoxic reactive oxygen species (ROS) such as singlet oxygen and/or free radicals) [8–10].

The process is initiated when a ground state PS (S0) absorbs light of an appropriate wavelength and is converted into an electronic excited singlet state (S1). Many PS molecules in this shortlived state can decay back to the ground state with the emission of light (fluorescence) or heat (internal conversion) [11], but some can also be transformed by intersystem crossing into a much longer-lived excited triplet state (T1, T3). The excited triplet state PS can do one of two things; it can react with molecular oxygen by energy transfer generating singlet oxygen (a process which is termed a type II reaction) or else it can undergo an electron transfer reaction to form PS radical ions which in turn react with oxygen to produce cytotoxic species such as superoxide, hydrogen peroxide, and/or hydroxyl radicals (which is termed a type I reaction) (Figure 2). The singlet oxygen or other reactive oxygen species (ROS) can cause damage to bacterial cells or other microbes through several mechanisms. These include oxidation of membrane lipids and amino acids in proteins, cross-linking of proteins and oxidative damage to nucleic acids with the subsequent disturbance of the normal functioning of the pathogen [12, 13].

It has been proposed that the ROS generated by light-activated PS can trigger microbial killing, and cell damage via three mechanisms: (a) damage of the cell membrane (or virus envelope); (b) inactivation of essential enzymes and proteins; and/or (c) damage to DNA [9].

The APDI-induced photo-damage to microbial constituents can result in considerable morphological and functional changes in the microbial cells themselves. Functional damage results from loss of enzyme activities, protein oxidation and formation of protein-protein

cross-links, and inhibition of cellular metabolic processes (e.g. DNA synthesis, glucose transport, etc). Morphological alterations include alteration of the mesosome structure. Direct damage to the cell membrane leads to leakage of cellular contents following inactivation of the membrane transport system [14].

There is a very wide variation in the cellular structure and organization among different classes of microbial cells. These structural variations influence the interaction of exogenous PS with different cellular components, and can also affect the effectiveness and the mechanism of action of the APDI with different pathogens. Differences in the cell walls of Gram-positive and Gram-negative bacteria play an important role in the susceptibility of bacteria to APDI. Grampositive bacteria have a thick and porous peptidoglycan layers that surround a cytoplasmic membrane, while Gram-negative bacteria possess an outer membrane, surrounding a thinner peptidoglycan layer, inside which is the cytoplasmic membrane [15, 16]. To perform efficient APDI, the PS needs to penetrate (or at least bind to) the cell wall of the bacteria and end up in the plasma membrane or in the cytoplasm; however, the membrane barriers of the bacterial cell limit the simple diffusion of PS into the bacterial cytosol [17, 18]. Therefore, APDI of Grampositive bacteria is definitely much easier to accomplish than that of Gram-negative bacteria. The cell walls of fungal cells have a structure that is intermediate in permeability between Gram-positive and Gram-negative bacteria. The outer part is a moderately porous layer of β-glucan and mannan polysaccharides.

3. Oxidative stress

Microbial life first evolved in a world without oxygen and was rich with reduced iron. By three billion years ago, microbial life forms shared basic biochemical mechanisms and a common metabolic system, which persist even today. The subsequent oxygenation of the atmosphere by photosynthetic organisms created a disaster for primitive life: oxygen is a reactive chemical species, and organisms had to develop strategies to defend themselves against it [19].

Molecular oxygen (O_2) is small and nonpolar, and it diffuses across typical biological membranes as quickly as it does through water [20]. Therefore, even the most active cells cannot consume through respiration rapidly enough to reduce the intracellular O_2 concentration considerably below the concentration immediately outside the cell. O_2 reacts poorly with cellular biomolecules, however its reactivity derives from the metabolic formation of ROS [21], which results from the uncontrolled addition of consecutive electrons to O_2, instead of the normal controlled addition to form water. This uncontrolled addition generates superoxide ($O_2 \bullet^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\bullet OH$) [22]. When the balance between ROS and their scavenger systems is disturbed, ROS accumulate within the cells leading to a condition called oxidative stress [23].

Some microorganisms escape oxidative stress by living in anaerobic microhabitats; all others must deal with the consequences of intracellular O_2. Different examinations show that the ability to do so varies widely: obligate anaerobes cannot tolerate oxygen at all, microaerophilic organisms require a relatively low-micromolar O_2 concentration, while

aerobes have adapted to grow in air-saturated fluids. However, almost all of these microorganisms suffer poor growth, elevated mutagenesis or even death when they are exposed to O_2 levels that exceed those of their native habitats [23].

Aerobic organisms use O_2 for respiration or oxidation of nutrients to obtain energy. Reactive by-products of oxygen, such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals ($\cdot OH$), are generated continuously in cells grown aerobically [24].

Environmental agents such as ionizing radiation, UV radiation, or numerous compounds that generate intracellular $O_2^{\bullet-}$, (redox-cycling agents such as menadione) can also cause oxidative stress, which arises when the concentration of ROS increases to a level that exceeds the cell's defense capacity [24]. High temperatures can result in high oxidative stress, leading to damage to proteins, DNA double-strand breaks and cell death [25, 26]. Moreover antioxidant enzymes function poorly at high temperatures. In addition, cold temperatures can cause oxidative stress; cells of the Antarctic bacterium *Pseudomonas fluorescens*, grown at 4°C, suffer an increasing amount of free radicals and show enhanced activity of two antioxidant enzymes [27]. Some immune cells (neutrophils and macrophages) use the NADPH oxidase enzyme, activated upon invasion by pathogenic bacteria, as a weapon to kill microbes during phagocytosis [24].

The biological targets for ROS are DNA, RNA, proteins and lipids. Much of the damage is caused by hydroxyl radicals generated from H_2O_2 via the Fenton reaction, which requires iron (or another divalent metal ion, such as copper) and a source of reducing equivalents (possibly NADH) to regenerate the metal. Moreover direct one-electron of H_2O_2 can give •OH. Lipids are major targets for damage caused during oxidative stress. Free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation. The initial effect of lipid peroxidation is a decrease in membrane fluidity, which changes the membrane properties and can significantly disrupt membrane-bound proteins. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of these products, such as the aldehydes (malondialdehyde and 4-hydroynonenal), are also very reactive and can damage molecules such as proteins [28]. Unlike reactive free radicals, aldehydes are rather long lived and can diffuse from the site of their origin and reach and attack targets which are distant from the initial free-radical event, acting as "toxic second messengers" of the complex lipid peroxidation chain reactions. DNA is also a main target; ROS attack both the base and the sugar moieties producing single- and double-strand breaks in the backbone, adducts of base and sugar groups, and cross-links to other molecules, lesions that block DNA replication [29, 30]. Several different kinds of oxidative damage to proteins have been documented [31, 32], including oxidation of sulfhydryl groups, oxidation of methionine to a sulfoxide, introduction of carbonyl groups, oxidative modification of amino acid residues close to metal-binding sites (via metal-catalyzed oxidation), addition of aldehydes to amino groups, modification of prosthetic groups or metal clusters, protein-protein cross-linking and peptide fragmentation. All these modifications are harmful to the cell, since they lead to a loss of function of membranes and proteins, and block DNA replication or cause mutations [24].

Singlet oxygen is the single most important ROS involved in APDI of microbial cells and the question arises of whether there are any antioxidant defense mechanisms devoted to protection against $^1O_2$? Certainly in green plants in which $^1O_2$ is routinely produced during photosynthesis (especially at high light intensities) by energy transfer from triplet excited chlorophylls in the chloroplasts, sophisticated defense mechanisms have evolved [33]. Carotenoids are considered to be the main $^1O_2$ quenchers in chloroplasts, and light stress can induce the oxidation of the carotenoid β-carotene in Arabidopsis plants, leading to the accumulation of oxidation products, such as β-cyclocitral. This compound was found to induce changes in the expression of a large set of genes, but had little effect on the expression of gene markers related to protection against H_2O_2 [34].

3.1 Oxidative stress sensing and response in bacteria

An effective defense response against oxidative stress is a required item in the basic survival kit of all aerobic organisms, as well as those anaerobes that exist in environments subject to transient exposures to oxygen [22].

When ROS levels exceed safe limits, bacteria have the ability to organize an inducible response, resulting in increased expression of ROS detoxification enzymes along with additional protective systems that repair oxidative damage, protect susceptible enzymes from inactivation, and control the levels of free Fe^{2+} [35].

The regulation of the expression of the genes involved in the bacterial defense response to oxidative stress is complex, and often under the control of regulators that can directly sense the levels of specific ROS and activate or de-repress transcription of their target genes. Three wellstudied examples of these transcriptional regulators are the peroxide responsive regulators OxyR, PerR, and OhrR [36]. OxyR and PerR are primarily sensors of H_2O_2, while OhrR senses organic peroxides (ROOH) and sodium hypochlorite (NaOCl). OxyR and OhrR sense oxidants by means of the reversible oxidation of specific cysteine residues in their structure. In contrast, PerR senses H_2O_2 via the Fe-catalyzed oxidation of histidine residues. These transcription regulators also affect complex biological phenomena, such as biofilm formation, the evasion of host immune responses, and antibiotic resistance via the direct regulation of specific proteins [37].

Oxidized OxyR (Figure 3A) binds to promoters co-operatively with the RNA polymerase in *E. coli* [38], and positively regulates a group of peroxide stress defense genes, such as *ahpCF*, *dps*, and *katG*, whose expression is also induced by H_2O_2 [39]. PerR (peroxide resistance regulator) is the major regulator of peroxide stress defense in Gram-positive bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, and in some Gram-negative bacteria, including *Campylobacter jejuni* and *Helicobacter hepaticus* [40–42]. As a member of the Fur (ferric uptake regulator) family of metallo-regulators, *B. subtilis* PerR senses the intracellular Fe/Mn ratio and requires metal ions, including Zn^{2+} as a structural component, and Mn^{2+} and Fe^{2+} as regulatory ions [43]. While Fe^{2+} mediates PerR regulation of peroxide defense genes, such as *katA*, *ahpCF*, and *mrgA* (a homolog of *dps*), the negative auto-regulation of perR involves Mn^{2+} in *B. subtilis*. Although *B. subtilis* PerR regulates peroxide defense genes, perR transcription is not affected by H_2O_2 [44]. Instead, conformational changes in the PerR protein caused by H_2O_2 stress contribute to the

regulatory function of PerR. Oxidation of one of two histidine residues (i.e., H37 and H91) by H_2O_2 in *B. subtilis* PerR results in the dissociation of Fe^{2+} from PerR, and the de-metallated PerR can no longer bind to DNA, so these conformational changes in PerR induce gene expression [45].

Another well-characterized regulatory system of oxidative stress defense is SoxRS, which is dedicated to the regulation of superoxide defense in *E. coli* and Salmonella [46]. SoxR (superoxide response regulator) was first identified as a genetic locus that positively regulates protein expression after exposure to superoxide-generating agents, such as paraquat [47, 48]. Redox-cycling compounds that directly generate the superoxide anion activate SoxR [49], and the activated SoxR stimulates expression of SoxS, which subsequently induces oxidative stress defense genes (Figure 3B) [50].

3.2 Defense mechanisms to oxidative stress

The emergence of oxygen in the atmosphere led to the development of defense mechanisms that either maintained the concentration of the O_2-derived radicals within acceptable levels, or else repaired oxidative damage. Iron plays a significant role in biology (transport, storage and activation of molecular oxygen, reduction of ribonucleotides, activation and decomposition of peroxides, and electron transport) and Fe^{2+} is required for the growth of almost all living cells. Due to its potential damaging effects, in bacteria, iron solubilization and metabolism is strictly regulated at two levels: (i) uptake into the cell by specific membrane-bound receptors; and (ii) storage inside the cell by two proteins, bacterioferritin (very similar to the eukaryotic ferritin) but presenting ferroxidase activity. Some molecules are constitutively present and help to maintain an intracellular reducing environment, or to scavenge chemically reactive ROS. These molecules comprise pools of non-enzymatic antioxidants such as NADPH and NADH, β-carotene, ascorbic acid, α-tocopherol, and glutathione (GSH). GSH, present at high concentrations, maintains a strong reducing environment inside the cell, and its reduced form is maintained by glutathione reductase using NADPH as a source of reducing power. In addition, specific enzymes decrease the steady-state levels of reactive oxygen. Two superoxide dismutases (SOD), which convert $O_2^{\bullet-}$ to H_2O_2 and O_2, have been described in *Escherichia coli*: an iron-containing enzyme, whose expression is modulated by intracellular iron levels [51], and a manganese containing SOD, the predominant enzyme expressed during aerobic growth, whose expression is transcriptionally regulated by at least six control systems [52]. A third SOD activity with properties similar to eukaryotic CuZn-SOD, has been found in the *E. coli* periplasmic space [53]. In *E. coli*, H_2O_2 is removed by two catalases (converting it to H_2O and O_2): hydroperoxidase I (HPI), which is present during aerobic growth and transcriptionally controlled at different levels [54], and hydroperoxidase II (HPII), which is induced during the stationary phase [55]. Glutathione peroxidase and DT-diaphorase are also ROS scavenging enzymes. Secondary defenses include DNA-repair systems, and proteolytic and lipolytic enzymes. DNA repair enzymes include endonuclease IV, which is induced by oxidative stress, and exonuclease III, which is induced in the stationary phase and in starving cells. Both enzymes act on duplex DNA by removing DNA 3' termini [56]. Prokaryotic cells contain catalysts able to directly repair some covalent modifications to the primary structure of proteins. One of the most frequent repair modifications is the reduction of oxidized

disulfide bonds: (i) thioredoxin reductase transfers electrons from NADPH to thioredoxin via a flavin carrier, (ii) glutaredoxin is also able to reduce disulfide bonds, but using GSH as an electron donor and, (iii) protein disulfide isomerase facilitates disulfide exchange reactions with large inactive protein substrates, besides having chaperone activity. Oxidation of methionine to methionine sulfoxide can be repaired by methionine sulfoxide reductase. Recent experimental data described that surface-exposed methionine residues surrounding the entrance to the active site were preferentially oxidized without loss of catalytic activity, and suggested that methionine residues could function as a "last-chance" antioxidant detection system for proteins [57].

3.3 Oxidative stress induced by APDI

The most relevant molecules produced by APDI that can induce oxidative stress in bacteria are singlet oxygen, hydroxyl radicals, superoxide anions, and hydrogen peroxide. How much and in what quantity these different ROS are generated depends on the chemical origin of the given PS and its microenvironment [58].

The APDI-induced damage to bacteria is specific in terms of the localization of a given PS. That means, when a PS is only attached to the surface of a bacterium, oxidative damage of proteins and fatty acids appears only at the site of localization due to the high reactivity, short lifetime and limited diffusion of the generated ROS [59, 60].

4. Can resistance develop after sub-lethal APDI?

It is known that repeated usage of antimicrobial agents at low (sub-lethal) concentrations may lead to the development of bacterial population that are more resistant (or at least more tolerant) to higher concentration of these agents [61].

If APDI was used in the treatment of infections and the PS would reach the target site at only sub-lethal concentrations, and was therefore activated by light producing sub-lethal of ROS, any microorganism viable at the site of infection would be exposed to doses of APDI that would not result in total cell death, i.e., sub-lethal doses of APDI (sAPDI), exposing survivors to ROS stress. Since aPDI can kill all microbial cells if the dose is high enough, increased tolerance would be manifested by the need for much higher concentrations of PS and/or higher doses of light. It is clear that ROS can damage DNA. Such stress also leads to increased mutational events, which could lead to selection for survival of more resistant or less susceptible strains [62].

The most frequently employed method of testing for a microbe's ability to become resistant to a particular agent is by subjecting the said microbe to routine continual exposure to a particular agent. Giuliani et al. analyzed whether the Gram-positive bacteria *Staphylococcus aureus*, and the Gram-negative bacteria *Pseudomonas aeruginosa* could become resistant to APDI via repeated exposure to tetracationic PS Zn(II) phthalocyanine derivative in concert with 30 J/cm^2 of 600–700 nm light. It was shown that after 20 consecutive APDI treatments at PS concentrations corresponding to the previously determined minimum inhibitory concentrations (MIC), *S. aureus,* and *P. aeruginosa* were all incapable of developing resistance to APDI. However, when the 20 exposures were repeated without light, their

results showed that the MIC of Zn (II) phthalocyanine derivative for *S. aureus* in the dark did increase. This shows that *S. aureus* may be able to develop some ability to protect itself against the dark toxic effect of a PS, perhaps by up-regulating efflux pumps or altering its membrane structure [63].

In order to assess the possible development of tolerance to antimicrobial blue light alone in *P. aeruginosa*, 10 repeated cycles of sub-lethal inactivation of bacteria in vitro, followed by bacterial re-growth, were carried out by Amin et al [64]. This tolerance study showed no evidence of the development of tolerance by *P. aeruginosa* to blue light after 10 consecutive cycles of sub-lethal inactivation. Their finding was in agreement with that obtained before when an *Acinetobacter baumannii* strain was treated with blue light for 10 consecutive cycles of sub-lethal inactivation [65].

Tavares et al. also attempted to model APDI resistance in bioluminescent *E. coli* and *Vibrio fischeri* by repeated exposure to 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)-porphyrin triiodide (TriPy+-Me-PF) excited with broad-band white light. After an initial exposure to APDI conditions in suspension (4 $\log_{10}$ reduction in cell viability), cells were plated, re-grown overnight, and repeatedly exposed to APDI 10 times thereafter using the aforementioned protocol. Their results demonstrated that neither *E. coli* nor *V. fischeri* could develop resistance to APDI [66].

Cassidy *et al.* exposed *S. aureus* and *P. aeruginosa* to the PS meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP) and methylene blue (MB) for 72 h in an effort to "habituate" cells. It was shown that sub-lethal APDI did not decrease the susceptibility to commonly employed antibiotics. Similarly, habituation with sub-lethal APDI did not reduce susceptibility of *P. aeruginosa* isolates to APDI protocols previously determined as lethal (eradication). A reduction in susceptibility to APDI following habituation was apparent for two *S. aureus* isolates with MB and for 1 S. aureus isolate with TMP as the photosesnitizer [67].

Pourhajibagher et al. studied the effects of APDI with toluidine blue O (TBO) and light emitting diode irradiation, on virulence features and expression profiling of genes encoding potential virulence factors in a colistin-resistant extensively-drug resistant (XDR) clinical isolate of *A. baumannii* (CR-XDR-AB) and in *A. baumannii* ATCC 19606 by studying the cells surviving APDI. Their results showed that the sAPDI could lead to modulation of the virulence of *A. baumanii* strains in surviving cells in planktonic growth mode by suppressing the expression of the genes (*csuE*, *epsA*, and *abaI*) associated with biofilm formation, but not *blsA* (the gene corresponding to attenuation in biofilm formation) at the transcriptional level. Following sAPDI, there was a significant up-regulation of *blsA* in CR-XDR-AB, but no up-regulation in the 19606 strain. Insignificant changes in the gene encoding bacterial heat shock protein (*dnaK*) and DNA repair gene (*recA*) in surviving cells demonstrated that sAPDI did not induce the typical cell stress fund with other antibacterial treatments, and there was no observable DNA damage when applied to *A. baumanii* strains in the planktonic growth mode. CR-XDR-AB cells surviving sAPDI showed a reduction of cell metabolic activity, increase in outer membrane permeability, and inhibition of efflux

pump systems. sAPDI reduced the minimum inhibitory concentrations of the most tested antimicrobials by 2-fold in CR-XDR-AB strain [68].

Pourhajibagher et al. also evaluated whether *Enterococcus faecalis* cells exposed to indocyanine green (ICG), TBO, and MB-sAPDI exhibited changes in metabolic activity and biofilm formation. The anti-metabolic and anti-biofilm potential at sub-lethal APDI doses mediated by ICG, TBO, and MB against *E. faecalis* was analyzed using the XTT reduction assay, crystal violet assay, and scanning electron microscopy. Their results showed that higher doses of sAPDI adversely affected biofilm formation ability and metabolic activity. ICG-, TBO-, and MB-APDI at a maximum sub-lethal dose clearly reduced the formation of biofilm up to 42.8%, 22.6%, and 19.5%, respectively. ICG-, TBO-, and MB-sAPDI showed an obvious reduction in bacterial metabolic activity by 98%, 94%, and 82%, respectively. ICG-APDI showed a stronger inhibitory effect on biofilm formation in *E. faecalis* than MB- and TBO-APDI at sub-lethal levels. Interestingly, a gradual increase in metabolic activity and biofilm formation were observed upon exposure to a lower dose of sAPDI [69].

Kashef et al. study aimed to determine the effect of sAPDI on the antibiotic susceptibility of clinical *S. aureus* isolates. Forty clinical *S. aureus* isolates were exposed to sAPDI with TBO and MB. After exposure, susceptibility of surviving organisms to a range of antibiotics was determined and compared with the susceptibility of an untreated control. It was observed that the effect of sAPDI on antibiotic susceptibility patterns of isolates was strain-dependent. Thirty-five (85.4%) isolates were resistant to oxacillin but after exposure to sub-lethal TBO-APDI, only 13 (31.7%) remained resistant to this antibiotic ($p < 0.001$). In contrast, only 9 (22%) isolates were resistant to erythromycin, however, sub-lethal TBO-APDI increased resistance to this antimicrobial agent (61%), ($p < 0.001$). Exposure to sub-lethal TBO-APDI also increased resistance to amoxicillin-clavulanate. Exposure to sub-lethal MB-APDI decreased resistant to oxacillin and piperacillin. A reduction in susceptibility to amikacin and erythromycin following sAPDI was apparent with MB [70].

It is very important to emphasize one severe limitation of these studies. APDI is designed to be a relatively brief clinical procedure, as opposed to antibiotics, which are applied for relatively prolonged periods. Accordingly, each of these aforementioned studies exposed microbial samples to APDI conditions (light and PS) for brief periods of time, then removed these conditions and allowed the remaining bacteria to re-grow. This approach to APDI resistance, fails to address a serious problem: when microbes acquire resistance to antimicrobial agents, they must continuously grow in the presence of low-levels of antimicrobial agents. This is best exemplified by the emergence of antibiotic-resistance microbial isolates from the widespread employment of antibiotics in livestock feedstuffs [71].

The approach of chemical antimicrobials is quite opposite to that of APDI. APDI is inherently designed to be a brief process in which excess PS is washed away and after delivery of light of the appropriate wavelength and desired fluence, light is removed from the region exposed to APDI. Thus, a more realistic way to establish a possible increased tolerance or resistance to APDI would be by allowing microbial samples to grow continuously under sAPDI conditions; i.e. exposed to defined sub-lethal levels of PS and

light. This also, has obvious limitations in that APDI is not designed to be an extended procedure; however, such a protocol could be clinically rationalized by considering extended microbe-PS interactions; antimicrobial PSs are generally cationic molecules such that there is sufficient interaction between the PS and the anionic cell envelope of Gram-positive and Gram-negative bacteria. If a patient were to receive an APDI treatment, removal of all excess PS after light exposure would prove entirely impossible. This then follows that as the patient goes about his or her daily life, exposure to ambient light sources may be unavoidable, thus creating quasi-sub-lethal PDI stress.

5 Mechanisms determining bacterial susceptibility to APDI

Studies have focused on bacterial protection from radical-type oxygen species such as superoxide and hydrogen peroxide. However, the mechanism by which bacteria respond to APDI-mediated stresses remains relatively unknown [72].

5.1 Protective pigments

In recent years, many reports about the efficacy of APDI against *P. aeruginosa* have been published, and it has become clear that *P. aeruginosa* is particularly tolerant to photodynamically induced oxidative stress [73–75]. The ability of *P. aeruginosa* to produce pigments, combined with the ability to elicit an oxidative stress response [76], may thus contribute to survival to APDI-induced oxidative stress.

Orlandi et al. [77] investigated the role of pigments in tolerance of *P. aeruginosa* to APDI-induced oxidative stress. To this end, differentially pigmented transposon mutants of *P. aeruginosa* PAO1 were isolated and submitted to APDI with two different PS, a phenothiazinium dye, TBO, that acts mainly via a type I mechanism, and a porphyrin dye, 5,10,15,20-tetrakis-(1-methyl-4-pyridyl)-21H,23-porphine tetra-p-tosylate (TMPyP), that acts mainly via a type II mechanism. In general, in the presence of pigments a higher tolerance to APDI-induced photo-oxidative stress was observed. Hyper-production of pyomelanin makes the cells much more tolerant to stress caused by either radicals or singlet oxygen generated by different PS upon photo-activation. Phenazines, pigments characteristic of *P. aeruginosa* (pyocyanin and phenazine-1-carboxylic acid) produced in different amounts depending on the culture conditions, are able to counteract both types of APDI-elicited ROS. Hyper-production of pyoverdin, caused by a mutation in a quorum-sensing gene, made *P. aeruginosa* more tolerant to a PS that generates mainly singlet oxygen, although in this case the observed tolerance to photo-oxidative stress cannot be completely attributed to the presence of the pigment.

Carotenoids are naturally occurring terpenoid pigments, formed from isoprene residues making up a polyene chain of conjugated double bonds. These pigments are responsible for the wide variety of orange-red colors seen in nature, and absorb light in the wavelength range of 300– 600 nm [78].

For many years the role of carotenoids as accessory pigments in photosynthesis was thought to be the only function in photosynthetic organisms. However, Sistrom et al. [79] suggested that carotenoids might be acting as protective agents against photodynamic action in

bacteria. They studied the wild type and an isogenic mutant of *Rhodopseudomonas spheroides* ("blue-green") that did not produce colored carotenoids but instead accumulated the colorless carotenoid precursor, phytoene. These workers found that when the blue-green mutant was grown in the presence of light and air, growth stopped in the light, chlorophyll was destroyed and the cells were killed. On the other hand, the wild type, with its normal level of carotenoid pigments, is not injured by growth in light and air; in the presence of air, chlorophyll synthesis stops and the organism grows by aerobic metabolism. It should be noted that the deletion of the carotenoid pigments did not markedly affect the ability of the blue-green mutant to grow photosynthetically in the presence of light, but in an atmosphere of nitrogen (bacterial photosynthesis is anaerobic). Only when both light and air were present did the lack of carotenoids lead to photosensitization. The authors were able to show that it was the bacteriochlorophyll that was responsible for the lethal photosensitization of the blue-green mutant and that both oxygen and light were necessary for the destructive reaction to occur (photodynamic action). They concluded that the carotenoid pigments functioned as protective agents against this photodynamic killing and that the bacteriochlorophyll was the endogenous photosensitizer. These findings that carotenoids could protect against chlorophyll photosensitization, have been confirmed in other photosynthetic bacteria and algae [80].

Most *S. aureus* strains produce an orange membrane-bound carotenoid pigment known as staphyloxanthin. It is a typical secondary metabolite. It is not necessary for the growth and reproduction of *S. aureus* but might serve a role in survival in infected hosts and in avoiding the immune system [81, 82]. Liu et al. showed that a *S. aureus* mutant with disrupted carotenoid biosynthesis was more susceptible to oxidant killing, indicating that carotenoids could act as a virulence factor [83]. Carotenoids could reduce the penetration of singlet oxygen by decreasing membrane fluidity [84].

5.2 Capsular polysaccharide

Due to the short diffusion distance of APDI-generated ROS, its efficiency to kill bacteria depends on the degree to which the PS binds to bacteria, and whether the PS can penetrate to a sensitive intracellular site. Capsular polysaccharide could either increase or decrease the binding of the PS to the bacterial cell and, independently of binding, could also act as a barrier to penetration of the PS into the interior of the microorganism, where the generation of ROS would be more likely to lead to cell death. Gad et al. used two isogenic pairs of wild-type and transposon mutants deficient in capsular polysaccharide and slime production generated from strains of *Staphylococcus epidermidis* and *S. aureus* to examine the effects of extracellular slime on susceptibility to APDI mediated by two cationic PS (a polylysine-chlorin e6 conjugate, pL-ce6, and MB) and an anionic molecule, free ce6, and subsequent exposure to 665-nm light. Free ce6 gave more killing of mutant strains than wild type, despite the latter taking up more PS. The cationic pL-ce6 and MB gave similar uptakes and killing despite a 50-fold difference in incubation concentration. Differences in susceptibility between strains observed with free ce6 largely disappeared with the cationic compounds despite significant differences in uptake. These data suggested that slime production can be a problem against APDI for gram-positive bacteria but that this problem can be overcome by using cationic PS [85].

5.3 Efflux pumps

One possible mechanism determining the efficiency of APDI of bacteria might be its dependence on multi-drug resistant efflux pumps [86]. Efflux pumps (summarized in Figure 4) are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment [87]. Such pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds.

Phenothiazinium dyes have long been established as nontoxic and clinically useful compounds both for staining living tissues [88] and for some pharmacological indications [89, 90]. This consideration together with their ready availability has been important in their selection as antimicrobial PSs for the few clinical indications in which APDI is carried out. Tegos and Hamblin demonstrated for the first time that phenothiazinium-based PSs were substrates of multi-drug resistant efflux pumps in bacteria. This discovery raised the possibility of combining the phenothiazinium dyes with one of range of possible efflux pump inhibitors [86].

Efflux pump inhibitors (EPI) had a dramatic effect in potentiating the killing effect of APDI with phenothiazinium dyes [91]. Killing of *S. aureus* mediated by TBO and red light was greatly increased by coincubation with known inhibitors of the major facilitator pump NorA (diphenyl urea INF271, reserpine, 5'-methoxyhydnocarpin, and the polyacylated neohesperidoside, ADH7). The potentiation effect was greatest in the case of mutants that overexpressed NorA and least in NorA null cells. Addition of the EPI before TBO, had a bigger effect than addition of the EPI after TBO. Cellular uptake of TBO was increased by EPI. EPI increased APDI mediated by other phenothiazinium dyes, such as methylene blue and dimethylmethylene blue, but not that mediated by non-phenothiazinium PS, such as Rose Bengal and benzoporphyrin derivative. Killing of Gram-negative *P. aeruginosa* mediated by TBO and light was also potentiated by the resistance nodulation division pump (MexAB-OprM) inhibitor phenylalanine-arginine betanaphthylamide but to a lesser extent than for *S. aureus*. Kishen et al. [92] evaluated the ability of MB and rose bengal (RB) to inactivate biofilms of *E. faecalis*. The role of a specific microbial EPI, verapamil hydrochloride in the MB-APDI of *E. faecalis* biofilms was also investigated. The results showed that *E. faecalis* biofilms exhibited significantly higher resistance to APDI when compared with *E. faecalis* in suspension. APDI with cationic MB produced superior inactivation of *E. faecalis* strains in a biofilm along with significant destruction of biofilm structure when compared with anionic RB. The ability to inactivate biofilm bacteria was further enhanced when the EPI was used with MB. These experiments demonstrated the advantage of MB combined with an EPI to inactivate biofilm bacteria and disrupt biofilm structure.

Grinholc et al. [93] investigated whether the uptake and activity of the multi-drug resistance pumps might influence their previously observed variations [94] among the clinical strains to protoporphyrin-derived, amphipilic protoporphyrin diarginate mediated APDI. Using a new set of four selected methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) clinical strains as well as ATCC *S. aureus,* they confirmed that the bactericidal effect of the APDI was straindependent as it showed a wide variation ranging from 0 to 5 $\log_{10}$-unit

reduction in viable counts. However, neither variation in levels of PS uptake nor the pharmacological inhibition of efflux pump explained such a phenomenon.

5.4 Antibiotic resistance mechanisms

Another study by Grinholc group [95] aimed to provide relevant evidence as to whether the response of MRSA strains to APDI differs significantly from that of MSSA, and whether it results from antimicrobial resistance mechanisms or susceptibility to routinely used antimicrobials. *S. aureus* response to protoporphyrin IX (PPIX)-mediated photo-inactivation was studied for 424 MRSA/MSSA isolates. VITEK 2 Advanced Expert System was used to detect antimicrobial resistance mechanisms and strains' susceptibility to antibiotic therapy. Their results demonstrated that MRSA were significantly more resistant to photo-inactivation than MSSA strains; however, the difference observed did not result from antimicrobial susceptibility or resistance mechanisms. Moreover, it was determined that the ability to form biofilms in vitro did not explain the observed differences between MRSA and MSSA strains.

Tang et al [96] compared APDI with pL-ce6 and TBO in clinical MRSA and ESBL producing *E. coli*, together with their corresponding ATCC reference strains. pL-ce6 at 8 μM, 30 J/cm2 gave 5 logs killing for ESBL-producing *E. coli* and *E. coli* (ATCC 25922); 4 log killing for MRSA, and 3 log killing for *S. aureus* (ATCC 25923). TBO at 80 μM, 30 J/cm^2 only gave 3 logs killing of MRSA and 2 log killing of *S. aureus* (ATCC 25923). TBO (400 μM, 30 J/cm^2 induced equal killing for both *E. coli* strains The MRSA isolate responded better than reference *S. aureus* to both PS.

5.5 Anti-oxidant enzymes

To understand if the antioxidant enzyme status may be involved in the *S. aureus* response to APDI, Nakonieczna et al. [97] checked survival rates of the isogenic *sod* mutant and wild-type strains of *S. aureus* and compared the effect of SOD in the response to APDI at both the protein as well as gene expression levels. Superoxide dismutase forms the first line of bacterial defense against oxidative stress, therefore it was expected that a correlation might exist between the SOD status in the cell and the response to APDI. The effectiveness of APDI towards *S. aureus* and its SOD isogenic mutants deprived of either of the two SOD activities, namely SodA or SodM or both of them showed similar results, regardless of the SOD status cultured in TSB medium. On the contrary, in the special CL medium (without Mn2+ ions) the double SodAM knockout mutant was highly susceptible to APDI. Among 8 clinical isolates of *S. aureus* analyzed (4 MRSA and 4 MSSA), strains, highly resistant and strains highly susceptible to APDI were identified. They observed that SOD activity, as well as sodA and sodM transcript level increased after protoporphyrin IX-based APDI, but only in APDI-sensitive strains. They confirmed that porphyrin-based photo-killing efficacy was a strain-dependent phenomenon. They showed that oxidative stress sensitivity caused by the lack of both SOD enzymes can be relieved in the presence of Mn^{2+} ions and partially in the presence of Fe^{2+} ions. The fact that SOD activity increased was observed only in APDI-susceptible cells emphasizes that this is probably not a direct factor affecting *S. aureus* vulnerability to porphyrin based APDI.

5.6 Agr status

As a global transcription regulator, the accessory gene regulator (Agr) plays a central role in gene regulation in *S. aureus* [98]. Activation of the Agr system down-regulates genes associated with bacterial adhesion and up-regulates genes for exoprotein production [99]. Park et al. [100] wanted to gain insight into the mechanism of bacterial response to APDI by exploring the genetic response of *S. aureus* to APDI combined with the photosensitizer Ce6 and laser light. It was shown that Agr was involved in *S. aureus* response to oxidative stress induced by APDI. Transcriptional profiling revealed that sAPDI induced a general stress response and also activated Agr-dependent gene regulation. Moreover, mutant *S. aureus* lacking Agr function showed hyper-susceptibility to two different APDI regimens with higher energy densities, which led to the hypothesis that the function of the Agr was necessary for protection of *S. aureus* from APDI-mediated oxidative stress.

S. aureus expresses a large variety of virulence factors that are accurately regulated by a complex network of different transcriptional factors (e.g.: *mgr*A, σ^B) and several two-component systems (e.g.: *sae*) [101, 102]. The *sae* system up-regulates a wide variety of virulence factors including proteins that link bacteria to the extracellular matrix of the host, like the adhesins Eap or FnBPs [103]. Moreover, both *agr* and *sae* systems have been found to be involved in the response to oxidative stress [104, 105]. Gándara et al. [106] studied the differential susceptibility to TBO-APDI of *S. aureus* mutants in different key regulators of virulence factors involved in planktonic lifestyles, and which have been related (to some extent) to oxidative stress. They showed that the two-component system *sae* impaired the response to TBO-APDI through a mechanism not related to the Eap adhesin. They also showed that the *agr* locus does not seem to be related to TBO-APDI sensitivity. However, by employing *S. aureus* mutants lacking the *agr* system, it was shown that *agr* induced a certain degree of resistance against APDI employing protoporphyrin diarginate and chlorin e6 as PS [100, 107]. It is therefore important to consider that different PS elicit photo-damage by different mechanisms, and this may be related to the different strain responses to APDI.

5.7 Heat shock proteins

Heat shock proteins (HSPs) are indicative of stressful conditions that may affect cell viability. They are a group of ubiquitous chaperone proteins responsible for the refolding, repair and recycling of damaged proteins, and stabilization of lipid membranes during cellular stress [108– 110]. In microbial cells, the heat shock proteome has best been characterized in *E. coli*, and it has been found that two major HSP families—GroEL / GroES and DnaK/ DnaJ / GrpE—are mainly responsible for protection against stress in both Gram-negative and Gram-positive bacteria [111–114]. Up-regulation of HSPs during oxidative, antibiotic, osmotic and acid stress is associated with resistance to these stresses, and up-regulation of HSPs prior to subsequent stress enables bacterial cells to acquire "tolerance" to that particular stress [114].

To date, only two studies have looked at the bacterial heat shock response to APDI. The first of these studies was performed by Bolean et al. [115]. They tested whether the expression of GroEL by *Streptococcus mutans* was enhanced after APDI with rose bengal. Higher HSP expression was detected in bacteria after APDI treatment as compared with light alone or PS

alone. The expression of HSP after APDI was similar to that induced by osmotic stress. No DNA degradation was observed after APDI of *S. mutans*. They concluded that APDI may cause effects similar to those of other stressful conditions in *S. mutans*, and cell death induced by this treatment reflects the inability of the bacteria to protect itself sufficiently against the lethal effects of APDI with rose bengal.

St Denis et al. [116] investigated whether APDI induced protective responses such as HSPs in bacteria. Using TBO at sub-lethal APDI conditions, a sevenfold increase in bacterial HSP GroEL and a three-fold increase in HSP DnaK were observed in *E. coli* post APDI. Pretreatment with 50°C heat for 30 min reduced APDI killing in both *E. coli* and in *Enterococcus faecalis*. Inhibition of the highly conserved chaperone DnaK using a small molecule benzylidene lactam HSP inhibitor potentiated (but not significantly) the effect of APDI in *E. faecalis*; however, this effect was not observed in *E. coli* presumably because inhibitor could not gain access due to the Gram-negative permeability barrier. They concluded that induction of HSPs may be a mechanism whereby bacteria could become tolerant to APDI and called for the need for further study in the application of dual APDI-HSP-inhibition therapies.

6. Biofilm resistance to APDI

A structured consortium of microbial cells (mostly bacteria or fungi) attached onto a living or inert surface is formed by the cells sticking to each other where they are surrounded by the self-produced extracellular polymeric matrix (composed of polysaccharides, proteins, lipids, and extracellular DNA) and is known as a biofilm (Figure 5). The formation of biofilm is considered an adaptation of microbes to hostile environments [117, 118]. Bacteria that live in a biofilm community possess several advantages, including structural stability, firm adherence to biotic or abiotic surfaces, increased virulence, and resistance to both antimicrobial therapy and the host immune response [18, 119, 120].

The principal hypotheses to explain reduced antibiotic susceptibility of bacteria in biofilms are the possibility of slow or incomplete penetration of the antibiotic into the biofilm (due to biofilm matrix components proving a mechanical barrier to diffusion), modification of microbial enzymes and efflux pumps, an altered chemical microenvironment within the biofilm (low oxygen concentration and slow growth), a unique, and highly protected, phenotypic state (persister cells), and high cell density due to quorum sensing (Figure 6) [121, 122].

It has been shown that bacteria growing as biofilms are less susceptible to APDI compared with their equivalent planktonic forms. The dye concentration and the light dose needed for the photo-inactivation of biofilms are considerably higher than those required to inactivate planktonic bacterial suspensions [123–125]. In fact, cells growing in biofilms differ from their planktonic counterparts in a number of aspects, such as the cell wall structure and composition, rate of growth, and presence of polysaccharide intercellular adhesin (PIA), which may block both the uptake of the PS and the penetration of light, and thereby reduce the photosensitizing efficiency [126, 127]. Generally, longer pre-incubation times (up to 24 h), higher concentrations (up to 25 times) and longer light exposure times (up to 30 min) are

required to reach a phototoxicity of 3 log10 steps against biofilm growing bacteria compared to their planktonic counterparts [58]. To date no specific regulatory process is known for biofilm tolerance to APDI.

6.1 Can Nanotechnology Potentiate APDI of Biofilm?

The use of nanotechnology is an attractive approach to improving the effectiveness of APDI against resistant microbial cells [128–133] and to improve PS delivery to biofilms [134–136]. This goal may be achieved in different ways, for instance by enhancing the delivery of PS to microorganisms (encapsulating the PS in nanoparticles) [137] or by increasing the $^1O_2$ yield of the PS (by covalently binding the PS to the surface of the nanoparticles [138], or simply by mixing the nanoparticles and PS together). In some cases the nanoparticles themselves (for instance TiO2, fullerenes or quantum dots) have been shown to act as PS themselves and are capable of photodynamically inactivating the microorganisms [139].

Shrestha and Kishen assessed the antibacterial effect of a novel PS (Rose Bengal functionalized chitosan nanoparticles [CSRBnp]) to eliminate bacteria in the presence of various root canal constituents that are known to inhibit the antibacterial efficacy of root canal disinfectants. Chitosan is an attractive material from which to make antimicrobial nanoparticles as it is a naturally occurring polymer, is biodegradable and biocompatible, and moreover it posseses an intrinsic cationic charge [140]. The antibacterial effect of CSRBnp was tested on planktonic *E. faecalis* with or without pretreatment by using different inhibiting agents such as dentin, dentin-matrix, pulp tissue, bacterial lipopolysaccharides, and bovine serum albumin (BSA). Pulp and BSA inhibited the antibacterial effect of CSRBnp (without photoactivation) significantly (P < 0.05) even after 24 hours of interaction. CSRBnp completely eliminated the bacteria after 24 hours of interaction after photodynamic therapy [132].

Chitosan (CS) nanoparticles were also used in a study by Chen et al. to investigate their ability to potentiate the activity of erythrosine (ER) against bacteria and yeast. CS nanoparticles loaded with ER were prepared by an ionic gelation method and tested for their APDI efficacy against planktonic cells and biofilms of *S. mutans*, *P. aeruginosa* and *Candida albicans*. Significant phototoxicity was observed when the cells were exposed to light irradiation after treatment with free ER or ER/CS nanoparticles. The antimicrobial activity of ER/CS nanoparticles was significantly higher than ER in free form [129].

Pagonis et al. studied the *in vitro* effects of poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with MB and light against *E. faecalis* (ATCC 29212). *E. faecalis* species were sensitized in planktonic phase and in experimentally infected root canals of human extracted teeth with MBloaded nanoparticles followed by exposure to red light at 665 nm. The synergism of light and MB-loaded nanoparticles led to approximately 2 and 1 log_{10} reduction of colony-forming units (CFUs) in planktonic phase and root canals, respectively. In both cases, mean log_{10} CFU levels were significantly lower than controls and MB-loaded nanoparticles without light [141].

Conjugating PSs to the surface of gold nanoparticles (GNPs) is a modern approach to increase APDI efficacy. Darabpour et al. aimed to determine whether GNPs could enhance

the efficiency of MB-APDI of MRSA in a mature biofilm. Positively charged MB was immobilized onto the negatively-charged GNPs through electrostatic interaction. Four day-old biofilms of MRSA were treated with MB-conjugated GNPs and subsequently exposed to red light. MB-conjugated GNPs showed significant photo-inactivation against 4-day-old biofilm of MRSA (>5 $\log_{10}$ CFU reduction) while free MB-APDI resulted in less than 1 log10 CFU reduction [142].

6.2 Potentiation of APDI by Inorganic Salts

We have discovered that APDI can be potentiated (up to 6 logs of extra killing) by addition of simple inorganic salts, depending on the PS structure and photochemical mechanism. The most powerful and versatile salt is potassium iodide [143], but potassium bromide [143], sodium thiocyanate [144], sodium azide [145] and even sodium nitrite have also shown potentiation. The mechanism of potentiation with iodide is believed to be singlet oxygen addition to iodide to form iodine radicals, hydrogen peroxide and molecular iodine [146]. Another mechanism involves two-electron oxidation of iodide/bromide taking place in titanium dioxide photocatalysis to form hypohalites [147, 148]. A third mechanism involves a oneelectron oxidation of azide anion to form azide radical [145].

The addition of iodide has been shown to improve the performance of aPDT in vivo using several animal models of localized infection. Potentiation of MB-APDT using red light, by addition of iodide was shown in 3rd degree burn wound in mice infected with bioluminescent MRSA [143]. Another study used a cationic fullerene (LC16) with a deca-quaternary chain and a second attached chain of ten tertiary amino groups excited by either white or UVA light [149]. A mouse model of a partial thickness skin abrasion infected with bioluminescent *A. baumannii* gave an increased rate of loss of bioluminescence signal when iodide (10 mM) was combined with LC16 and then illuminated with either UVA or with white light. Finally addition of iodide was tested in a mouse model of oral candidiasis infection mediated by MB and red light [150]. Although we have not yet specifically published a paper looking at the effect of iodide on potentiating APDI destruction of bacterial biofilms, preliminary data have shown that KI makes a surprisingly large difference to the number of logs of killing. Studies are ongoing.

7 Conclusion

APDI is an exciting new approach for the selective inactivation and eradication of microbial pathogens. As antibiotic resistance becomes an even greater issue, APDI will likely be introduced more widely into the clinical setting, however, it is crucial that APDI efficacy be practically evaluated.

The studies mentioned in this work have attempted to model and evaluate possible bacterial resistance mechanisms to APDI. While antibiotics generally work on a "key-and-lock" principle, with each drug having a single target in the bacterial metabolism, APDI is very different. APDI produces ROS that can damage a host of microbial biomolecules, most of which can be lethal, rather than just inhibiting growth. Therefore the chances that microbes can develoip tolerance or resistance to APDI must be considered highly unlikely. Although it seems that there is no universal agreement that can be reached at this time, one thing is

certain: more evaluation is needed. The scientific community should be cautious and not get too excited over numerous in vitro studies reporting the killing of a wide variety of species. Nevertheless, in vivo studies of APDT continue to show promise for carefully chosen types of infection [151, 152].

Acknowledgments

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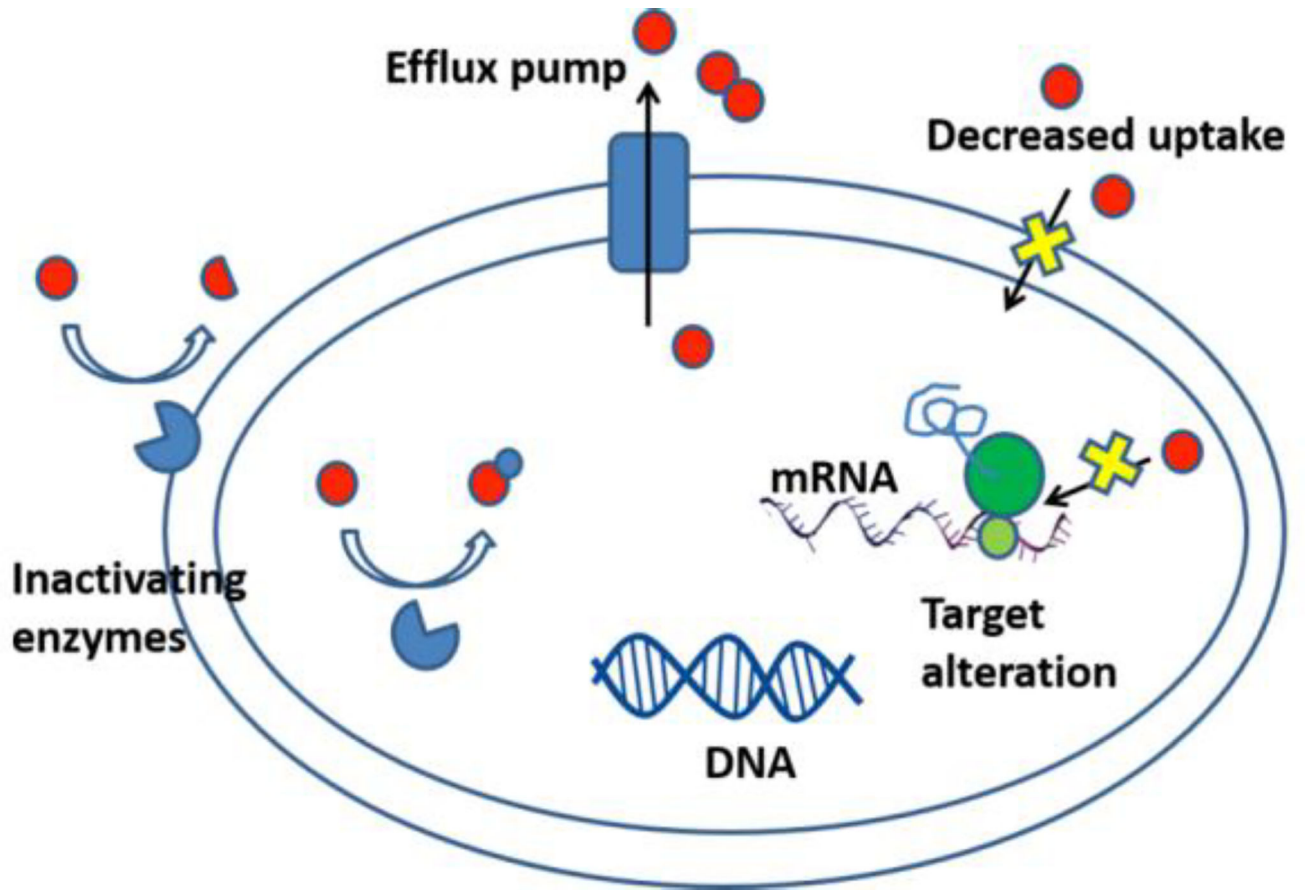
Figure 1.
Antibiotic resistance mechanisms in bacteria
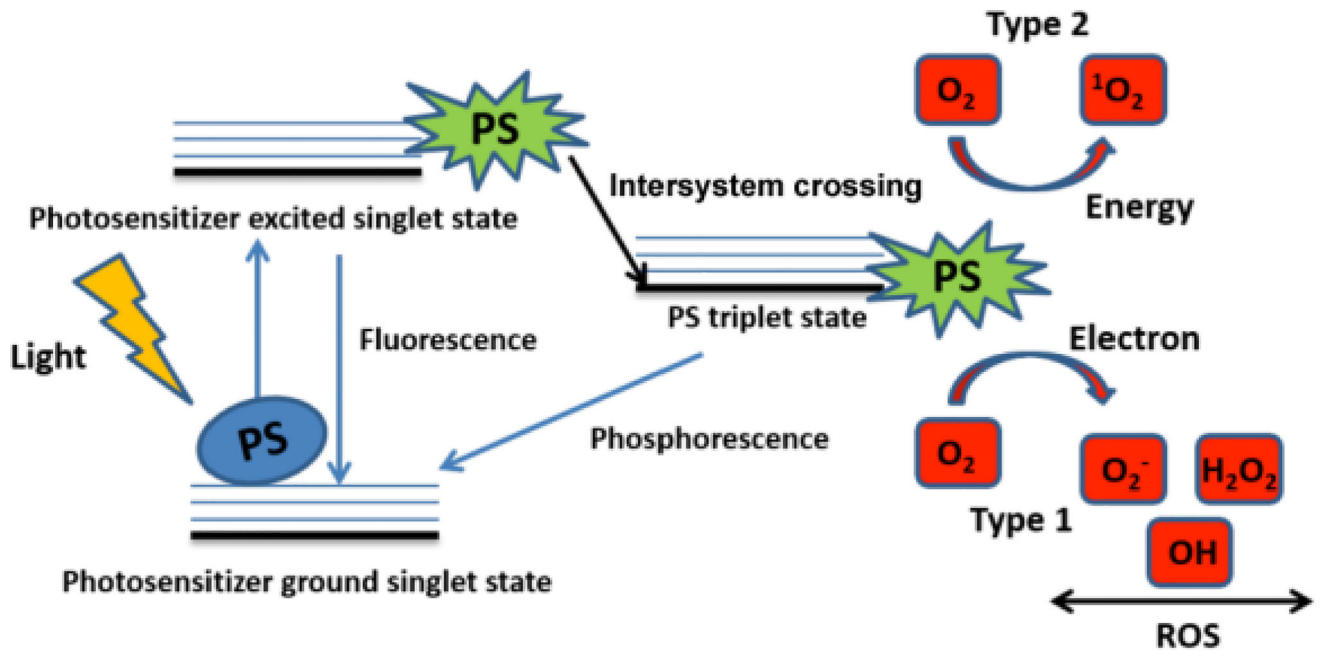
Figure 2.
Principals of Antimicrobial Photodynamic Inactivation shown in a Jablonski Diagram
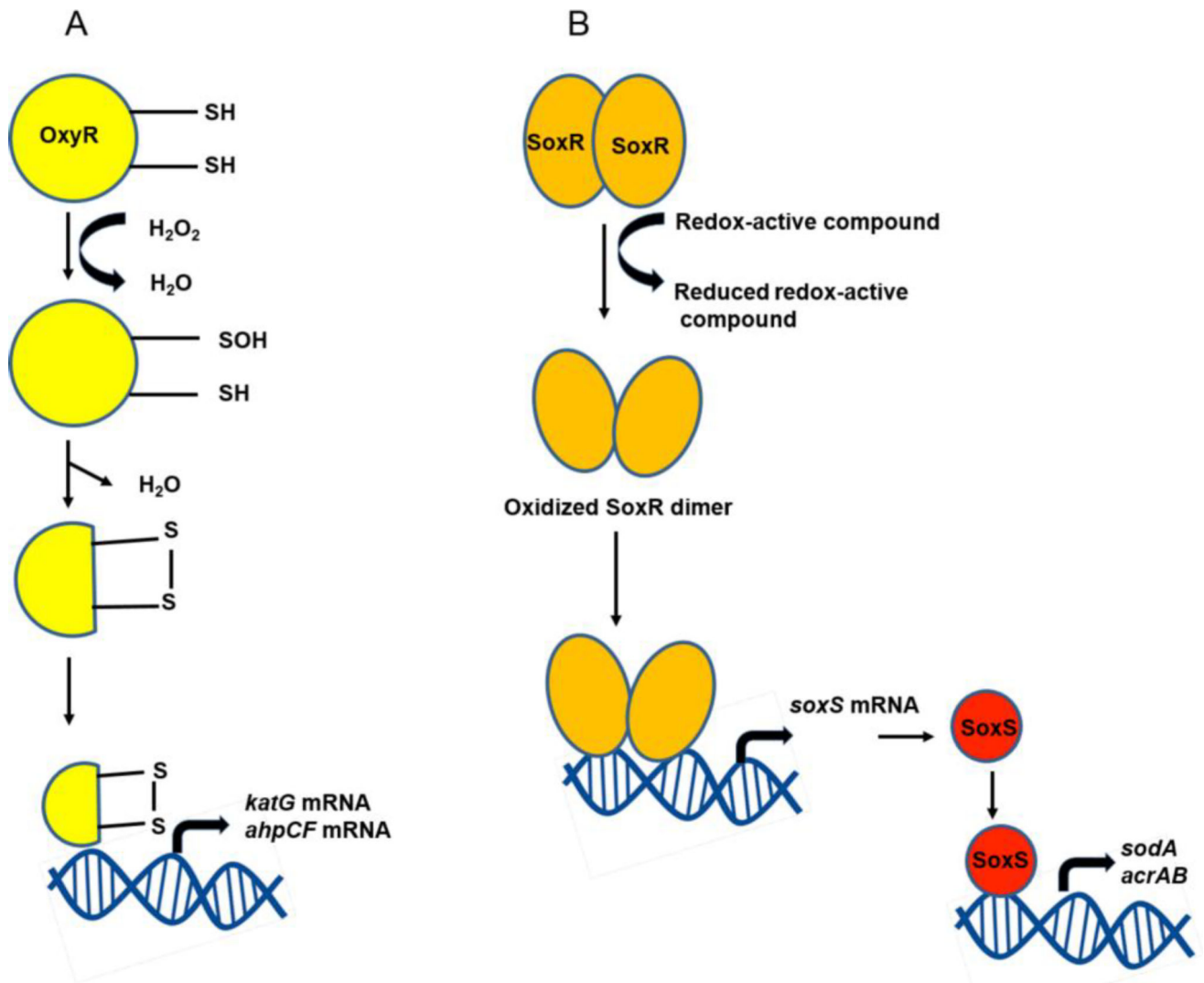
Figure 3.
Activation of redox-sensitive transcriptional regulators in *Escherichia coli*.
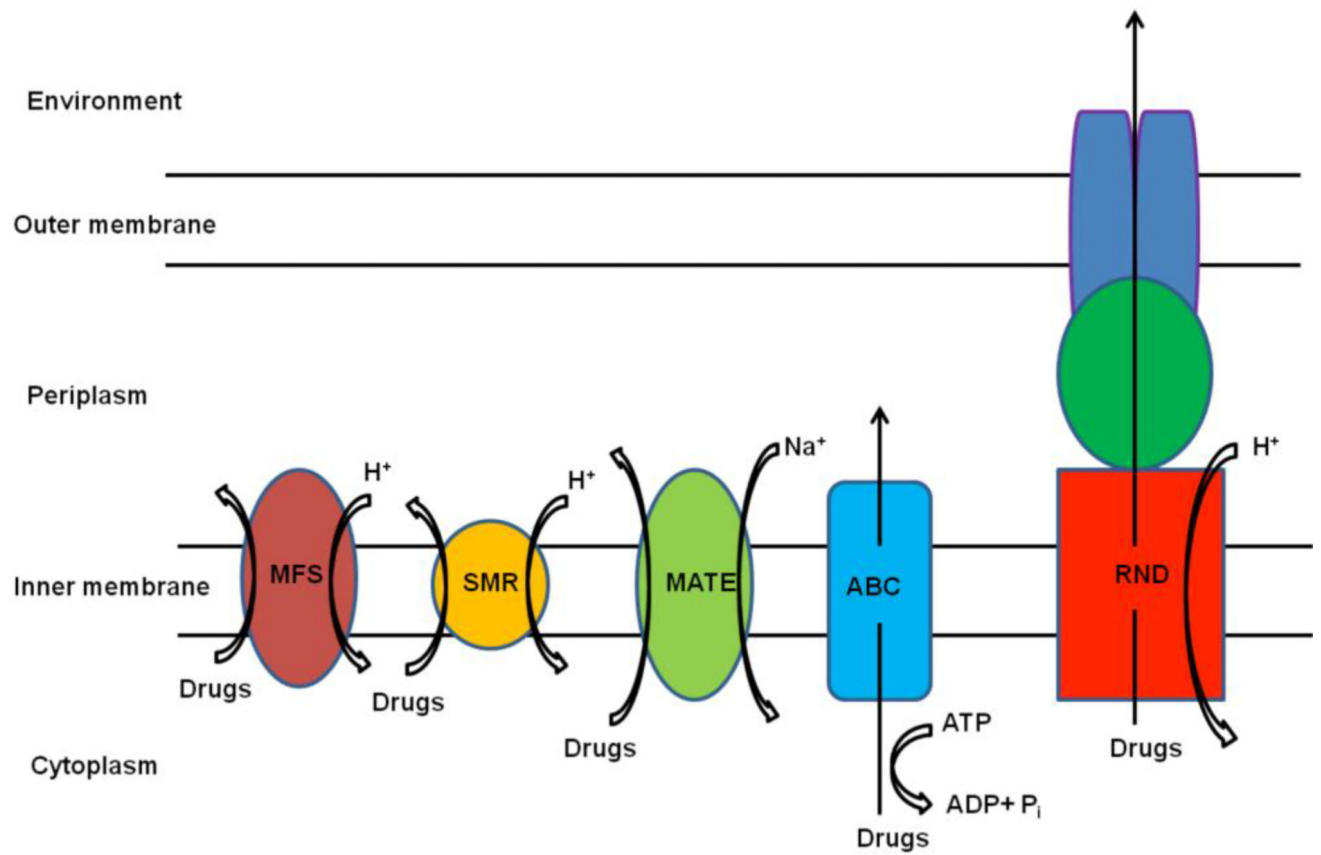
Figure 4.
Schematic illustration of the main types of bacterial drug efflux pumps (MFS: major facilitator superfamily, SMR: small multi drug resistance, MATE: multi drug and toxic compound extrusion, ABC: ATP-binding cassette family, and RND: resistance-nodulation-division family)
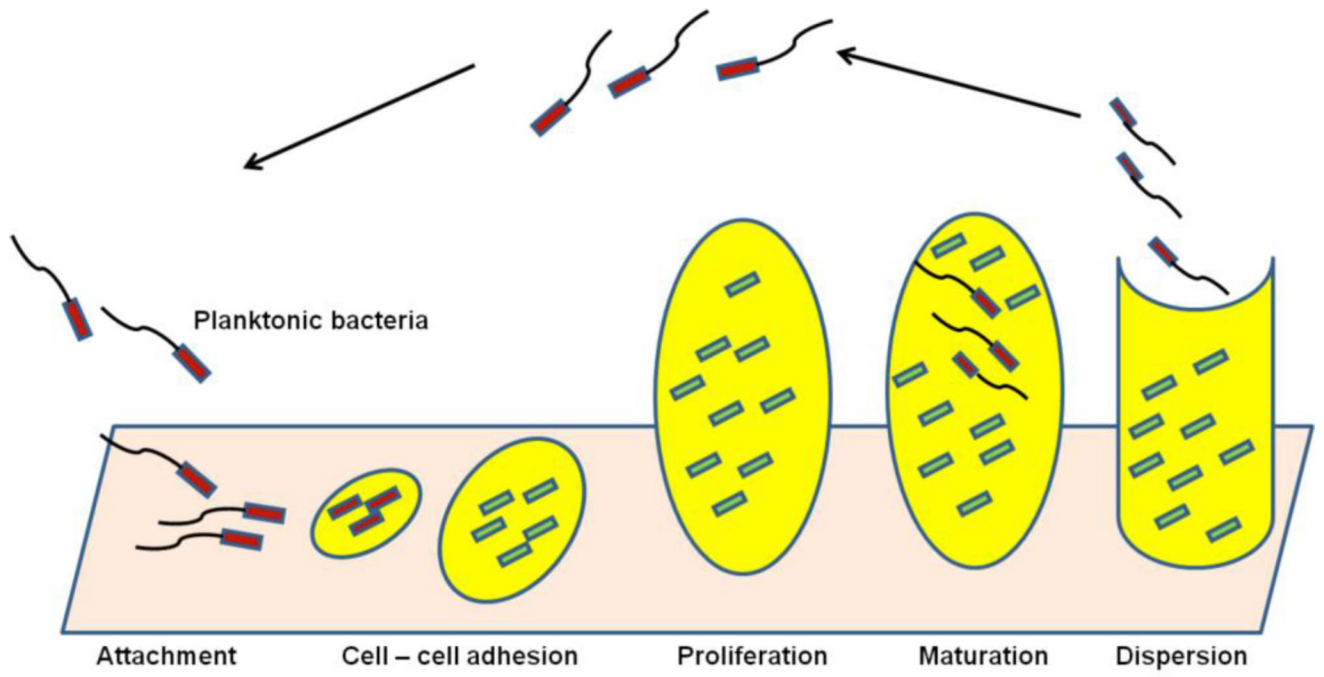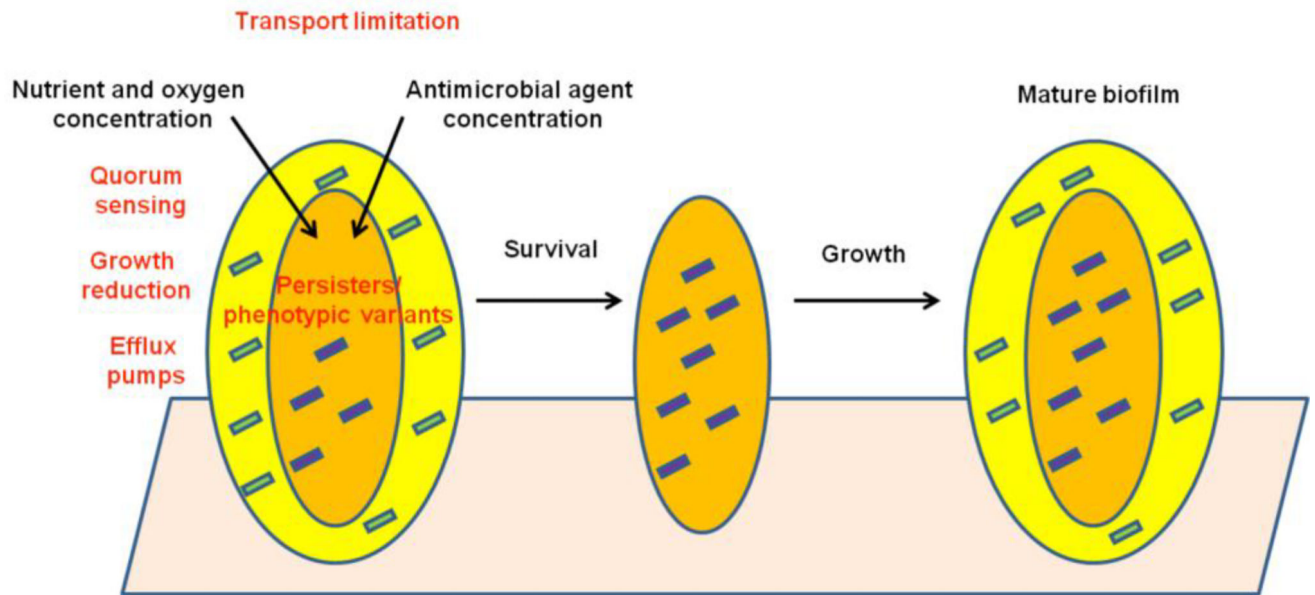
Figure 5.
Stages of biofilm development

Figure 6.
Mechanisms of reduced antimicrobial susceptibility of bacteria in biofilm (1-Antimicrobial agents kill susceptible outer cells, 2- Inner persister cells survive, 3- Entire biofilm regenerates)