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Antimicrobial strategies centered around reactive oxygen species - bactericidal antibiotics, photodynamic therapy and beyond

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Abstract

Reactive oxygen species (ROS) can attack a diverse range of targets to exert antimicrobial activity, which accounts for their versatility in mediating host defense against a broad range of pathogens. Most ROS are formed by the partial reduction of molecular oxygen. Four major ROS are recognized comprising: superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), but they display very different kinetics and levels of activity. The effects of $O_2^{\bullet -}$ and H_2O_2 are less acute than those of $^{\bullet}OH$ and $^1O_2$, since the former are much less reactive and can be detoxified by endogenous antioxidants (both enzymatic and non-enzymatic) that are induced by oxidative stress. In contrast, no enzyme can detoxify $^{\bullet}OH$ or $^1O_2$, making them extremely toxic and acutely lethal. The present review will highlight the various methods of ROS

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formation and their mechanism of action. Antioxidant defenses against ROS in microbial cells and the use of ROS by antimicrobial host defense systems are covered. Antimicrobial approaches primarily utilizing ROS comprise both bactericidal antibiotics, and non-pharmacological methods such as photodynamic therapy, titanium dioxide photocatalysis, cold plasma and medicinal honey. A brief final section covers, reactive nitrogen species, and related therapeutics, such as acidified nitrite and nitric oxide releasing nanoparticles.

1. Introduction

Since the evolution of oxygen-based life as we know it, all living organisms have had to cope with the possibility of their critical molecular components being damaged by reactive oxygen species (ROS). Low levels of ROS such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are produced as a consequence of aerobic respiration and metabolism, but sensing mechanisms combined with constitutive and inducible antioxidant defenses (superoxide dismutase and catalase) have evolved to largely neutralize these ROS without damage occurring. However larger concentrations of $O_2^{\bullet -}$ and H_2O_2 may overwhelm these defenses, and small amounts of ROS against which organisms do not have adequate defenses such as hydroxyl radical (**.**OH) and singlet oxygen ($^1\Delta gO_2$, commonly abbreviated as $^1O_2$; an abbreviation that will be used throughout our manuscript) may rapidly prove fatal, especially to microorganisms.

Oxidizing agents have long been used as disinfectants and antiseptics, but have not found widespread applications as anti-infectives due to their perceived lack of specificity towards microbial cells when compared with host mammalian cells; in other words they are considered likely to cause unacceptable damage to normal tissue. In recent times however, new ways of generating ROS have been discovered that allow therapeutic application to be considered for actual infections, both systemically and topically. The discovery that microbicidal antibiotics produce their killing effect by inducing the formation of hydroxyl radicals within bacterial cells (Kohanski, *et al.*, 2007), together with the known use of ROS generation by host defense cells such as neutrophils and macrophages has spurred new efforts to utilize ROS and oxidative stress as an active anti-infective strategy. Furthermore nitrosative stress and reactive nitrogen species are relative newcomers to the therapeutics arena, but may also have interesting roles to play.

2. Reactive oxygen species in living organisms

ROS is a collective term used to denote molecules and reactive intermediates with a highly positive redox potential (pE or E_h). The standard reduction potential is defined relative to a standard hydrogen electrode reference electrode, which is arbitrarily given a potential of 0.00 volts. It is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced (or in other words to act as an oxidizing agent). Reduction potential is measured in volts (V), or millivolts (mV). Each species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and its strength as an oxidizing agent. Table 1 provides a compiled list of ROS and their known redox potentials for the appropriate half-reactions; the list includes ROS, reduced oxygen molecules, secondary ROS radicals, reactive nitrogen species, high valence transition metals and other reactive species. The table is organized in order of decreasing redox potential to give what Buettner called a "pecking order" (Buettner, 1993). In principle each species can acquire an electron from a species below it in the list, but must donate an electron to each species above it. However it must be stated that it is not only the electrode potential that governs whether a reaction can proceed or not, the activation energy is also important.

In eukaryotic cells mitochondria are the power-house of the cell; they are the major consumer of oxygen and also the major sources of ROS (Droge, 2002). During aerobic-energy generation, mitochondria reduce molecular oxygen (O_2) to water (oxidative phosphorylation). In principle, the cytochrome c oxidase has to catalyze the transfer of four electrons to an oxygen molecule without any intermediates, but, in reality, during this process partially reduced oxygen species are produced as "by products"; thus, leakage of electrons from the electron transport chains (located on the inner membrane of mitochondria) is the source of $O_2^{\bullet -}$, which then produces H_2O_2 by dismutation (Fridovich, 2004). Further reaction to give $^{\bullet}OH$, and peroxynitrite ($ONOO^-$) can follow (Inoue, *et al.*, 2003, Buonocore, *et al.*, 2010). Figure 1 shows a schematic diagram illustrating the generation and interactions of naturally occurring ROS produced from the mitochondria can damage host mammalian cells.

Other potential endogenous sources include cytochrome P450 metabolism, peroxisomes, microsomes, inflammatory cell activation, monoxygenase system, nitric oxide synthase, and several other enzymes that are involved in inflammatory processes (Inoue, *et al.*, 2003, Authen & Davis, 2009). ROS are continuously generated through variety of pathways, involving both enzyme-catalyzed and non-enzymatic reactions. For instance, during the respiratory burst process, taking place in activated phagocytes, ROS damaging effects (as byproducts of photosynthesis and byproducts-components of several cellular enzymes, including but not limited to flavins in NADPH oxidases (NOX1-3 in smooth muscle and vascular endothelium), xanthine oxidase (XO), and uncoupled endothelial nitric oxide synthase (eNOS), can be observed in the components of the cell membranes (Droge, 2002).

Besides mitochondria, there are other endogenous sources of cellular ROS, such as neutrophils, eosinophils, and macrophages (Conner & Grisham, 1996). Free radicals can also be produced by a host of exogenous processes such as environmental agents and xenobiotics (metal ions, radiation, barbiturates) (Klaunig JE, 1997) and bacterial invasion. Oxidative stress and a higher ROS production can be also induced by stress factors such as tumor necrosis factor a (TNF-a) and the increase in ROS production can be recognized by various redox sensors; this recognition triggers a redox cascade which leads to the activation of both pro-survival and pro-cell-death factors (Pourova, *et al.*, 2010). On that basis alone one would expect these organelles to produce substantial amounts of the partially reduced oxygen products of $O_2^{\bullet -}$ and H_2O_2.

Furthermore the products of reaction of ROS with organic molecules can themselves function as ROS, such as lipid hydroperoxides that can initiate free radical chain reactions. It is well known that ROS readily attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction (Mylonas & Kouretas, 1999), and since lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage (Mylonas & Kouretas, 1999). This pathway of lipid peroxidation is illustrated in Figure 2.

When we look closely into the energy generation process in mitochondria, we see stepwise reduction of ROS "byproducts" (through electrons ($\mathbf{e}^-$) addition):

$$O_2 + e^- + H^+ \rightarrow HO_2^{\bullet} \quad (1\text{-}1)$$

$$HO_2^{\bullet} \rightarrow H^+ + O_2^{\bullet -} \quad (1\text{-}2)$$

$$O_2^{\bullet -} + 2H^+ + e^- \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + e^- \rightarrow HO^- + {}^{\bullet}OH \quad (3)$$

$${}^{\bullet}OH + e^- + H^+ \rightarrow H_2O \quad (4)$$

During this cascade of reactions, the hydroperoxyl radical ($HO_2^{\bullet}$) further dissociates to generate $O_2^{\bullet -}$ (eq 1-2).

$O_2^{\bullet -}$ is formed when an electron is captured by one of the π*2p orbitals of oxygen. Its biological effect stems from the fact that depending on the solution environment (i.e. pH and fluctuations) its reduction potential changes. In aqueous solution it is a weak oxidizing agent and as such able to oxidize ascorbic acid and thiols, as well as act as a very strong reducing agent, and reduce iron complexes of cytochrome c and ferric-EDTA. This anion radical transforms further via a well known dismutation reaction

$$2H^+ + O_2{}^{\bullet -} + O_2{}^{\bullet -} \rightarrow H_2O_2 + O_2 \quad (5)$$

Superoxide dismutase (SOD) enzyme with copper-zinc center accelerates this reaction and produces H_2O_2. There are other enzymes (such as urate oxidase, D-amino acid oxidase, and glucose oxidase) that catalyze the same reaction as well.

By nature H_2O_2 is a covalent and uncharged molecule that readily mixes with water, and is treated as such by the body; thus, it diffuses across cell membrane with ease. But, at the same time it has redox properties as well as capabilities to form highly reactive free radicals (in the presence of transition metal ions). As such it is imperative that this molecule is "neutralized" by the cells. The evolved body defense mechanisms operate via actions of selenium containing glutathione peroxidase, catalase, and certain other peroxidases.

Another major byproduct of high-energy ionization of water molecules is ${}^{\bullet}OH$

$$H_2O \rightarrow {}^{\bullet}OH + H^{\bullet} + e^-{}_{aq} \rightarrow H_2O_2 \quad (6)$$

This radical is an extremely aggressive oxidant and as such it can attack various biological molecules and impair their proper functioning. The multi-faceted Fenton reaction-cascade produces oxo-iron, ${}^{\bullet}OH$, and $HO_2^{\bullet}$ intermediates

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\bullet}OH \quad (7)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2{}^{\bullet} + H^+ \quad (8)$$

the overall reaction is as

$$\text{iron salt} + 2H_2O_2 \rightarrow 2H_2O + O_2 \quad (9)$$

One has to take into account the fact that in living organisms and at physiological pH, the ferrous ion (Fe^{2+}) (in presence of oxygen and phosphate ions) has a transient lifetime and it auto-oxidizes to ferric (Fe^{3+}) state. During this process an e^- is transferred from iron to the O_2 and generates a $O_2^{\bullet -}$. The unstable intermediates are perferryl ions ($Fe^{2+}O_2$ and $Fe^{3+}O_2^-$), where iron is at V oxidation state.

$$Fe^{2+} + O_2 \leftrightarrow Fe\,(II)\,O_2 \leftrightarrow Fe\,(III)\,O_2{}^- \leftrightarrow Fe^{3+} + O_2{}^{\bullet -} \quad (10)$$

Thus, it is clear that in aerobic life oxygen has a dual effect: it is a crucial source of energy, its reduction through the electron transfer system of mitochondria is a lifeline of survival, and at the same time the byproducts of the oxygen usage/metabolism generate the damaging molecules of $O_2^{\bullet -}$, $^{\bullet}OH$ and H_2O_2. These three species, together with unstable intermediates from the peroxidation of lipids (and referred to as ROS as well) have major life effecting impact. Many diseases (such as Alzheimer's, Parkinson's, atherosclerosis, myocardial infarction, autoimmune diseases, etc.) are linked to the damaging effects of ROS, and develop as a result of an imbalance between radical-generating and radical-scavenging systems, i.e. oxidative stress condition. SOD activity of erythrocuprein together with the fact that almost all mammalian cells contain SOD suggests the physiological importance of $O_2^{\bullet -}$ as a ROS (McCord & Fridovich, 1969).

$^1O_2$ is deemed to be one of the most damaging ROS. It is a highly reactive form of oxygen where the electron spin restriction that prevents ground state triplet oxygen reacting with other singlet molecules is lifted, thereby increasing its oxidizing ability (Halliwell & Gutteridge, 2010). Formation of $^1O_2$ is extremely important “natural” processes with enormous biological implications. There are few mechanisms through which living organisms generate the $^1O_2$: (a) in photosynthetic cells- energy is transferred from a light-excited photosensitizer (i.e. a light-sensitive molecule) to ground state oxygen. This process of light excitation of pigments or chlorophylls/ bacteriochlorophylls (in photosynthetic microorganisms) is the most common source of $^1O_2$ (Borland, *et al.*, 1988, Cogdell, *et al.*, 2000, Krieger-Liszkay, 2005, Telfer, 2005, Krieger-Liszkay, *et al.*, 2008, Ziegelhoffer & Donohue, 2009, Tomo, *et al.*, 2012); (b) in non- photosynthetic cells- the main endogenous photosensitizers are flavins, porphyrins, rhodopsins, and quinones (Ryter & Tyrrell, 1998). Since these compounds are innately present in the cells, in events of simultaneous presence of oxygen and light, $^1O_2$ is generated (proteo- and bacteriorhodopsins are considered to be light driven proton pumps). A wide variety of heterocyclic aromatic compounds can generate $^1O_2$ as well such as naphthalene, anthracene, and their derivatives, dyes such as methylene blue, rose bengal, etc., herbicides, and various therapeutic agents) (Paul, *et al.*, 2004, Sun, *et al.*, 2006, Grandbois, *et al.*, 2008). c) light independent $^1O_2$ generation can be due to respiratory burst of macrophages (where NADPH oxidases generate $O_2^{\bullet -}$ that spontaneously or enzymatically dismutates to H_2O_2 which then is reduced to hypochlorous acid (HClO) via chloro-, lacto-, or myeloperoxidase catalyzes, and finally, the spontaneous reaction of H_2O_2 with HClO forms the $^1O_2$) (Ryter & Tyrrell, 1998, Tarr & Valenzeno, 2003, Davies, 2004). Other light independent $^1O_2$ sources are lipid peroxidation processes.

3. Antioxidant defense against ROS

As mentioned above the reliance of most life on aerobic metabolism, together with the role of ROS in intracellular signaling, host defense against invading microbes, and in regulatory mechanisms, has meant that defense against excessive ROS has needed to evolve to deal with the harmful effects of ROS, such as lipid peroxidation (damaging cell membranes), oxidative damage to proteins (Davies, 2003), mutations to DNA, and activation of pro-cell death factors.

When ROS produced overwhelm the cellular antioxidant defense system, either by increased ROS generation or decreased cellular antioxidant capacity, oxidative stress occurs. Thus, oxidative stress is an imbalance occurring due to excessive ROS or over-generated oxidants exceeding the capability of the cell to mount an effective antioxidant response. It is well known that oxidative stress results in macromolecular damage and is implicated in various diseases including and not limited to atherosclerosis (Paravicini & Touyz, 2006), diabetes (Paravicini & Touyz, 2006), carcinogenesis and tumor metastasis through gene activation (Ishikawa, *et al.*, 2008, Trachootham, *et al.*, 2009), neurodegeneration (Andersen, 2004,

Shukla, *et al.*, 2011), and aging (Haigis & Yankner, 2010). ROS destructive effect is so significant and defense against them such a priority that protective enzymes (such as SOD, catalase and GSH-peroxidase), substrates (such as GSH), and vitamin E, all, tend to be in higher concentration in locations where ROS damage is more likely to occur and be potentially more damaging (Moslen, 1994). Figure 3 shows how a wide range of physiologically relevant primary and secondary ROS and RNS are produced from superoxide as the principle initial species.

Complicating matters further, it is known that ROS can also serve in various critical cellular signaling pathways needed for optimum cellular function. There is good data to demonstrate that oxidative stress has effects on signaling pathways. But how about the direct interaction between ROS and elements of the signaling pathways? What happens at the “oxidative interface”, how is ROS initiating the signaling cascade(s)? By now it is known that ROS regulates several signaling pathways via interaction with the signaling molecules affecting areas of cell proliferation, survival, differentiation and metabolism through ASK1, PI3K, PTP, Shc pathways; anti-inflammatory/anti-oxidant responses via TRX, Ref1-Nrf2 pathways; DNA damage via ATM pathway; iron homeostasis via IRP pathway. In order to understand these complicated interactions, and resulting functional alterations, the issue of ROS-protein interaction needs to be addressed. The oxidative interface functions mainly via redox regulation of redox-reactive cysteine (cys) residues of the proteins. Oxidation of these residues generates reactive sulfenyl moiety (-S-OH) that then form disulfide bonds within the nearby cysteines (-S-S-) (it is well known that disulfide bonds can be reduced back to the free thiol moiety under physiological intracellular conditions (Paravicini & Touyz, 2006, Miki & Funato, 2012, Ray, *et al.*, 2012)) or undergo further oxidation to form sulfinyl moiety (-SO-OH) and then via further oxidation sulfonyl moiety (-SOO-OH) acids. The sulfenyl moiety may react with the nitrogen atom in the peptide bond and form a sulfenylamide bond, which can be reduced to a thiol moiety under physiological conditions). All these reversible oxidative changes/modifications end up changing the structure and because of that the function of the protein (Janssen-Heininger, *et al.*, 2008, Winterbourn & Hampton, 2008, Roos & Messens, 2011).

The action of ROS is implicated in wound-healing as well (Miki & Funato, 2012); it was shown that upon local injury of the tail-fin of zebrafish, H_2O_2 is produced extensively at the wounded area due to ROS-generated enzyme Duox, H_2O_2 diffuses from the wound site and penetrates into neutrophils and oxidizes the Cys466 of Lyn; the oxidatively activated Lyn induces the migration of neutrophils to the wound site (Miki & Funato, 2012).

It is important to know not only how ROS signaling in diseases affects function, but also how ROS initiate/propagate cellular signaling in homeostatic conditions as well (Valko, *et al.*, 2007).

Cells have developed elaborate defense mechanisms to detoxify/neutralize the ROS and to preserve life (Droge, 2002). These defense mechanisms can operate at several different (even overlapping) levels within the cells: a) preventing radical formation; b) scavenging or quenching the radicals by reaction with antioxidants; c) destroying the ROS using enzymatic action; d) repairing the caused oxidative damage; e) expediting the elimination of damaged molecules; f) not repairing the extensively damaged molecules in order to minimize the introduction of possible mutations.

3.1. Antioxidant vitamins and membrane defenses

Among the natural antioxidants that function effectively as ROS quenchers are ascorbic acid, carotenoids and tocopherols (Devasagayam & Kamat, 2002). Considering that the structure of membranes have a hydrophobic domain in the interior of the lipid membrane is

separate from the aqueous milieu of the intracellular medium, the radicals that are formed in membranes are also lipophilic and are different from those formed in the aqueous cytoplasm. Therefore the defense molecules (effective antioxidants) are also of different types. The α-tocopherol (commonly known as vitamin E) is a lipid-soluble (thus a poor antioxidant in aqueous milieu) chain-breaking molecule and β-carotene is a lipid-soluble radical scavenger and $^1O_2$ quencher, etc.

In photosynthetic microorganisms and in chloroplasts, antioxidants provide protection against $^1O_2$ via either quenching the $^1O_2$ directly or quenching the excited chlorophylls/bacteriochlorophylls (Cogdell, *et al.*, 2000, Trebst, 2003, Telfer, 2005, Krieger-Liszkay, *et al.*, 2008). Non-photosynthetic microorganisms may also use the carotenoids as quenchers of $^1O_2$ (Di Mascio, *et al.*, 1989, Di Mascio, *et al.*, 1990) while some oxygenic phototrophs (such as cyanobacteria) can use tocopherol (Krieger-Liszkay & Trebst, 2006) and plastoquinone (Kruk & Trebst, 2008). $^1O_2$ scavengers include: amino acids (such as L-histidine, tryptophan) (Davies, 2004), polyamines (such as cadaverine, spermidine, spermine, and putrescine) (Das & Misra, 2004), thiols (such as glutathione) (Devasagayam, *et al.*, 1991), thioredoxin (Das & Das, 2000), mycosporine lysine (Suh, *et al.*, 2003).

There have been many clinical trials of antioxidant vitamins as supplements for a wide variety of diseases (particularly in the cardiovascular system) that were thought to involve oxidative stress (Jha, *et al.*, 1995, Lonn & Yusuf, 1997). By and large these trials have mostly failed (Sugamura & Keaney, 2011). The reasons for this failure are usually attributed to the aforementioned beneficial role of low levels of ROS in cell signaling and homeostasis (Mishra, 2007). However another possible reason can be appreciated when it is realized that free radicals react with antioxidant vitamins to give less reactive but longer-lived and more stable free radicals (Mortensen & Skibsted, 1997, El-Agamey, *et al.*, 2004).

3.2 Intracellular defenses

SOD (with Cu, Zn, or Mn active centers) catalytically remove $O_2^{\bullet -}$; glutathione peroxidase (with Se active center) removes H_2O_2 when it is at low and steady state concentrations; catalases (with 4 NADPH molecules and active Fe centers) remove H_2O_2 when it is in high concentrations; formation of cytochrome oxidase with Cu centers (in preventing the formation of H_2O_2, $^{\bullet}OH$, and $O_2^{\bullet -}$) during which there is no release of oxygen molecules while the reduction of O_2 to H_2O. Enzymes also are employed to remove the intermediates/byproducts generated during oxygen metabolism speedily, specifically, and with high efficiency. Enzymes such as SOD rapidly trigger dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2 with much faster rates than uncatalyzed one. The H_2O_2 byproduct can be destroyed by the two enzymes, catalase and glutathione peroxidase. During normal oxidation metabolism, these two enzymes are employed to eliminate the toxic intermediates of oxygen reduction inside the cells. The system is so finely tuned that this system at the same time allows a small amount of low-molecular mass iron to exist safely, since its presence is imperative for signaling functions and synthesizing DNA, and iron-containing proteins.

3.3. Extracellular defense systems

Molecules functioning at this level include glutathione peroxidases and SOD employed as glycosylated extracellular proteins (transferrin to bind ferric ions, lactoferrin to bind ferric ions at lower pH, extracellular-SOD (EC-SOD) removing $O_2^{\bullet -}$ catalytically, extracellular-$GSHP_X$ (EC-$GSHP_X$) removing H_2O_2 and H-O-$O^{\bullet}$ catalytically). To provide both optimum function and protection the system is finely tuned to allow limited survival of some ROS (such as $O_2^{\bullet -}$, $NO^{\bullet}$, and H_2O_2) in the extracellular fluid milieu to use them as signaling, messenger, or triggering molecules while the rest is safely removed/neutralized. There is another safety layer in this system, where $O_2^{\bullet -}$ and H_2O_2 are not allowed to come in contact

with the reactive extracellular copper and iron (copper and iron are kept at poorly- or non-reactive states).

3.4 Antioxidant defense against ROS in bacteria

Due to their relative simplicity, bacteria have been widely used to study and explain the processes of oxidative stress. Bacteria offer several advantages due to the relatively easy ability to modify and manipulate them (and their external environmental conditions) to study the mechanisms that induce ROS formation and their impact on cells. The absence of intracellular organelles makes possible a more accurate quantization of oxidants generated in the reactions. Researchers have been able to generate knockout mutants of several enzymes that deal with $O_2^{\bullet -}$ and H_2O_2 stresses. The manipulation of bacterial growth conditions has allowed identifying the molecules more sensitive to radical species. Moreover using *Escherichia coli (E. coli)*, that can grow in an anaerobic environment, has been possible to investigate on the effect of oxygen introduction in mutants lacking oxidative defense (Imlay, 2008).

Under aerobic conditions bacteria are more likely to experience oxidative stress and ROS production (Storz & Imlay, 1999, Imlay, 2003) that damage various cellular sites, including cysteine, methionine protein residues, DNA, and iron-sulfur clusters (Imlay & Linn, 1988, Storz & Imlay, 1999, Imlay, 2003). With bacteria, ROS is a natural byproduct of cellular activity- oxygen utilization and/or exposure. Flavin cofactors, found in the NADH dehydrogenase II of the respiratory chain, during their autooxidation and reaction with molecular oxygen produce H_2O_2 and $O_2^{\bullet -}$ (Eq. (11)) (Messner & Imlay, 1999), and highly reactive $^{\bullet}OH$ is formed when the H_2O_2 reacts with free ferrous ion via Fenton reaction (Eq. (7)) (Imlay, *et al.*, 1988, Glaeser, *et al.*, 2011). Bacteria in their use of N_2 during the denitrification process produce RNS as byproducts, in this case nitric oxide (Eq.(12)) (Zumft, 1997).

$$O_2 + e^- \rightarrow O_2^{\bullet -} \quad (\text{with FAD}) \quad (11)$$

$$NO_3^- \rightarrow NO_2^- \rightarrow NO\bullet \rightarrow N_2O \rightarrow N_2 \quad (12)$$

The gene regulatory response of *E. coli* to oxidative stresses (due to H_2O_2 and $O_2^{\bullet -}$) is through induction of SOD and catalase. $O_2^{\bullet -}$ is dismutated to form H_2O_2 (Eq.(13)) (McCord & Fridovich, 1969). In contrast to mammalian cells that have only two SOD isoforms, *E. coli* uses three types of SOD that have different metal cationic centers MnSOD (sodA), FeSOD(sodB), and CuZnSOD(sodC).

$$2\,O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \ (\text{SOD}) \quad (13)$$

Catalases dismutate H_2O_2 to H_2O and O_2 (Eq.(14)) (McCord & Fridovich, 1969), and *E. coli* has two catalases to do just that (hydroperoxidase I (HPI) and hydroperoxidase II (HPII))

$$2H_2O_2 \rightarrow 2H_2O + O_2 \ (\text{catalase}) \quad (14)$$

In *E. coli* genes that regulate the SOD and catalases are members of two major oxidative stress regulons, the OxyR and SoxRS, in addition to the “general stress regulon” RpoS, a fact in itself signifying the importance of ROS removal. *E. coli* has been used as a model system to study the effects of these regulons functioning in a vide range of bacterial phyla,

from *Proteobacteria* to *Actinobacteria*. Clearly, in bacteria the oxidative stress response regulation (through specific regulators) is a key survival factor during episodic exposure to exogenous ROS as well as ROS from normal respiration. It is interesting that despite the huge genomic diversity of bacteria and the presence of other oxidative stress regulators (such as PerR, RpoS), these regulators are functionally conserved in wide range of bacterial species from diverse niches. Apparently, even in anaerobic species of *Bacteroidetes* that lack catalase homologs, OxyR is important for H_2O_2 tolerance (Diaz, *et al.*, 2006, Honma, *et al.*, 2009). In bacteria OxyR is also implicated in protection against heat stress (Christman, *et al.*, 1985), singlet oxygen (Kim, *et al.*, 2002), lipid peroxidation-mediated cell damage (Yoon, *et al.*, 2002), neutrophil-mediated killing (Staudinger, *et al.*, 2002), and protection against near-UV damage (Kramer & Ames, 1987).

In bacteria low molecular weight protein-thiols (such as small peptide glutathione that reduces disulfides in conjunction with glutaredoxin are one class of small proteins that are doing just that, promoting the degradation of ROS, reactive nitrogen species (RNS), and intermediates in order to repair oxidative and nitrosative altered proteins.

Enzymes that catalyze the degradation of H_2O_2 are named “catalases” and are widely spread in different bacterial species. In *E. coli* these enzymes include thiol peroxidase, bacterioferritin comigratory protein, glutathione peroxidase, cytochrome c peroxidase and rubrerythrins. However, their effects in *in-vivo* conditions remain unclear (Mishra & Imlay, 2012). Also, enzymes such as SOD and alkyl hydroperoxidase are known to limit the accumulation of ROS and to provide antioxidant defense for bacterial cells. The catalytic mechanism of the alkyl hydroperoxidase requires two peroxidases: AhpC and AhpD, where AhpD is known to play the role of an essential adaptor protein (Clarke, *et al.*, 2011) and AhpC is the catalytic subunit responsible for alkyl peroxide metabolism (Mongkolsuk, *et al.*, 2000). On the other hand, AhpC from *Mycobacterium tuberculosis* (MtAhpC) is known to be a major component in the NADH-dependent peroxidase and peroxynitrite reductase system, where AhpC directly reduces peroxides and peroxynitrite, and at the same time it is reduced by AhpD and other proteins. It has been shown that over-expression of MtAhpC in isoniazid-resistant strains of *M. tuberculosis* (that harbors mutations in the catalase/ peroxidase katG gene) provides antioxidant protection and it may even substitute for the lost enzyme activities (Guimaraes, *et al.*, 2005).

Interestingly, it has been shown that mutations in *E. coli* OxyR suppress sensitivity towards H_2O_2 which in turn cause rise in the levels of one of the three enzymes which are responsible for destroying organic ROS and H_2O_2, namely: catalase-hydroperoxidase I (the katG gene product), catalase hydroperoxidase II (controlled by katEF) or alkyl hydroperoxide reductase (specified by the Ahp genes). It appears that peroxides serve as mediators of toxicity for a variety of redox agents, and as such are produced in sufficient quantities during normal metabolism in order to generate substantial elevations in the levels of ‘spontaneous’ mutations in cells that lack adequate antioxidant defenses (Greenberg & Demple, 1988).

It is expected that for pathogenic mycobacteria to be able to persist in macrophages and in granulomatous caseous lesions, they must be equipped to withstand the action of toxic metabolites/ROS. In Gram-negative bacteria the OxyR protein is a critical component of the oxidative stress response where OxyR is not only a sensor for ROS but also a transcriptional activator, inducing expression of detoxifying enzymes such as catalase/ hydroperoxidase and alkyl hydroperoxidase (Sherman, *et al.*, 1995). Sherman et al. (Sherman, *et al.*, 1995) in their study characterized the responses of various mycobacteria to H_2O_2, both phenotypically and at the levels of gene and protein expression, and found that only the saprophytic *M. smegmatis* induced a protective oxidative stress response analogous to the OxyR response of

Gram-negative bacteria. They also found that under similar conditions, the pathogenic mycobacteria exhibited a limited, non-protective response, which in the case of *M. tuberculosis* was restricted to induction of a single protein, KatG. Their conclusion was that the response of pathogenic mycobacteria to oxidative stress differs significantly from the inducible OxyR response of other bacteria (Sherman, *et al.*, 1995).

ROS and RNS produce damage to iron-sulfur clusters, proteins and DNA (Chiang & Schellhorn, 2012). In bacteria, proteins are major biological targets for oxidative damage within cells owing to their high abundance and rapid rates of reaction with ROS/ RNS and numerous post-translational, reversible or irreversible modifications (Davies, 2004, Butterfield & Dalle-Donne, 2012). In these cases, oxidation can involve cleavage of the polypeptide chain, modification of amino acid side chains, and conversion of the protein to derivatives that are highly sensitive to proteolytic degradation (Stadtman, 2006). It has been shown that reaction of mainly aromatic and sulfur containing amino acid residues (such as tryptophan, tyrosine, histidine, cysteine, and methionine), both free and on proteins, due to ROS effect generate peroxides in high yield, which themselves may decompose into reactive intermediates (protein carbonyl derivatives) and induce damage to other targets, such as molecular oxidation of thiol moiety of cysteine (Butterfield & Dalle-Donne, 2012). Moreover, bacterial proteins can be modified by aldehydes and ketones produced during reactions of ROS with lipids and glycated proteins (Davies, 2003, Stadtman, 2006). This can result in the inactivation of cellular enzymes and the oxidation of other biological targets. Protein cross-linking and aggregation can also be induced by reactive species formed on oxidized proteins (Ray, *et al.*, 2012). The senescence-related oxidation targets are enzymes of the Krebs cycle, universal stress protein A, the Hsp70 chaperone DnaK, translation elongation factors, and histone-like proteins (Nystrom, 2002). Based on the identity of the oxidized proteins, it has been concluded that several different cell processes are targets for stasis-induced damage; these functions include peptide chain elongation, protein folding and reconstruction, large-scale DNA organization, gene expression, central carbon catabolism, and general stress protection (Dukan & Nystrom, 1999, Nystrom, 2002).

Oxidative stress responses coordinated by specific regulators ensure bacterial survival during ROS exposure. Recently, several ROS-sensing global regulators in both Gram-positive and Gram-negative pathogenic bacteria have been reported (Chen, *et al.*, 2011). Bacteria have several major regulators activated during oxidative stress, including bacterial transcription factors viz. OxyR, SoxRS, and RpoS. OxyR and SoxRS undergo conformation changes when oxidized in the presence of H_2O_2 and $O_2^{\bullet -}$ respectively, and subsequently control the expression of cognate genes. OxyR serves as a peroxide-sensitive thiol-based redox sensor and controls the expression of several genes involved in the antioxidant response. Among the genes controlled by OxyR are a peroxidase, AhpC, and its reductase, AhpF. AhpC belongs to the family of peroxiredoxin (Prx), which catalyzes the reduction of H_2O_2 and alkyl hydroperoxides through reversible disulfide formation (Lindahl, *et al.*, 2011). OxyR also has a role in protecting against heat stress, near-UV, $^1O_2$, lipid peroxidation-mediated cell damage and neutrophil-mediated killing. RpoS is a stationary phase and general stress response regulator (Dukan & Nystrom, 1999, Chiang & Schellhorn, 2012). Another example of redox regulation in bacteria is the oxidative activation of the molecular chaperone Hsp33, which contains four conserved cysteines prone to the formation of disulfide bridges (Kumsta & Jakob, 2009). The mechanisms that these redox sensitive regulatory proteins employ to sense redox signals through covalent modification of redox active amino acid residues or associated metalloprotein centers will suggest further understanding of bacteria pathogenesis, antibiotic resistance, and host–pathogen interaction (Chen, *et al.*, 2011).

3.5 Genetic responses to ROS and oxidative stress in microorganisms

Microorganisms that regularly face photooxidative stress have evolved specific systems to sense $^1O_2$ and tightly control the removal of $^1O_2$ reaction products (Glaeser, 2011). A general distinction for the molecular machinery responsible for these responses can be made between photosynthetic and non photosynthetic microorganisms.

Cyanobacteria for example are exposed to photooxidative stress due to chlorophyll-mediated singlet oxygen production and by the UV-B induced production of ROS (He, 2002). The key defense system implicated in response to photooxidation involves the five high light inducible (Rychlik) proteins from *Synechocystis* PCC6803. Hli proteins are essential for survival when the cells are absorbing excess excitation energy with a demonstrated crucial role in photoacclimation (He, 2001, Havaux, 2005). The homologs NblS (*Synechococcus elongatus* PCC7942) and DspA (*Synechocystis* PCC6803) control Hli protein expression synthesis and are required in survival in high light. In some cyanobacteria, iron starvation leads to the synthesis of proteins (IdiA, IsiA) and a regulatory system involving IdiB and Fur proteins as well as the isiAB genetic loci, which protect the photosystems against oxidative stress (Michel, 2004, Havaux, 2005, Dühring, 2006).

A complex network of different regulatory components controls the defense against photooxidative stress in anoxygenic photosynthetic bacteria. The photooxidation responses were first studied in anoxygenic prototrophs employing *Rubrivivax gelatinosus* and the characterization of mutants with impaired caretonoid synthesis.(Ouchane, 1997) Apart from this first pilot study, *R. sphaeroides* has been emerging as the model photosynthetic bacterium to dissect the photooxidative responses. Briefly it involves regulation of genes by the alternative sigma factors RpoE, RpoHII, and RpoHI, by small noncoding RNAs (Berghoff, 2009) and the RNA chaperone Hfq (Berghoff, 2011). In addition at least one two-component system comprising an OmpR-like DNA-binding protein and a yet unknown sensor histidine kinase are involved (Nuss, 2010). Proteomic studies revealed altered synthesis rate in about 60 soluble proteins in response to $^1O_2$ (Glaeser, 2007) with the majority of the corresponding genes controlled by the alternative sigma factors RpoHII and/or RpoHI whereas a small number were directly depended on the extracytoplasmic function (ECF) sigma factor RpoE (Nuss, 2009, Nuss, 2010). The comparison of the transcriptomic profiles between the wild type and a mutant lacking ChrR, the antisigma factor for RpoE revealed over 180 genes clustered in 61 operon with at least 3-fold difference in expression levels between the two strains (Anthony, 2005).

RpoE and ChrR homologs exist in the heterotrophic proteobacterium *Caulobacter crescentus* and contribute in the regulation of photooxidative stress in a similar fashion with the anoxygenic prototrophs although some mechanistic elements of the RpoE:ChrR dissociation are still unknown (Lourenço, 2009). A network of sigma factors, antisigma factors, repressor proteins, and antirepressors control the expression of carotenoid genes in response to light (Elias-Arnanz, 2010). The antisigma factor CarR together with the ECF sigma factor CarQ controls expression of the *carQRS* operon and the *crtI* gene in *Myxococcus xanthus*. CarS is an antirepressor of CarA and CarH which repress the car operon in the dark. CarR is associated with the inner membrane and is unstable in illuminated stationary phase cells (Browning, 2003). The core of information for the photooxidative responses of the non-photosynthetic bacteria comes mainly from the enteric pathogens *E. coli* and *Salmonella typhimurium*. The list includes:

1. The OxyR gene which function as a redox sensor, which is oxidized at elevated levels of H_2O_2, binds to DNA target sequences in its oxidized form and subsequently activates a small subset of genes (Storz, 1990, Storz, 1990). It was discovered in a screen for *Salmonella* mutants that were hyper-resistant to H_2O_2

(Christman, 1985). OxyR is the critical regulator both in oxidative stress but also an important coordinator in pathogenesis and virulence in a variety of human and plant pathogens. With regards the human pathogens, in *Klebsiella pneumoniae*, apart from the upregulation of defense mechanisms against oxidative stress it enhances biofilm formation, fimbrial expression, and mucosal colonization (Hennequin, 2009). In *Shigella flexneri*, the causative agent of bacterial dysentery mediates the regulation of the iron-sulfur cluster biosynthesis systems Suf and Isc (Runyen-Janecky, 2008). In the oral pathogens that encounters constant oxidative stress in the human oral cavity due to exposure to air and ROS from coexisting dental plaque bacteria as well as leukocytes, OxyR has been proven to be a critical regulator for both *Tannerella forsythia* (Honma, 2009) and *Porphyromonas gingivalis* (Meuric, 2008, Wu, 2008).

2. The earlier but comprehensive reports for *E. coli* hemH or hemG mutants which accumulate non-iron porphyrins to about 100 times higher level than the wild-type and respond to blue light at an intensity 100-fold lower than that required for the wild-type (Yang, 1995, Yang, 1996).

3. The SoxRS regulon mobilizes diverse functions to scavenge free radicals and repair oxidative damage in macromolecules. The SoxRS system in particular responds to the $O_2^{\bullet -}$ stress (González-Flecha, 2000). SoxR contains a (2Fe–2S) cluster that is oxidized by $O_2^{\bullet -}$ and subsequently activates transcription of SoxS, an AraC family protein (Ding, 1996, Watanabe, 2008). SoxS binds to its target promoters and activates genes which encode SOD, DNA repair enzymes and enzymes of the carbon metabolism (Pomposiello, 2002) It is likely that the SOD activated by SoxRS has a protective role against $^1O_2$ as it was demonstrated for the iron SOD of *Agrobacterium tumefaci*ens (Saenkham, 2008). A combinatorial approach employing DNA microarrays to evaluate the paraquat-induced global transcriptional response of *Bacillus anthracis* to endogenous superoxide stress and mutants lacking the SOD genes sodA1 and sodA2 was supportive for the protective role of the sod locus (Passalacqua, 2007). The exact stimuli and functionality of SoxRS as well as its partition in SOD activation is under investigation (Gu, 2011, Fujikawa, 2012). The role of SoxRS in microbial virulence and pathogenesis is pivotal and it has explored extensively in enteric bacteria (van der Straaten, 2004, Rychlik, 2005, O'Regan, 2010). The *E. coli*-based SoxRS paradigm does not hold in *P. aeruginosa* and the hypothesis fostered for a possible physiological role, involves the SoxR-mediated transcriptional regulation of quorum sensing genes and signaling through the phenazine pyocyanin (Palma, 2005, Dietrich, 2006). SoxR has been also proposed as the key regulator mediating oxidative stress and virulence capabilities of the prominent plant pathogenic bacteria *Erwinia chrysanthemi* (Nachin, 2001) *Xanthomonas campestris pv. campestris* (Mahavihakanont A, 2012).

As previously mentioned, small RNAs (sRNAs) and the RNA chaperone Hfq play a pivotal role in the regulation of the photoxidative stress responses in the anoxygenic prototrophic bacterium *R. sphaeroides.* sRNAs interact with mRNAs not only in photoxidative stress, but possess broader functionality as small noncoding post-transcriptional gene regulators. The mRNA targets are influenced by means of stability and/ or translation initiation (Repoila, 2009, Vogel, 2009). As the knowledge of the regulated genes increases it is possible to define categories of regulatory circuits which are similar to those described for protein transcription factors (Beisel, 2010). Most sRNAs function as regulators in stress responses. For example, the OxyS sRNA regulates expression of the general stress sigma factor RpoS in *E. coli* (Repoila, 2003). They are implicated in many phenomena of microbial physiology including pathogenicity and virulence (Pichon, 2005, Bradley, 2011, Kröger. C, 2012,

Pichon, 2012), catabolite repression, quorum sensing, nitrogen assimilation (De Lay, 2009). Hfq affects sRNA and mRNA stability as well as facilitates sRNA:mRNA interaction (Aiba, 2007). The role of Hfq in oxidative stress regulation have been explored in a variety of pathogens such as *Salmonella* sp (Bang, 2005) *Vibrio parahaemolyticus* (Su, 2010) *Francisella novicida* (Chambers, 2011) *F. tularensis* live vaccine strain (LVS) (Honn, 2012).

4. Host defense systems against microbes utilizing ROS

The mammalian innate immune system has evolved sophisticated mechanisms to recognize and kill foreign invaders such as bacteria, fungi, and parasites. This is mediated mainly by the process of phagocytosis, by which macrophages and neutrophils engulf these foreign cells and kill them by a combination of ROS, RNS and enzymatic digestion (Malle, *et al.*, 2007). The relatively sudden production of ROS when phagocytes have engulfed pathogens has become known as the “oxidative burst” (Freitas, *et al.*, 2009) or “respiratory burst” (Forman & Torres, 2001). Figure 4 shows how ROS produced in this manner form phagocytes, damage and inactivate invading microbial pathogens.

4.1. NADPH oxidase

The phagocyte NADPH oxidase is the principal source of ROS generation in activated neutrophils and macrophages (Segal, *et al.*, 2012). In addition to the phagocyte NADPH oxidase isoform (NOX2), isoforms of NADPH oxidase exist in several cell types and mediate diverse biological functions. The phagocyte NADPH oxidase complex comprises a cytochrome component consisting of gp91^{phox} (phagocyte oxidase) and p22^{phox} embedded in membranes. The cytoplasmic subunits p47^{phox}, p67^{phox}, and p40 ^{phox} and rac translocate to the membrane-bound cytochrome upon activation of the oxidase. NADPH is oxidized to NADP+, and electrons are transported down a reducing potential gradient that terminates when oxygen accepts an **e**− and is converted to $O_2^{\bullet -}$. The NADPH oxidase family is highly conserved being expressed in fungi, plants, and animals (Bedard, *et al.*, 2007).

Chronic granulomatous disease (CGD) is an inherited disorder of NADPH oxidase in which phagocytes are defective in generation of ROS. As a result of this defect, CGD patients suffer from recurrent life-threatening bacterial and fungal infections (Segal, *et al.*, 2011). Among CGD patients, the degree of impairment of NADPH oxidase in neutrophils correlates with clinical disease severity (Kuhns, *et al.*, 2010).

4.2 Myeloperoxidase

Myeloperoxidase (MPO) is an iron-containing heme protein localized in the azurophilic granules of neutrophil granulocytes and in the lysosomes of monocytes. MPO catalyzes the formation of HOCl from H_2O_2 and chloride ion which has a strong microbicidal action (Winterbourn, *et al.*, 2006). It plays a major role in the killing of several micro-organisms including bacteria, fungi, viruses, red cells, and malignant and nonmalignant nucleated cells (Stendahl, *et al.*, 1984, Nauseef, 1986, Lanza, 1998, Malle, *et al.*, 2003, Malle, *et al.*, 2007, van der Veen, *et al.*, 2009). Although HOCl dramatically enhances the microbicidal activity of H_2O_2, MPO appears to be nonessential for host defense (Prokopowicz, *et al.*, 2012), as MPO-deficient individuals do not have a high frequency of infections, with the exception of an increased susceptibility to fungal species like *Candida* (Lehrer & Cline, 1969). Despite the primary role of the oxygen-dependent MPO system in the destruction of certain phagocytosed microbes, the lack of an increased frequency of infections, may be because other MPO-independent microbicidal pathways compensate for the lack of MPO (Kwakman, *et al.*).

There is also some evidence that the microbicidal action of MPO may involve either •OH (Rosen, 1980) and/or $^1O_2$ (Klebanoff & Rosen, 1978). Furthermore, activated human neutrophils were shown to use MPO for conversion of nitrite into the oxidants nitryl chloride (NO_2Cl) and nitrogen dioxide ($•NO_2$) that can cause tyrosine nitration and chlorination of target molecules (Eiserich, *et al.*, 1998).

4.3 Xanthine oxidase (XO)

XO is a major protein component of the milk fat globule membrane (MFGM) surrounding fat droplets in milk and its enzymology is well characterized. The enzyme is widely distributed in mammalian tissues and is generally accepted to be a key enzyme of purine catabolism. It catalyzes the oxidation of a wide range of substrates and can pass $\mathbf{e}^-$ to O_2 to form $O_2•^-$, similar reduction of nitrite yields RNS. Using the $p47^{(phox-/-)}$ mouse model of CGD, Segal et al (Segal, *et al.*, 2000) evaluated the residual antibacterial activity of XO. Clearance of *B. cepacia*, a major pathogen in CGD, was reduced in $p47^{(phox-/-)}$ mice compared to that in wild-type mice and was further inhibited in $p47^{(phox-/-)}$ mice by pretreatment with the specific XO inhibitor allopurinol. In CGD, XO may contribute to host defense against a subset of reactive oxidant-sensitive pathogens. Harrison (Harrison, 2004) also suggested that XO played a role in anti-microbial defense particularly in the gastrointestinal tract.

4.4 Inducible nitric oxide synthase

The discovery of nitric oxide ($NO^•$) led to one of the most highly studied and important biological molecules (Ohshima, *et al.*, 2003). $NO^•$ plays an important role as a cell-signaling molecule, anti-infective agent and, as most recently recognized, an antioxidant. The metabolic fate of $NO^•$ gives rise to a further series of compounds, which are collectively known as RNS (Moncada, *et al.*, 1991, Rubbo, *et al.*, 1996, McAndrew, *et al.*, 1997). $NO^•$ is produced by macrophages as a cytotoxic agent in the immune or inflammatory response (Hibbs, *et al.*, 1988, Marletta, *et al.*, 1988, Moncada, *et al.*, 1991). $NO^•$ is also believed to be the key mediator of macrophage induced cytotoxicity as $NO^•$ scavengers block the cytotoxic effect of macrophages (Hibbs, *et al.*, 1987, MacMicking, *et al.*, 1997). The innate immune systems relies the high-output isoform of inducible nitric oxide synthase (iNOS or NOS2) and this high-output NO pathway has evolved to protect the host from infection (MacMicking, *et al.*, 1997). All NOS isoforms convert the amino acid L-arginine and O_2 to L-citrulline and NO, and require NADPH, FAD, FMN, tetrathydrobiopterin (BH4) and a thiol donor as cosubstrates and cofactors. Mice deficient in iNOS had an increased mortality in the cecal ligation and puncture model of sepsis (Cobb, *et al.*, 1999) which was considered surprising as NO has been proposed as the main cause of septic shock.

There is some debate on exactly how $NO^•$ exerts its antimicrobial effects (Vallance & Charles, 1998). Since the direct antimicrobial action of pure $NO^•$ is limited it is thought that downstream reactions of $NO^•$ RNS such as $ONOO^-$, S-nitrosothiols (RSNO), nitrogen dioxide ($NO_2^•$), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), and dinitrosyl–iron complexes (DNIC). $ONOO^-$ is formed from the rapid interaction of $NO^•$ and $O_2•^-$, from the combination of H_2O_2 and nitrous acid (HNO_2), which would exist in equilibrium with nitrite (NO_2^-) within an acidified phagolysosomal vacuole (Klebanoff, 1993), or from the interaction of nitroxyl anion (NO^-) and O_2 (Kirsch & de Groot, 2002). S-nitrosothiols such as S-nitrosoglutathione can be formed from $NO^•$ and reduced thiols in the presence of an $\mathbf{e}^-$ acceptor (Gow, *et al.*, 1997). The potent oxidant $NO_2^•$ can be formed by the autooxidation of $NO^•$, or possibly by the oxidation of NO_2^- by MPO and H_2O_2 (Eiserich, *et al.*, 1996).

5. Bactericidal Antibiotics

Antibiotics can be divided into two broad classes; bacteriostatic drugs that inhibit bacterial growth (Pankey & Sabath, 2004) and bactericidal drugs, which kill bacteria. Induction of ROS following treatment of bacteria with bactericidal antibiotics has been well documented using different types of antibiotic such as aminoglycosides (Kohanski, *et al.*, 2008), quinolines (Goswami, *et al.*, 2006, Dwyer, *et al.*, 2007), rifampicin (Kolodkin-Gal, *et al.*, 2008), and chloramphenicol (Kolodkin-Gal & Engelberg-Kulka, 2008). These antibiotics induce oxidative stress regardless of their specific targets. The general mechanism induced from bactericidal antibiotics involves tricarboxylic acid cycle metabolism that can cause a transient depletion of NADH, destabilization of iron-sulfur clusters, and iron misregulation (Kohanski, *et al.*, 2007). In a follow-up study, it was observed that using aminoglycoside antibiotics, the production of $^{\bullet}OH$ was generated by mistranslation and misfolding of membrane–associated proteins, producing stress-response (Kohanski, *et al.*, 2008). Furthermore, it was more recently reported that oxidation of the guanine nucleotide pool to 8-oxo-guanine underlies cell death of bactericidal antibiotics (Foti, *et al.*, 2012). In another study the role of gyrase was investigated and mechanisms were proposed that can contribute to gyrase inhibitor-mediated cell killing. Gyrase is involved in bacterial chromosomal supercoiling of DNA. Gyrase inhibitors are known to induce cell death by stimulating DNA damage formation, impeding lesion repair and blocking replication processes (Drlica & Zhao, 1997, Couturier, *et al.*, 1998). Dwyer *et al.* showed that oxidative damage by $O_2^{\bullet -}$ and $^{\bullet}OH$ contributes to bacterial cell death following gyrase inhibition (Dwyer, *et al.*, 2007). Indole is a bacterial signaling molecule that is synthesized from tryptophan via the action of tryptophanase (TnaA). A study (Kuczynska-Wisnik, *et al.*, 2010) demonstrated that the treatment of *E. coli* biofilm with bactericidal antibiotics that generate ROS, could inhibit biofilm formation via indole signaling. The increase of the expression of indole is a consequence of overexpression of tryptophanase (TnaA). The inhibition or lack tryptophanase (TnaA) restored the generation of *E. coli* biofilm. However, although it has been demonstrated that, ROS generated from treatment with bactericidal antibiotics play a key role in killing bacterial, several studies have demonstrated that ROS are also directly involved in induction of mutagenesis and resistance. In a recent study, Kohanski and colleagues showed that sub-lethal levels of ROS have an important role in mutagenesis inducted by bactericidal antibiotics. The increase of ROS was correlated to mutagenesis and also, mutagenesis was prevented by inhibiting ROS formation (Kohanski, *et al.*, 2008).

6. Antimicrobial photodynamic inactivation

Photodynamic therapy (PDT) involves the use of photosensitizers (PS) in combination with visible light of the correct wavelength to excite the PS. In the presence of molecular ground (triplet) state oxygen ($^3O_2$), the excited state PS transfers energy or electrons to producing reactive oxygen species (ROS) that are able to kill cells (Dai, *et al.*, 2009). PDT has mostly been developed as a cancer therapy (Agostinis, *et al.*, 2011), but recently has been proposed as an antimicrobial therapy (Hamblin & Hasan, 2004). PDT is an approach that has several favorable features for the treatment of infections caused by microbial pathogens, including a broad spectrum of action, the efficient inactivation of multi-antibiotic-resistant strains, the low mutagenic potential, and the lack of selection of photo-resistant microbial cells (Jori, *et al.*, 2006).

The PS molecule has a stable electronic configuration in the singlet state with the most energetic electrons in the highest occupied molecular orbital (HOMO) (St Denis, *et al.*, 2011). Following absorption of a photon of light of the specific wavelength according to its absorption spectrum (Figure 5 shows a Jablonski diagram illustrating the process), an electron in the HOMO is excited to the lowest unoccupied molecular orbital (LUMO),

causing the PS to reach the unstable and short-lived excited singlet state. In this state, several processes may rapidly occur such as fluorescence and internal conversion to heat, but the most critical of these to PDT, is the reversal of the spin of the excited electron (known as intersystem crossing) to the triplet state of the PS. This excited triplet state is less energetic than the excited singlet state, but has a considerably longer lifetime, as the excited electron, now with a spin parallel to its former paired electron, may not immediately fall back down (as it would then have identical quantum numbers to that of its paired electron, thus violating the Pauli Exclusion Principle). This much longer lifetime (many microseconds as compared to a few nanoseconds) means the triplet PS can survive long enough to carry out chemical reactions which would not have been possible with the excited singlet PS. Dyes without a significant triplet yield may be highly absorbent or fluorescent but are not good PS.

The photochemical reactions of the triplet state can be divided into two different pathways, either the Type I mechanism involving $\mathbf{e}^-$ or hydrogen atom transfer from one molecule to another, or the Type II mechanisms involving energy transfer to molecular oxygen. It should be noted that both these mechanisms can occur at the same time but the relative proportions may depend on the PS structure and also on the microenvironment.

The type I pathway can involve an electron-transfer reaction from the PS to O_2 in the triplet state which results in the formation of toxic oxygen species such as $O_2{\bullet}^-$ that can further transfer to form ROS, such as H_2O_2 and $^{\bullet}OH$, which are formed by the Fenton reaction in the presence of divalent metal ions as Fe^{2+} (Glaeser, *et al.*, 2011) (Eq. 7-9). Another possible mechanism has been proposed that may operate in cases where the triplet state PS is a good $\mathbf{e}^-$ donor. Here H_2O_2 (formed from $O_2{\bullet}^-$) can undergo a one $\mathbf{e}^-$ reduction to form $^{\bullet}OH + {}^-OH$ (the redox potential is only +0.32V, see Table 1).

The two most prevalent damaging ROS ($^{\bullet}OH$ and $^1O_2$) are able to react with many biomolecules in microbial cells. The exact targets and reaction mechanisms involved depend on the following considerations. Firstly the localization of the PS generation is critical because most of the ROS are highly reactive and cannot travel far from their site of production before disappearing. Secondly the relative abundance of the target biomolecule is important. Davies et al (Davies, 2003) calculated the following % reactions of $^1O_2$ in leukocytes: protein 68.5%, ascorbate 16..5%, RNA 6.9%, DNA 5.5%, beta-carotene 0.9%, NADH/NADPH 0.6%, tocopherols 0.5%, reduced glutathione 0.4%, lipids 0.2%, cholesterol 0.1%. It should be noted that the distribution of $^1O_2$ may be different in bacterial cells from that found in leukocytes. Thirdly we have the question of whether Type 1 or Type 2 mechanisms produce the ROS in question. In Table 2 we have tried to point out differences in products that are formed depending on whether $^{\bullet}OH$ and $^1O_2$ are the predominant ROS involved.

The amino-acids that are susceptible to oxidation and the products formed are listed in Table 3. Lipid peroxidation starts with a ROS “stealing” an electron from the lipid in the cell membrane and the process continues via a free-radical chain reaction mechanism proceeding through initiation, propagation, and termination steps as shown in Figure 2. The peroxidation reaction is especially affective in polyunsaturated fatty acids containing non-conjugated double bonds, since the methylene groups (-CH2-) between double bonds are especially susceptible to ROS attack.

PS are usually organic delocalized aromatic molecules consisting of a central chromophore with auxiliary branches (auxochromes) that are responsible for further electron delocalization of the PS, thus altering the absorption spectra of the PS. Due to extensive electron delocalization, PS tend to be deeply colored. This means that the energy required to

excite the $\mathbf{e}^-$ in the HOMO to the LUMO is low compared with less delocalized molecules and therefore, the absorption bands are in the longer wavelength (red) spectral region and are large, reflecting the high probability of excitation. Acridine orange was the first photodynamic agent used for microbial killing (Von Tappenier, 1900) but since then an astonishing array of compounds have been tested (Sharma, *et al.*, 2011). Many of the PS that have been employed for the treatment infections (and indeed of cancer as well) are based on the tetrapyrrole nucleus, with emphasis on the use of porphyrins, chlorins, bacteriochlorins and phthalocyanines. Broadly speaking the PS based on the tetrapyrrole architecture tend to largely undergo Type 2 photochemical mechanisms, i.e. generate $^1O_2$ (Maisch, *et al.*, 2007, Regensburger, *et al.*, 2010). The one exception to this rule appears to be bacteriochlorins, which have been reported (Vakrat-Haglili, *et al.*, 2005, Silva, *et al.*, 2010, Dabrowski, *et al.*, 2012) to generate $^{\bullet}OH$, and other ROS/RNS generated from $O_2^{\bullet -}$ (Ashur, *et al.*, 2009). It is thought that. PS with different molecular frameworks (non-tetrapyrrole based) are more likely to undergo Type I photochemistry as well as Type 2 and examples of these PS include phenothiazinium salts, such as toluidine blue O (TBO) (Martin & Logsdon, 1987) and methylene blue (MB) (Sabbahi, *et al.*, 2008)), perylenequinones such as hypericin (Lopez-Chicon, *et al.*, 2012), functionalized buckminsterfullerenes (e.g., C60) (Mroz, *et al.*, 2007), halogenated xanthenes such as Rose Bengal (Lambert & Kochevar, 1997) etc. Various methods have been employed to distinguish between the different types of ROS produced during PDT. Some of these methods however, frequently attract criticisms based on the perceived lack of selectivity for different ROS (Chaudiere & Ferrari-Iliou, 1999). The most popular method has been the use of various quenchers or scavengers. These are molecules that can be added to the illuminated microbial suspension containing the PS, and will then either consume the ROS or deactivate it thus decreasing the extent of microbial killing. Azide has been used as a selective deactivator of $^1O_2$ by physically removing the energy from the excited state and has been used to reduce microbial killing in several PDT studies (Maisch, *et al.*, 2005, Spesia, *et al.*, 2010, Tavares, *et al.*, 2011, Huang, *et al.*, 2012) and to explore to what extent Type 1 and Type 2 mechanisms operate. Interestingly Huang et al (Huang, *et al.*, 2012) recently reported that azide anion could paradoxically potentiate the killing of both Gram-(+ve) and Gram-(−ve) bacteria by MB and light via the generation of azidyl radicals. Other quenchers that have been used for $^1O_2$ include histidine (Banks, *et al.*, 1985) and beta-carotene (Bohm, *et al.*, 2012). The increase of $^1O_2$ lifetime in deuterium oxide giving greater microbial killing has been used as evidence of Type 2 photochemistry (Ragas, *et al.*, 2010). Quenchers that have been used for $^{\bullet}OH$ include mannitol (Rywkin, *et al.*, 1992), thiourea (Martin & Logsdon, 1987), and dimethyl sulfoxide (Martin & Logsdon, 1987). Another popular approach is to use various compounds whose reaction with ROS can be followed by either absorption or fluorescence spectrometry. These techniques have often been used to determine relative $^1O_2$ quantum yields (Φ_Δ) (Redmond & Gamlin, 1999) by comparison of reaction rates with reference compounds whose Φ_Δ is known (RB and MB were often used for this purpose in the past, but now it is known that these PS are not exclusively Type 2). Examples of these compounds are 1,3-diphenylisobenzofuran (DPBF) (Ma, *et al.*, 1994), 2-amino-3-hydroxypyridine (AHP) (Ma, *et al.*, 1994), 4-nitrosodimethylaniline (RNO) (Matthews & Cui, 1990) and various anthracene derivatives (Kuznetsova, *et al.*, 2001). In recent years the use of fluorescent ROS probes has become popular and $^1O_2$ sensor green (SOSG) (Lin, *et al.*, 2012) and hydroxyphenyl fluorescein (HPF), specific for $^{\bullet}OH$, are becomingly popular (Price, *et al.*, 2009, Price & Kessel, 2010).

Some methods have do exquisite selectivity for different specific ROS and are therefore preferred as gold standards, but do require specialized equipment that may be considered expensive. More laboratories are now using the 1270-nm luminescence emission from $^1O_2$ now that photomultiplier tubes from Hamamatsu that are sensitive to photons at that wavelength are commercially available (Niedre, *et al.*, 2002). Perhaps the most accepted gold standard method is the use of electron paramagnetic resonance (EPR) spectroscopy

combined with spin trap probes (spin-trapping) (Augusto & Muntz Vaz, 2007). If the correct spin-trap is chosen then products can be generated with hyperfine splitting patterns that can be unequivocally attributed to $^1O_2$, $^{\bullet}OH$, and $O_2^{\bullet -}$, not to mention other possible free radicals that may be involved (Villamena & Zweier, 2004). The azidyl radical referred to previously was identified by EPR spin-trapping using DMPO (Huang, *et al.*, 2012). Girotti's laboratory (Geiger, *et al.*, 1997) developed an HPLC system based upon the formation of different cholesterol hydroperoxides. Cholesterol 5-alpha-, 6-alpha-, and 6-beta-OOH were characterized as $^1O_2$ adducts, while 7-alpha- and 7-beta-OOH were products of free radical oxidation.

Various studies have shown a fundamental difference in susceptibility to PDT between different classes of microorganisms (fungi, Gram-(−ve (−) bacteria, Gram-(+ve) bacteria and protozoa). This difference is explained by their physiology and morphology. Jori et al (Jori, *et al.*, 2006) distinguished two broad groups of microbial cells that interact differently with PS Group 1 involves Gram-(+ve) bacteria and protozoa in the trophozoic stage and a direct translocation of the PS through the relatively permeable outer cell wall to the plasma membrane occurs. Group 2 consists of Gram-(−ve) bacteria, fungi, and protozoa in the cystic stage, and an initial increase in the permeability of the outer cell wall is required to allow the PS to penetrate to sensitive cellular locations such as the plasma membrane. Figure 6 shows how the structures of the cell wall and their associated permeability barriers vary between Gram--(−ve) bacteria, Gram--(+ve) bacteria and fungi. Although the first reports used chemical methods of increasing this Gram--(−ve) outer membrane permeability such as treating bacteria with polymyxin B nonapeptide (Nitzan, *et al.*, 1992) with Tris-EDTA to remove lipolysaccharide (Coratza & Molina, 1978), with high calcium to produce competence (Bertoloni, *et al.*, 1984) or with toluene (Villamena & Zweier, 2004), it has now become apparent that the most simple and effective method of achieving effectiveness against these classes of microbe is to choose or synthesize PS with pronounced cationic charges (Costa, *et al.*, 2012). This desirable goal can be achieved in two different ways. Firstly PS used for other diseases such as cancer and bearing neutral or anionic charges can be chemically conjugated to carriers that that have cationic charges at physiological pH such as poly-L-lysine (Hamblin, *et al.*, 2002) or polyethylenimine (Tegos, *et al.*, 2006) or encapsulated in liposomes with cationic charges on the outside (Bombelli, *et al.*, 2008). The second approach is simply to have constitutive cationic charges on the PS molecule itself by using quaternary ammonium or phosphonium groups (Sharma, *et al.*, 2011). Furthermore the class of dyes typified by phenothiazinium salts (MB, TBO etc) have constitutive cationic charges as part of their chemical structure (Wainwright, *et al.*, 1997). Although there is general agreement that cationic charges on the PS are important for broad spectrum antimicrobial activity, it may be the case that different amounts of cationic charges can be optimal for different classes of microbial cell. For instance when studying a set of four bacteriochlorins Huang et al (Huang, *et al.*, 2010) found that six quaternary groups was optimal for Gram-(−ve) bacteria, while two quaternary groups were best for Gram-(+ve) bacteria and two basic groups (no constitutive cationic charges) were best for Candida fungal cells.

It is quite clear that although microbial cells that have formed biofilms can still be killed by PDT, they are significantly more resistant than planktonic cells (Mantareva, *et al.*, 2011). Studies that have dissociated bacterial biofilms into planktonic cell suspensions before subjecting them to PDT, and comparing the results to those obtained with logarithmic phase planktonic cells have shown that the resistance of biofilms mainly consists of difficulties faced by PS in penetrating through the exopolysaccharide matrix, rather than intrinsic changes in gene expression found in biofilm cells affecting susceptibility to ROS. Furthermore it may be the case that the highly effective polycationic PS find it more difficult to penetrate the anionic biofilm matrix because they bind too strongly to it compared with

less effective PS with fewer cationic charges. The use of cationic PS also has a second highly beneficial property besides the property of broad spectrum activity (because they are taken up by all classes of microbial cells). This second property allows selectivity for microbial cells over host mammalian cells that will be present in an infected lesion (Mantareva, *et al.*, 2011). This is because the binding and uptake of cationic compounds by microbial cells is rapid while the uptake of cationic compounds by mammalian cells is a slow process. Cationic compounds are taken up into mammalian cells by the slow process of absorptive endocytosis as contrasted with the rapid process of diffusion through the plasma membrane that occurs with neutral lipophilic compounds. Therefore if the drug-light interval is short (few minutes) after the cationic PS have been topically applied to an infected lesion, microbial cells will be selectively killed, while if the drug-light interval is long (hours) the possibility of harming surrounding host tissue is much higher. Furthermore it is generally accepted that the appropriate manner to deliver antimicrobial PS to infected lesions is by topical or local delivery into the infected lesion rather than the intravenous, oral or intraperitoneal systemic delivery routes that are mostly used for cancer (both in animal models and in patients).

This selectivity of cationic PS for microbial cells has been demonstrated many times both in vitro and in vivo. Our laboratory has devised mouse models of localized infections caused by bacteria and fungi that have been genetically engineered to emit bioluminescence allowing monitoring of the infection by real-time non-invasive optical imaging (Demidova, *et al.*, 2005). These models have allowed us to demonstrate the ability of PDT to effectively treat wound, burn and soft-tissue infections using a variety of PS including pL-ce6 (Gad, *et al.*, 2004), PEI-ce6 (Dai, *et al.*, 2009), cationic fullerenes (Lu, *et al.*, 2010), phenothiazinium salts (Ragas, *et al.*, 2010), and a cationic porphycene (Ragas, *et al.*, 2010).

Several clinical applications of PDT for localized infections have emerged or are in the process of being tested (Kharkwal, *et al.*, 2011). The most successful is “Periowave” for periodontitis (Berakdar, *et al.*, 2012) in which MB and red light are introduced into the dental pocket (Atieh, 2010). MRSAaid is a PDT nasal decontamination system (http://mrsaid.com/) and applications of PDT for chronic sinusitis (Biel, *et al.*, 2011), endotracheal tube disinfection (Biel, *et al.*, 2011) and infected leg ulcers (Clayton & Harrison, 2007) are also under investigation.

7. Photocatalytic disinfection

The ability of titanium dioxide (TiO_2) to act as a photocatalyst has been known for 90 years (Hashimoto, *et al.*, 2005). In 1972 Fujishima and Honda (Fujishima & Honda, 1972) first reported the photoelectrolysis of water at a semiconductor electrode, and this property was then utilized to catalyze the oxidation of pollutants (Carey, *et al.*, 1976, Frank & Bard, 1977). Photocatalytic surfaces can be manufactured into construction and building materials (Chen & Poon, 2009) and commercial uses include self-cleaning windows and self-cleaning glass covers for road lights (Hashimoto, *et al.*, 2005)

The mechanism of photocatalysis relies upon the fact that TiO_2 is a semiconductor. The adsorption of a photon with sufficient energy promotes an electron from the valence band to the conduction band leaving a positively charged hole in the valence band. The bandgap energy (energy required to promote an electron) of anatase TiO_2 is approximately 3.2 eV, which means that photocatalysis can be activated by photons with a wavelength shorter than 385 nm (i.e. UVA). The hole may be filled by migration of an electron from an adjacent molecule, leaving that molecule with a hole, and so on. A recent paper (Schrauben, *et al.*, 2012) suggested that the mechanism would be better characterized as “proton-coupled electron transfer”. Electrons and holes may recombine and neutralize each other (bulk

recombination), or when the electrons reach the surface, they can react O_2 to give $O_2^{\bullet -}$. As discussed previously $O_2^{\bullet -}$ can react further to form H_2O_2 and $^{\bullet}OH$. It is possible to apply an electric field to enhance charge separation of the $\mathbf{e}^-$ and holes and reduce the likelihood of bulk recombination termed photoelectrocatalysis (Harper, *et al.*, 2000, Zhang, *et al.*, 2008)

There are three main polymorphs of TiO_2: anatase, rutile and brookite. The majority of studies show that anatase was the most effective photocatalyst and that rutile was less active; the differences are probably due to differences in the extent of recombination of $\mathbf{e}^-$ and hole between the two forms (Lilja, *et al.*, 2012). However, studies have shown that mixtures of anatase and rutile were more effective photocatalysts than 100% anatase and were more efficient for inactivating viruses (Lilja, *et al.*, 2012).

The utility of photocatalytic disinfection is limited by the requirement for UVA light for activation. Catalysts can be modified by doping with metals such as Sn, Pd, and Cu to reduce the band gap and extend the useful spectrum into the visible region (Rehman, *et al.*, 2009, Lilja, *et al.*, 2012). Zinc oxide can also be used as a photocatalyst (Giraldi, *et al.*, 2011) but even though it has a higher band gap (3.37 eV) than TiO_2, it was found to perform better in sunlight (Sakthivel, *et al.*, 2003). Zhang et al (Zhang, *et al.*, 2010) prepared mixed nanoparticles containing both TiO2 and ZnO

Since the efficiency of generation of ROS by photocatalysis depends on the ratio of surface area to mass, it is not surprising that a wide range on nanoparticles have been tested for this application (Di Paola, *et al.*, 2012). These have included nanotubes (Roy, *et al.*, 2011, Xu, *et al.*, 2012), nanorods (Ma, *et al.*, 2011), nanowires (Wang, *et al.*, 2011), nanopyramids(Li, *et al.*, 2011), and core-shell nanocomposites (Zhang, *et al.*, 2012), Matsunaga and colleagues (Matsunaga, *et al.*, 1985, Matsunaga, *et al.*, 1988) were the first to use TiO_2 photocatalysis to kill microorganisms. This subject area has recently been comprehensively reviewed (McCullagh, *et al.*, 2007, Foster, *et al.*, 2011) and the effect of key variables on the effectiveness has been studied (Cushnie, *et al.*, 2009). The applications that have been proposed include sterilization and decontamination of wastewater (Praveena & Swaminathan, 2003), manufacture of antibacterial fabrics (Kangwansupamonkon, *et al.*, 2009), self-sterilizing urinary catheters (Sekiguchi, *et al.*, 2007), self-sterilizing lancet for blood glucose determination (Nakamura, *et al.*, 2007), antibacterial food packaging film (Chawengkijwanich & Hayata, 2008), antibacterial dental implants (Suketa, *et al.*, 2005), antibacterial surgical implants (Lilja, *et al.*, 2012) and many more.

8. Cold atmospheric plasma

Plasma is considered the fourth state of matter and in fact is the commonest form of matter in the universe by both mass and volume. It forms the material that stars are composed of and (at very low density) fills the interstellar space. Plasma contains ionized atoms and $\mathbf{e}^-$ and responds to magnetic fields and conducts electricity. The degree of ionization reflects its temperature; plasmas with temperatures of millions of degrees are completely ionized, while cold or non-thermal plasmas may only have 1% of the gas molecules ionized. Cold plasma is generated by an pulsed high voltage electric discharge between electrodes between which is flowing a gas (often a mixture of helium and oxygen). The primary chemical process is ionization of oxygen molecules which can then react with nitrogen in the air to produce a cocktail of reactive species including ($^1O_2$, $^{\bullet}OH$, $O_2^{\bullet -}$, O_3, $NO^{\bullet}$, $NO^{\bullet}{}_2$, etc.), charged particles ($\mathbf{e}^-$, ions), and UV photons (4 peaks between 300-400 nm derived from emission from excited nitrogen molecules) (Maisch, *et al.*, 2012). All of these species are well known to have antimicrobial activity (Laroussi, 2005). Wu et al (Wu, *et al.*, 2012) used electron spin resonance to demonstrate the presence of $^1O_2$ and $^{\bullet}OH$ in cold plasma and by using quenchers suggested that $^1O_2$ contributed most to the killing. There have been efforts to

introduce the new concept of “plasma medicine” and international conferences have been held on this topic (http://www3.interscience.wiley.com/journal/121376857/issue). A new journal entitled “Plasma Medicine” has also been launched in 2011.

Several devices have been described; one of which was termed the “plasma pencil” (Laroussi & Lu, 2005, Peplow, 2005). The device (illustrated in Figure 7) emits a cold plasma plume several centimeters in length. The plasma plume can be touched by bare hands and can be directed manually by a user to come in contact with delicate objects and materials including living tissue without causing any heating or painful sensation. It was reported to be highly effective at killing bacteria (Laroussi, *et al.*, 2006). Another device termed the “FlatPlaSter” used a 6.75 kHz pulsed voltage with a peak-to-peak of approximately 7 kV to excite ambient air (Maisch, *et al.*, 2012). Other devices use radiofrequency voltages (for instance 13.56MHz) to generate cold plasma in N_2 or N_2/O_2 mixtures (Sureshkumar, *et al.*, 2010). Cold argon plasma is widely used for coagulation in endoscopic GI surgery (Manner, 2008), but can also be used as an antimicrobial therapy (Fricke, *et al.*, 2012).

One of the primary applications of cold plasma is to kill microrganisms for sterilization of foods (Niemira, 2012) or for dermatological (Heinlin, *et al.*, 2011) and dental (Yang, *et al.*, 2011) uses. This microbial killing occurs via several mechanisms: (a) permeabliisation of the cell wall/membrane, leading to leakage of potassium; (b) intracellular damage due to oxidative or nitrosative damage to critical proteins; (c) direct chemical damage to DNA. There have been several studies directed towards using cold plasma as a specfic approach for inactivating microbial biofilms (Joaquin, *et al.*, 2009). Biofilm inactivation has been shown with *Pseudomonas aeruginosa* (Alkawareek, *et al.*, 2012),*Enterococcus faecalis* (Jiang, *et al.*, 2012), and *Candida albicans* (Sun, *et al.*, 2012). Cold plasma has been used to inactivate bacterial endospores from Bacillus and Clostridum species (Tseng, *et al.*, 2012).

One of the advantages claimed for cold plasma as an antimicrobial treatment is its non-toxicity towards host tissue. For instance Maisch et al (Maisch, *et al.*, 2012) demonstrated a reduction of 5 log10 in CFU of *S. aureus* (both methicillin sensitive and resistant) and *E. coli* on ex vivo pig skin without morphological changes or histological signs of necrosis or apoptosis as determined by the TUNEL-assay.

Isbary et al (Isbary, *et al.*, 2010) conducted a prospective randomized controlled trial to decrease bacterial load using cold atmospheric argon plasma on chronic wounds in 36 patients. Patients received 5 min daily applications of cold atmospheric argon plasma in addition to standard wound care. Analysis of 291 treatments in 38 wounds found a highly significant (34%, $P < 10(-6)$) reduction of bacterial load in treated wounds, regardless of the species of bacteria. No side-effects occurred and the treatment was well tolerated.

9 Medicinal honey

Honey has been used in treatment of wounds since ancient times (Zumla A., 1989, Kwakman, *et al.*, 2011). However with the emergence of antibiotics, the clinical application of honey was abandoned in modern Western medicine. In recent times, the development of antibiotic resistance has increased interest in alternative antimicrobial agents and honey is regaining its popularity again. In several studies, honey has been shown to be bacteriocidal or bacteriostatic against organisms such as; *B, subtilis, S. aureus,* methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, extended-spectrum β-lactamase producing E. coli, E. faecium, Enterobacter cloacae, K. oxytoca, ciprofloxacin-resistant *P. aeruginosa,* and vancomycin-resistant *E. faecium* (VRE), *C. albicans, A. baumannii* and Leishmania parasites (Zeina, *et al.*, 1997, Kwakman, *et al.*, 2008, Tan, *et al.*, 2009, Kwakman, *et al.*, 2010, Alzahrani, *et al.*, 2012). Manuka and Revamil® are the two major types of medical-

grade honeys currently used for clinical application. The high sugar concentration, action of glucose peroxidase to produce H_2O_2 and $^{\bullet}OH$, low pH, lysozyme, antibacterial aromatic acids, trans-10-hydroxy-decene-(2)-acid-(1) (10-HAD, the main royal jelly acid), methylglyoxal (MGO) and the antimicrobial peptide bee defensin-1 have been identified as important antibacterial components in honey (Russel, 1988, Isidorov, 2011, Chen, *et al.*, 2012, Kwakman & Zaat, 2012).

Different microbial species have varying susceptibility to different types of honey since H_2O_2, MGO, and bee defensin-1 differentially contribute to each type of honey and in turn to their antimicrobial activity (Kwakman, *et al.*, 2010, Kwakman, *et al.*, 2011). For instance, it has been shown that *E. coli* and *P. aeruginosa* are substantially less susceptible to manuka honey than *S. aureus* and *B. subtilis* (Kwakman, *et al.*, 2010). Bee defensin-1 and H_2O_2 were shown to be the major factors involved in rapid bactericidal activity of Revamil source honey, however these factors were absent in manuka honey, and instead manuka honey contained 44-fold higher concentrations of methylglyoxal than Revamil source honey (Kwakman, *et al.*, 2010).

Brudzynski et al. reported that the damaging effects of honey H_2O_2 were strongly influenced by the microbial sensitivity, defense mechanisms to oxidative stress, the growth phase (exponential vs stationary) and survival strategy (non-spore forming vs spore forming) (Brudzynski, *et al.*, 2011). Glucose oxidase, a carbohydrate-metabolizing enzyme secreted from the hypopharyngeal glands of honey bees and added to nectar, converts glucose into H_2O_2 and gluconic acid under aerobic conditions (White, 1963, Bang, *et al.*, 2003). The function of H_2O_2 is assumed to be prevention of spoilage of unripe honey when the sugar concentration has not yet reached sufficient levels to prevent microbial growth (Kwakman & Zaat, 2012). During the ripening of honey, glucose oxidase is inactivated, due to lack of free water, however its activity is restored when honey is diluted with water, providing a slow, sustained release of H_2O_2 (Chen, *et al.*, 2012, Kwakman & Zaat, 2012). H_2O_2 accumulation is highest in the range of 30–50% honey in water, and below 30%, it starts declining rapidly(Schepartz & Subers, 1964). Furthermore, accumulation of H_2O_2 varies among different honeys (Kwakman, *et al.*, 2011, Chen, *et al.*, 2012, Kwakman & Zaat, 2012). Even though a functional relationship between H_2O_2 produced in honey and antibacterial activity strongly suggests that H_2O_2 is the main contributor to this activity (White, *et al.*, 1963, Bang, *et al.*, 2003, Brudzynski, 2006, Chen, *et al.*, 2012), recent new findings question this view. One reason is that molecular H_2O_2 is a relatively weak oxidant and it requires high concentrations to exert its cytotoxic effect (Finnegan, *et al.*, 2010) however as shown by Brudzynski et al. the average H_2O_2 content in honey is over 900-fold lower than that observed in H_2O_2 disinfectant and yet even at these low concentrations, honey effectively inhibited bacterial growth and caused DNA strand breaks (Molan, 1992, Brudzynski, *et al.*, 2011). Secondly, the oxidative stress of endogenous H_2O_2 was clearly augmented by the action of unknown honey components (Brudzynski, *et al.*, 2011). Thirdly, even though honey samples with little or no H_2O_2 have a correspondingly low ability to inhibit bacteria and fungi, when present, the level of H_2O_2 and the degree to which the honey is antimicrobial do not necessarily correlate (Chen, *et al.*, 2012), and lastly, Brudzynski et al. demonstrated for the first time that honeys of high bacteriostatic activity possessed significantly higher levels of phenolic compounds with radical scavenging activities than honey with average bacteriostatic activity, suggesting that a coupling chemistry between H_2O_2 and polyphenols, rather than H_2O_2 alone, may exert the oxidative effect causing bacterial growth arrest and DNA degradation (Brudzynski, 2012).

Although high oxygen radical quenching capacity of polyphenols are usually indicative of their efficient antioxidant abilities (Cao, *et al.*, 1997, Price, *et al.*, 2006), the same polyphenols have the potential to turn into powerful prooxidants when oxidized. Oxidized

polyphenols further generate H_2O_2 and in the presence of transition metals such as Cu(I) or Fe(II) they drive the generation of $^{\bullet}OH$ from H_2O_2 via the Fenton reaction (Puppo, 1992, Hanasaki, *et al.*, 1994, Cao, *et al.*, 1997, Brudzynski & Lannigan, 2012). Honey possesses all necessary substrates for the Fenton reaction; H_2O_2, polyphenols as well as transition metal ions (Bogdanov, 2007).

When two bacterial strains of MRSA and VRE were incubated with dilutions of buckwheat honey, there was a steady accumulation of $^{\bullet}OH$ up to the 16-fold dilution, and the peak of $^{\bullet}OH$ generation corresponded to the MIC of honey (Brudzynski & Lannigan, 2012). However, further honey dilutions resulted in a decrease of $^{\bullet}OH$ levels and loss of growth inhibition (Brudzynski & Lannigan, 2012). These findings support a causal relationship between $^{\bullet}OH$ generation and bacterial growth inhibition (Brudzynski & Lannigan, 2012) and suggest that the oxidative stress caused by honey action on bacterial cells may result from $^{\bullet}OH$ rather than from the action of molecular H_2O_2 itself and the redox capacity of honey polyphenols plays a role (Brudzynski, 2012). However, in a subsequent study $^{\bullet}OH$ radicals could not be identified as inducers of bacterial cell death (Brudzynski, *et al.*, 2011) which suggests that other free radicals generated from the initial Fenton reaction may be effector molecules as well (Brudzynski, 2012). Lastly, it is important to note that the $^{\bullet}OH$ based mechanism of honey action is equally effective against antibiotic-sensitive and antibiotic-resistant bacteria (Brudzynski, *et al.*, 2011, Brudzynski & Lannigan, 2012).

A synthetic honey containing glucose and glucose oxidase has been developed with antimicrobial activity. It was based on a technology that allows for the enzyme catalysed synthesis of H_2O_2 to be in a stable sustained release format. This product has demonstrated better antibacterial efficacy than manuka honey in-vitro. The organisms which have been shown to be susceptible include MRSA, Campylobacter spp., S. pyogenes, P. aeruginosa, S. aureus, E .coli, B. cereus and *Propionibacterium acnes.* The technology has also demonstrated significant antifungal activity against C. albicans, Saccharomyces cerevisiae, Botrytis cinerea and a number of unidentified mould isolates.

In terms of clinical applications, best evidence regarding the efficacy of honey has been obtained for treatment of mild to moderate superficial and partial thickness burn wounds, where honey was found to be more effective than conventional treatment for reduction of microbial colonization and improved healing (Moore, *et al.*, 2001, Jull, *et al.*, 2008). Johnson et al. also demonstrated that, application of honey resulted in bacteremia-free period of hemodialysis catheters compared to that obtained with mupirocin treatment (Johnson, *et al.*, 2005). Surgical wounds (Chirife, *et al.*, 1982, Greenwood, 1993), decubitus ulcers (Van der Weyden, 2003), medium for storing skin grafts (Subrahmanyam, 1993) are among its other potential applications.

10. Miscellaneous ROS

There are a set of miscellaneous techniques that exert an antimicrobial effect (amongst other activities) mediated by ROS or oxygen and its derivatives. These are sometimes referred to as “oxygen therapies” (Altman, 2007) but use of this term carries a connotation of alternative and unproven approaches (Cassileth, 2009).

10.1 Hyperbaric oxygen

Hyperbaric oxygen therapy (HBOT) involves exposure to and inhalation of 100% oxygen at supra-atmospheric ambient pressure (2-3 atmospheres). HBOT has been used for many different medical applications throughout the years including infections (Cimsit, *et al.*, 2009). HBOT is well established to be effective when used as either a primary or adjunctive treatment in the management of infections such as gas gangrene (Slack, *et al.*, 1969),

necrotizing fasciitis (Wilkinson & Doolette, 2004), diabetic foot infections (Chen, *et al.*, 2010), refractory osteomyelitis (Andel, *et al.*, 1998), neurosurgical infections (Larsson, *et al.*, 2008) and fungal infections (Bitterman, 2007). HBOT has been proposed to act as a bactericidal/bacteriostatic agent against both aerobic, and particularly anerobic, bacteria by increasing the formation of free oxygen radicals (Cimsit, *et al.*, 2009). HBOT also restores the bacterial-killing capacity of leukocytes in hypoxic wounds by increasing tissue oxygen tensions (Clark & Moon, 1999). At present it is uncertain whether the direct ROS-mediated killing of microbial cells or the potentiation of antimicrobial host defense by HBOT is most important for its success in lowering the infectious burden in the tissue.

10.2 Ozone and polyatomic oxygen

It is well known that ozone is poisonous, that it is an important factor in air pollution particularly affecting children, and that inhalation can damage the lungs with possible serious consequences (Mathieu-Nolf, 2002). However (in common with many other therapies that induce ROS) at very low doses (Bocci, 2004) the outcome of treatment with ozone can be beneficial rather than damaging (Valacchi, *et al.*, 2005, Bocci, *et al.*, 2009). Therapeutic applications of ozone to prevent or control infection in animal models have comprised repetitive pneumo-peritoneum with ozonized oxygen to reduce the severity of sepsis developing after cecal ligation and puncture in rats (Schulz, *et al.*, 2003, Silva, *et al.*, 2009). Treatment with perfluorocarbons loaded with ozonized oxygen was applied to infected wounds in rats (Dmitrieva, *et al.*, 2009). Intraperitoneal ozone administration reduced parasite burden in a mouse model of *Schistosomiasis mansoni* infection (Thabet, *et al.*, 2007).

Clinical applications have included intra-operative delivery during surgery for total hip plasty to prevent infection (Bialoszewski, 2003) and the combination of ozone with ultrasound in the treatment of purulent wounds (Lipatov, *et al.*, 2002).

There was an alternative medicine treatment approach called “Polyatomic oxygen and polyatomic apheresis” that was championed by Basil Earle Wainwright, and which involved treatment of blood with oxygen containing both O_3 and O_4 (Viebahn-Haensler, 1999). This approach was largely discredited after Wainwright was prosecuted for claiming his system could cure AIDS (Farley, 1994).

10.3 Hydrogen peroxide

Hydrogen peroxide (3% v/v) has long been used as a topical antiseptic to be applied to wounds and as a disinfectant for various applications. In many dental applications such as endodontics (Poggio, *et al.*, 2012) and periodontitis (Wennstrom, *et al.*, 1987) H_2O_2 is employed as a topical antimicrobial. In endodontics H_2O_2 can be combined with sodium hypochlorite as an irrigant for the infected root canal (Grossman, 1943); this reaction is known to produce $^1O_2$ (Tsukagoshi, *et al.*, 2006). A Japanese group reported a disinfection system that relied upon the photolysis of H_2O_2 to form $^{\bullet}OH$ (as shown by ESR spin-trapping), which was catalyzed by violet light (405 nm) either from a laser (Ikai, *et al.*, 2010) or from a LED array (Hayashi, *et al.*, 2012). The system was designed to be used in dental infections and was tested in the oral mucosa and wounded skin of rats with no evidence of injury and with some evidence of improved wound healing (Yamada, *et al.*, 2012). Another paper from this group showed a synergistic effect between photolysis of H_2O_2 and elevated temperature (up to 55°C) (Shirato, *et al.*, 2012). Again it should be mentioned that there is a substantial movement in alternative medicine to use systemic H_2O_2 therapy (30% food grade) for treating cancer, AIDs and infections (Douglass, 2003). Although conventional medical opinion remains that H_2O_2 is poisonous and can kill by causing oxygen gas embolisms (Watt, *et al.*, 2004).

10.4 Superoxidized water and atomic oxygen radical anion

Superoxidized water is a solution of sodium chloride, which has been electrolyzed by passage over titanium electrodes at 9 amp. The product that is produced has a pH of 5.0–6.5, an oxidation potential of >950 mV and is mainly HClO at a concentration of approximately144 mg/L, Depending on the manufacturer, superoxidized water can be called “Sterilox” (Selkon, *et al.*, 1999), “Microcyn” (Landa-Solis, *et al.*, 2005), “Optident” (Martin & Gallagher, 2005), or “Oxum” (Abhyankar, *et al.*, 2009). These solutions have been used in wound sterilization where Oxum was found to be superior to povidone iodine (Kapur & Marwaha, 2011) for care of the diabetic foot (Peters, *et al.*, 2012). One study (Wang, *et al.*, 2007) reported that “atomic oxygen radical anion” or $O^{\bullet -}$ was present in superoxidized water and killed *E. coli.* This chemistry of this species ($O^{\bullet -}$) has been comprehensively reviewed (Lee & Grabowski, 1992) and it was also reported (Lee & Grabowski, 1992) to be released from the surface of a crystal composed of $12CaO{:}7Al_2O_3$

11. RNS-based approaches

Nitric oxide ($NO^{\bullet}$) serves multiple physiological functions in the cardiovascular, respiratory, gastrointestinal, genitourinary, and central and peripheral nervous systems. However synthesis of $NO^{\bullet}$ also contributes to host defense and has cytostatic and cytotoxic effects against certain pathogens. $NO^{\bullet}$ is a naturally occurring short lived free radical that itself is not a ROS. The sphere of influence of $NO^{\bullet}$ is around 100μm from its origin and it is likely that it only affects a few cells (Miller & Megson, 2007). The dual action effect so characteristic of ROS/RNS is observed in $NO^{\bullet}$ as well: in nanomolar concentrations (generated by constitutive NO synthase (NOS) isoforms) $NO^{\bullet}$ acts as a cell signaling molecule and interacts preferentially with its physiological target enzymes—the most significant of which seem to be soluble guanylyl cyclase and possibly cytochrome C oxidase; whereas at higher concentrations (micromolar and higher), when the cytokine-induced isoform of NOS (iNOS) is expressed in cells, $NO^{\bullet}$ is cytotoxic. Thus it is necessary to find practical delivery systems for $NO^{\bullet}$ in order to exert its therapeutic antimicrobial effect in infections.

11.1. Acidified nitrite

$NO^{\bullet}$ apparently plays an important role in adult stomachs as well. It is found that human breast milk contains high concentrations of nitrate and nitrite in the early postpartum period. By acidification the nitrite leads to the generation of $NO^{\bullet}$ in the gastric lumen. Breast milk thus regulates the mucosal blood flow and gastric motility and achieves bacteriostasis via induction of $NO^{\bullet}$ generation in the neonatal stomach (Iizuka, *et al.*, 1999). Xu et al. studied the bactericidal effects of $NO^{\bullet}$ on *E. coli* and lactobacilli. They demonstrated the bactericidal effect and chemical reactions of acidified nitrite under conditions simulating the stomach, suggesting its role in the ecology of the gastrointestinal tract and in host physiology (Xu, *et al.*, 2001).

Major et al (Major, *et al.*, 2010) demonstrated acidified nitrite could kill the cystic fibrosis pathogens *P. aeruginosa, S. aureus*, and *B. cepacia* under anaerobic planktonic and biofilm conditions. Shlaq et al (Schlag, *et al.*, 2007) showed that staphylococcal biofilm formation and polysaccharide intercellular adhesion synthesis can be inhibited by nitrite and preformed biofilm removed. Dave et al (Dave, *et al.*, 2012) developed a hydrogel based dressing containing citric acid cross-linked cotton gauze and sodium nitrite loaded gelatin. The dressing showed significant antimicrobial activity against both planktonic and biofilm forms and was effective during consecutive re-uses.

Omerod et al (Ormerod, *et al.*, 2011) carried out an observational prospective clinical trial of topical acidified nitrite cream (4.5% citric acid and 3% sodium nitrite) for killing MRSA in

contaminated wounds. Nine of 15 wounds (60%) and 3 of 8 patients (37%) were cleared of infection. A similar preparation was used to treat onychomycosis (Finnen, *et al.*, 2007) in 13 patients with positive mycological cultures for *Trichophyton* or *Fusarium* species for 16 weeks. The formation of S-nitrosocysteine was shown throughout the treated nail and >90% of the nails became culture negative.

11.2. NO• releasing nanoparticles

There have been several published studies describing the use of NO• releasing nanoparticles (NO-np) for eradication of soft tissue and skin infections NO-np was first introduced by Freidman et al and this nanoparticle has many of the critical characteristics that an ideal NO• carrier is supposed to have (Englander & Friedman, 2010). They developed a new biocompatible platform based on hydrogel/glass composites in order to prepare NO-np. The sucrose and trehalose were doped with glucose or tagatose for preparation of these composites and they act as the thermal electron source during the thermal conversion of nitrite to NO•. The data demonstrated that therapeutic levels of NO• in a controlled and sustained manner were released following exposure of these nanoparticles to moisture. The release profiles for the NO• from these new platforms can be easily tuned by adding of specific additives in the structure of these composites.

Hetrick and et al (Hetrick, *et al.*, 2009) investigated the ability of NO-releasing silica nanoparticles to eradicate biofilms of *P. aeruginosa, E. coli, S. aureus, S. epidermidis,* and *C. albicans.* The results showed that this approach was more effective and more than 99% of cells from each type of biofilm were eliminated. Furthermore, investigators observed that Gram-negative bacteria were more susceptible than Gram-positive species. Based on these results, they concluded that using nanoparticles for delivery of antimicrobial agents such as NO• to microbial biofilms could be a promising approach.

Recently, NO-np was applied in order to treat wounds infected with *A. baumannii* (Ab) (Mihu, *et al.*, 2010). The results demonstrated that NO-np significantly promoted healing of infected wounds in murine model. Additionally, in the treated model where nanoparticles were used, less inflammation, microbial burden and degradation of collagen was observed. These findings suggested that NO-np was a promising candidate for the treatment of Ab wound infections. Han and et al (Han, *et al.*, 2012) investigated the effects of NO• releasing nanoparticles on wound healing in mice. Their results showed that wound healing was accelerated through application of NO-np due to its promoting effects on fibroblast migration and collagen deposition. Moreover, NO-np modified leukocyte infiltration and increased angiogenesis and TGF-beta production in the wound area.

In the study of (Friedman, *et al.*, 2011), the efficacy of a NO-np against certain Gram-negative and Gram-positive bacteria isolated from clinical samples was investigated. Samples were tested against both antibiotic sensitive and resistant strains. The results showed that NO-np were uniformly effective against all clinical isolates obtained i.e. E. faecalis, S. pyogenes, K. pneumonia, P. aeruginosa and *E. coli,* and concluded that NO-np was a very powerful antimicrobial agent with a wide spectrum antimicrobial activity.

An innovative therapeutic method based on NO-np for *C. albicans* burn infection was developed by Macherla et al (Macherla, *et al.*, 2012). *C. albicans* growth and morphogenesis was arrested by using NO-np; thus its antifungal activity was demonstrated in a murine burn model. The results revealed that hydrogel/glass nanoparticles containing NO• enabled acceleration of wound healing in cutaneous burn infections, through modification of leukocyte migration, minimizing the fungal burden and reducing collagen destruction. Based on these results, it can be suggested that NO-np can be emerged as a novel antifungal approach for treatment of cutaneous burn infections and wounds (Macherla, *et al.*, 2012).

Antimicrobial activity of NO-np against MRSA abscesses in a mouse model was demonstrated by Han et al (Han, *et al.*, 2009). The involved area and bacterial burden was reduced following topical or intradermal administrations of NO-np in abscesses, and skin architecture was preserved through inhibition of collagen degradation. Furthermore, it was observed that NO-np stimulated cytokine response in the mice model as quantities of TGF-β, MCP-1, IL-12, IL-1β, TNF-α and IFN-γ increased following of treatment of infected mice with NO• releasing nanoparticles.

Schairer et al (Schairer, *et al.*, 2012) compared the effectiveness of NO-np and vancomycin for treatment of intramuscular abscesses infected with MRSA. The results showed that treatment with topical (TP) and intralesional (IL) NO-np administration in combination with vancomycin was able to accelerate resolution of the abscesses, and both TP and IL administration of NO-np were more effective than systemic treatment with vancomycin alone.

Recently, a simple and reproducible method was developed for synthesis of NO-ruthenium nanoparticles. This water-soluble derivative of a novel NO-np could deliver NO• to reduced myoglobin following light irradiation in aqueous media. It is supposed that this new kind of NO-np may find some applications in biomedicine (Ho, *et al.*, 2011). In order to generate NO• in a controlled and sustained manner, a new platform based on hydrogel/glass hybrid for preparation of NO-np was synthesized. The requirement for stimulation of release of NO• from these nanoparticles is moisture environment, so when the prepared NO-np is exposed to moisture, the therapeutic levels of NO• start being released in a slow controlled manner. Later on, the NO• was trapped within the dry nanoparticle following thermal reduction of nitrite to NO•. The advantages of NO-np relative to other NO• releasing compounds is the sustained release of NO• controlled by the rate of hydration and no necessity for chemical decomposition nor enzymatic catalysis (Cabrales, *et al.*, 2010, Muzykantov, 2010).

12. Conclusion

ROS and RNS are attractive weapons to kill pathogenic microbial cells. They can be produced by a wide variety of mechanisms ranging from the use of standard antibiotics and disinfectants, to naturally occurring honey, to more hitech routes such as PDT, photocatalysis, cold plasma and NO• releasing nanoparticles. The selectivity for microbial cells over host mammalian cells appears to be favorable for many of these delivery routes. Although microbial cells can develop resistance by upregulating defenses to some types of ROS such as $O_2^{\bullet -}$ and H_2O_2, they are unable to defend themselves against others such as •OH and $^1O_2$. The involvement of RNS in host defense and gastric pathogen destruction has been known for some time but only recently has it been proposed as a therapy with NO• releasing nanoparticles. The relative future development of these diverse ROS/RNS based strategies as clinical therapies remains difficult to predict, but at least one of them (and possible more) should make it to the clinic.

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One sentence summary

Reactive oxygen species are involved in cell signaling and disease causing damages, and in the antimicrobial arena mediate killing via bactericidal antibiotics, photodynamic inactivation, titania photocatalysis, cold plasma, medicinal honey, and nitric oxide releasing nanoparticles.

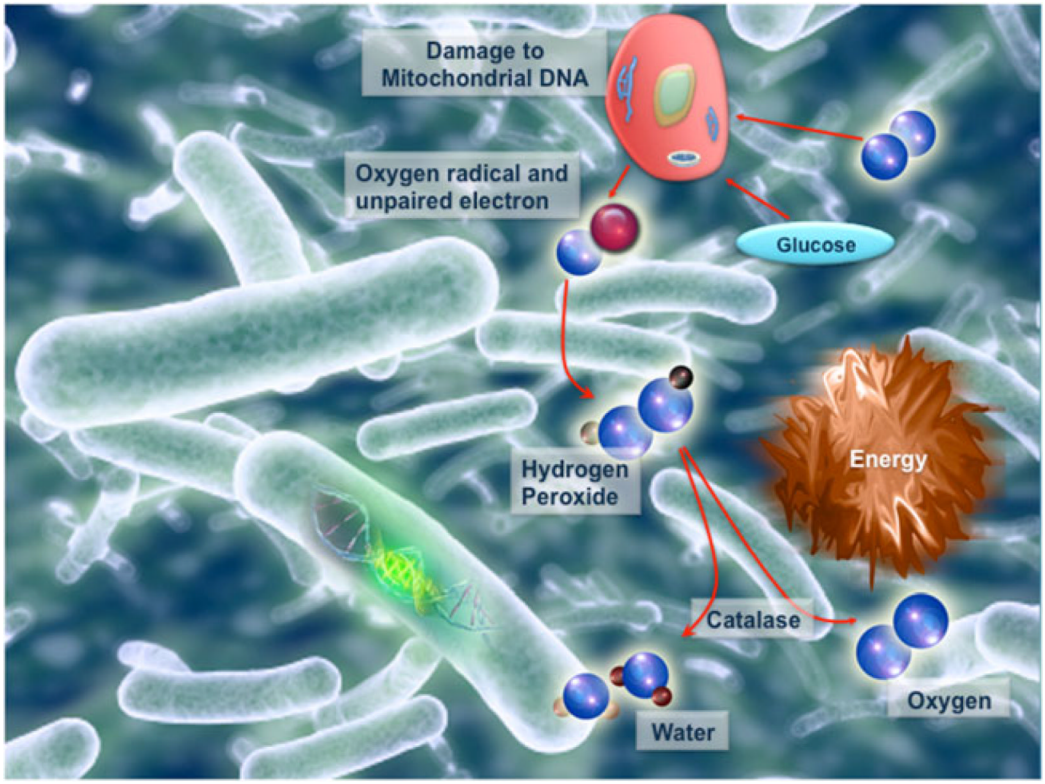

Figure 1. Production of ROS via leakage of O_2•– from mitochondrial respiratory chain
Further ROS (H_2O_2 and •OH) are formed and defense systems such as catalase and SOD can be induced to mitigate the resulting damage and prevent excessive oxidative stress.

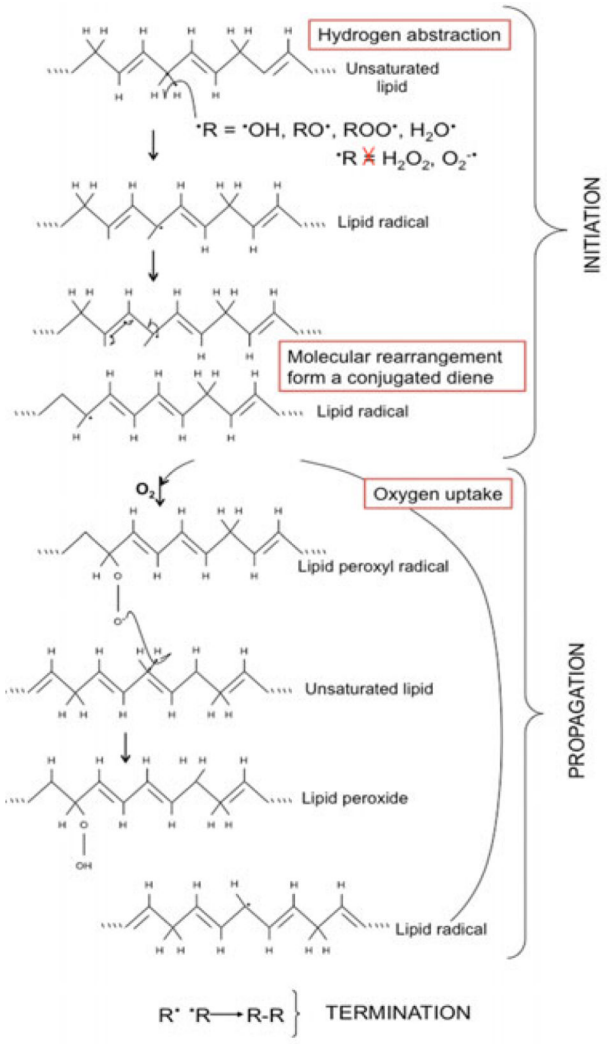

Figure 2. Oxidative degradation of lipids

Lipid peroxidation, starts with an ROS "stealing" an electron from the lipid cell membrane and the process continuous via free-radical chain reaction mechanism. The peroxidation reaction is especially affective in polyunsaturated fatty acids, since they contain multiple double bonds and in between methylene groups (-CH2-). This position of the methylene groups renders the hydrogen atoms especially reactive and susceptible to ROS attack. The degradation process constituting three major steps goes through initiation, propagation, and termination.

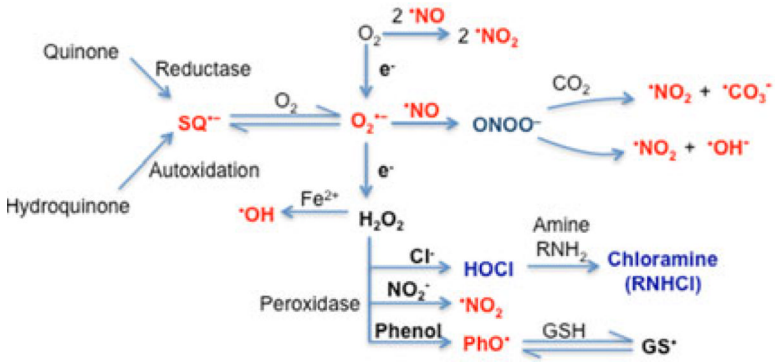

Figure 3. Generation and interconversion of physiologically relevant ROS

It is known that ROS sustain homeostasis but may also trigger cell death by apoptosis and/or necrosis (Pourova, *et al.*, 2010). Basal levels of ROS production in cells is beneficial for several physiological functions, however, excessive ROS production above basal levels can impair and oxidatively damage DNA, lipids and proteins, and consequently result in dysfunction of these molecules within cells and finally cell death (Winterbourn, 2008, Mohsenzadegan & Mirshafiey, 2012). Semiquinone-like radicals ($SQ^{\bullet-}$) are generated by autoxidation of a range of compounds including adrenaline and DOPA, or by enzymatic reduction of quinones such as ubiquinone or menadione. Flavonoids and other polyphenols can generate both semiquinone and phenoxyl radicals. Phenoxyl radicals ($PhO^{\bullet}$) are produced from tyrosine and other phenolic metabolites and xenobiotics. Aromatic amines and indoles are oxidized to radicals with similar properties. Glutathionyl radical ($GS^{\bullet}$) are generated from other thiols such as dihydrolipoic acid or cysteine residues. Only myeloperoxidases are capable of generating hypochlorous acid (HOCl).

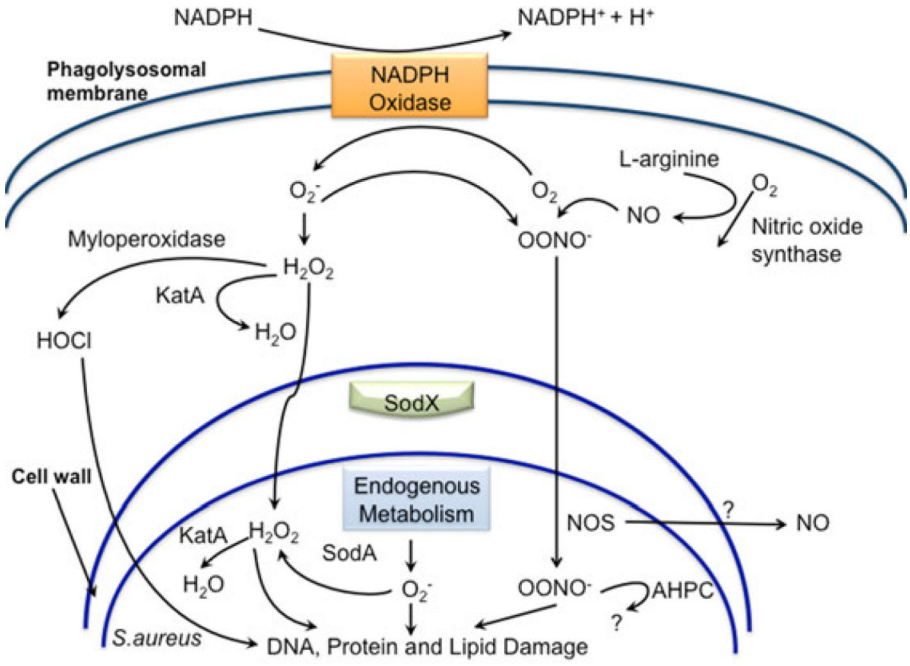

Figure 4. Antimicrobial host defense relies on ROS generation by macrophages and neutrophils

The microbial cells is engulfed into a phagolysome and this triggers NADPH oxidase generates O_2•–, while myeloperoxidase generates HOCl, and iNOS generates NO•. These ROS and RNS combine to kill the microbes.

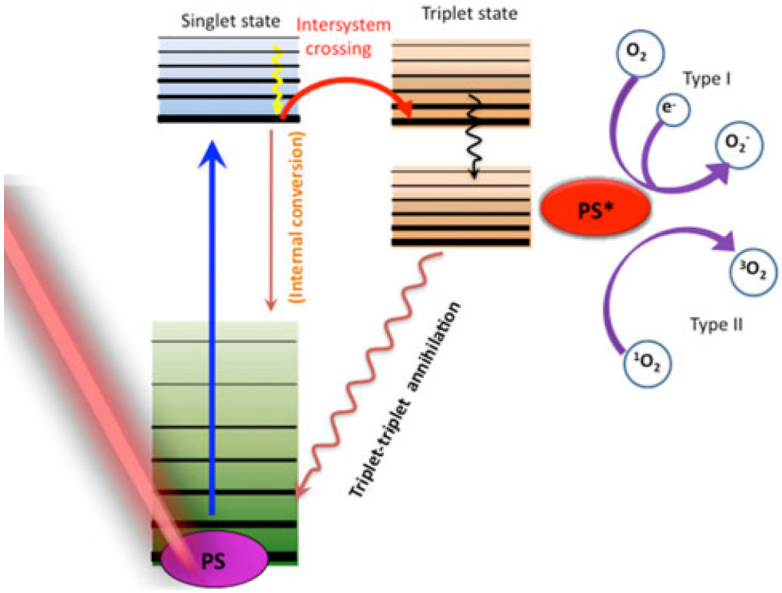

Figure 5. Jablonski diagram illustrating the mechanisms of PDT including Type I and Type II photoreactions

The PS absorbs photons from light and causes excitation to the singlet excited state (^{1} PS*). The singlet excited PS* can decay back to the ground state with release of energy in the form of fluorescence. It is possible for the singlet to be converted into the long-lived triplet excited state (^{3} PS*) which is able to transfer energy to another triplet (ground state oxygen) or alternatively carry out electron transfer to oxygen producing a range of ROS via superoxide.

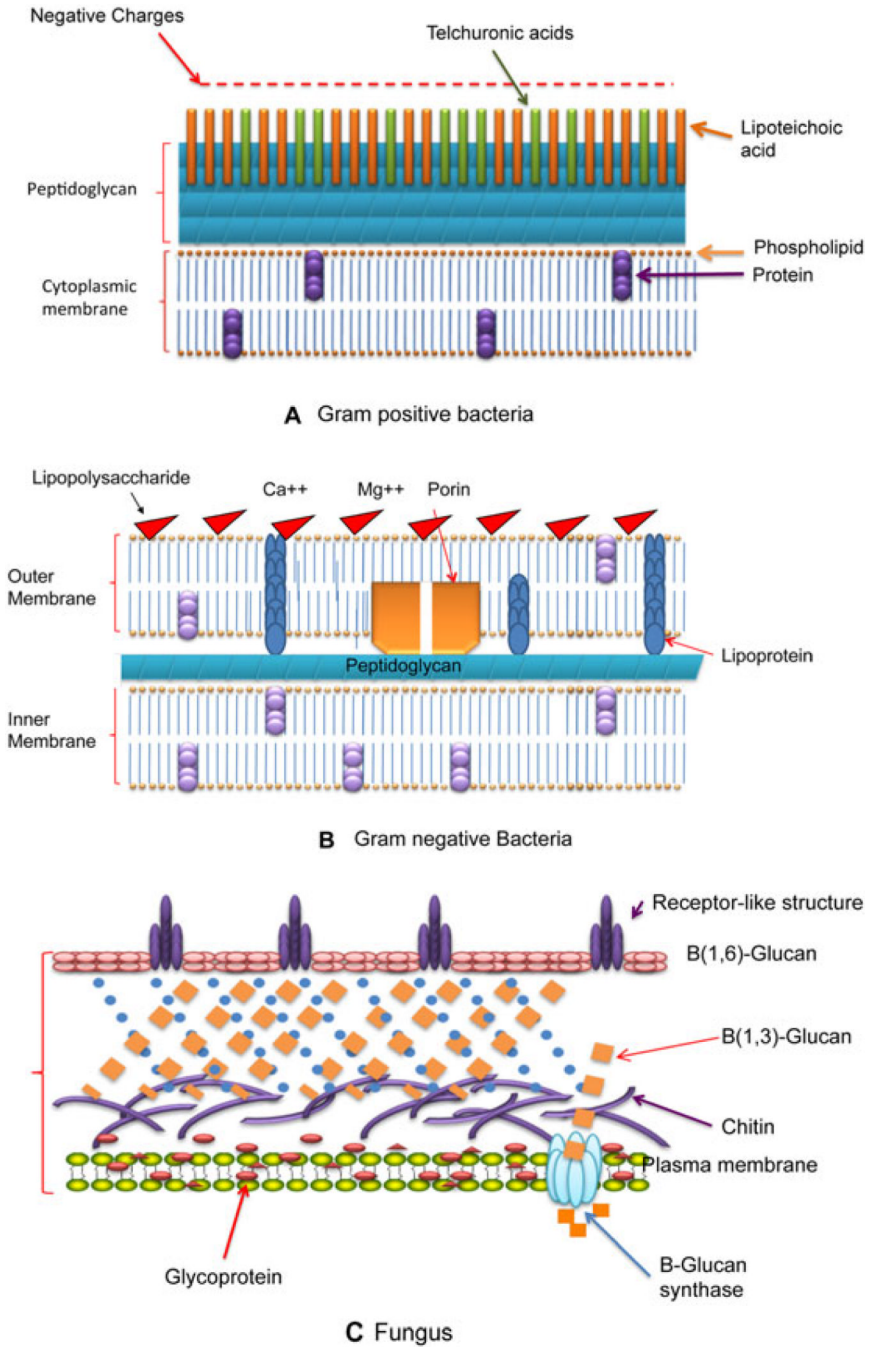

Figure 6. Structures of the cell walls of different classes of microbial cells

Gram-positive bacteria have a relatively porous outer cell wall composed of peptidoglycan, teichuronic acids and lipoteichoic acids. Gram-negative bacteria have a thin layer of peptidoglycan and then a second lipid bilayer incorporating lipopolysaccharide and providing a permability barrier. Yeast have a relatively impermeable cell wall composed of beta-gucan and chitin (Sharma, *et al.*, 2011).

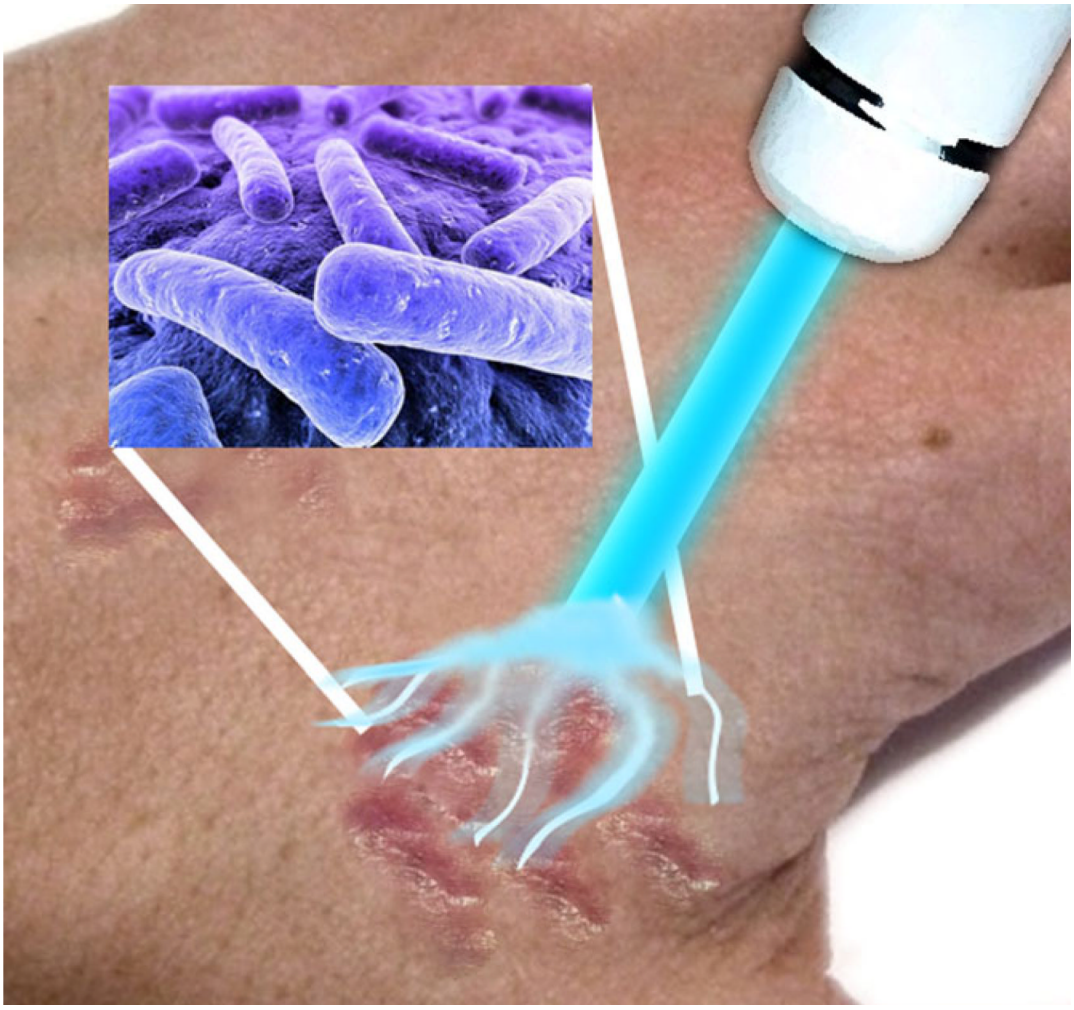

Figure 7. Plasma pencil

The five-centimeter-long plasma plume is generated when a stream of helium gas containing a trace of oxygen passes between two high-voltage copper electrodes. Helium is very difficult to ionize, but the plume's oxygen molecules break into two highly reactive oxygen atoms, which then attack the bacteria. The key to keeping the plasma pencil cool is its kilovolt electric field, which switches on and off thousands of times a second.

Table 1

Standard reduction potentials for biologically relevant molecules and reactive species

Half reaction	Electrode potential[a]
$HO\bullet + \mathbf{e}^- + H^+ \rightarrow H_2O$	+2.31V
$O_3 + 2\mathbf{e}^- + 2H^+ \rightarrow H_2O + O_2$	+2.075V
$O_3\bullet^- + \mathbf{e}^- + 2H^+ \rightarrow H_2O + O_2$	+1.9V
$Co(III) + \mathbf{e}^- \rightarrow Co(II)$	+1.82V
$CO_3\bullet^- + \mathbf{e}^- \rightarrow CO_3^{2-}$	+1.8V
$H_2O_2 + 2\mathbf{e}^- + 2H^+ \rightarrow 2H_2O$	+1.76V
$RO\bullet + \mathbf{e}^- + H^+ \rightarrow ROH$ (alkoxyl)	+1.6V
$O\bullet^- + \mathbf{e}^- + 2H^+ \rightarrow H_2O$	+1.46V
$N_3\bullet + \mathbf{e}^- \rightarrow N_3^-$	+1.3V
$^1\Sigma_g\ O_2 + \mathbf{e}^- \rightarrow O_2\bullet^-$	+1.27V
$N_2O_4 + \mathbf{e}^- \rightarrow NO\bullet + NO_3^-$	+1.2V
$HOCl + H^+ + 2\mathbf{e}^- \rightarrow H_2O + Cl^-$	+1.08V
$Fe(III)(1,10\text{-}phen)_3 + \mathbf{e}^- \rightarrow Fe(II)(1,10\text{-}phen)_3$	+1.06V
$HO_2\bullet + \mathbf{e}^- + H^+ \rightarrow H_2O_2$	+1.06V
$O_3 + \mathbf{e}^- \rightarrow O_3\bullet^-$	+1.03V
$ROO\bullet + \mathbf{e}^- + H^+ \rightarrow ROOH$ (alkylperoxyl)	+1.0V
$NO_2\bullet + \mathbf{e}^- \rightarrow NO_2^-$	+0.99V
O_3 (g) $+ \mathbf{e}^- \rightarrow O_3\bullet^-$	+0.91V
$^1\Delta_gO_2 + \mathbf{e}^- \rightarrow O_2\bullet^-$	+0.81V
$N_2O_3 + \mathbf{e}^- \rightarrow NO\bullet + NO_2^-$	+0.8V
$^1\Delta_gO_2$ (g) $+ \mathbf{e}^- \rightarrow O_2\bullet^-$	+0.64V
$SO_3\bullet^- \mathbf{e}^- \rightarrow SO_3^{2-}$	+0.63V
$H_2O_2 + \mathbf{e}^- + H^+ \rightarrow H_2O + HO\bullet$	+0.32V
$O_2\bullet^- + \mathbf{e}^- + 2H^+ \rightarrow H_2O_2$	+0.36V
$ONOO\bullet + \mathbf{e}^- \rightarrow ONOO^-$	+0.2V
$Cu(II) + \mathbf{e}^- \rightarrow Cu(I)$	+0.16V
$2H^+ + 2\mathbf{e}^- \rightarrow H_2$	0.00V (defined)
$O_2 + \mathbf{e}^- \rightarrow O_2\bullet^-$	−0.18V
O_2 (g) $+ \mathbf{e}^- \rightarrow O_2\bullet^-$	−0.33V
$NAD^+ + \mathbf{e}^- + H^+ \rightarrow NADH^+$	−1.58V
$H_2O + \mathbf{e}^- \rightarrow \mathbf{e}^-_{aq}$	−2.87V

[a] In aqueous solution (pH=7), unless otherwise stated (g), at 25°C and 1M concentration.

Table 2

Differences in reaction products between Type I reactions (HO•) and Type II reactions ($^1O_2$).

Target	Type 1 reaction	Type 2 reaction
Amino acids	Tyr (phenol coupling), Phe (hydroxylation), Trp	His (oxygen addition), Trp (oxygen addition) Cys (disulfide), Met (sulfoxide),
Proteins	Cross-linking, carbonylation	Cross linking, Proteolysis
Lipids	Radical chain reactions	Lipid hydroperoxides
Nucleic acids (2'-deoxyguanosine)	5-carboxamido-5-formamido-2-iminohydantoin	8-oxo-7,8-dihydroguanosine
Cholesterol	5- & 6-hydroperoxides	7-hydroperoxides

Table 3

Biological oxidation products of amino acids.

Amino Acid	Physiological oxidation products
Cysteine	Disulfides, mixed disulfides (*e.g.*, glutathiolation), HNE-Cys
Methionine	Methionine sulfoxide
Tyrosine	Dityrosine, nitrotyrosine, chlorotyrosines, dopa
Tryptophan	Hydroxy- and nitro-tryptophans, kynurenines
Phenylalanine	Hydroxyphenylalanines
Valine, Leucine	Hydro(pero)xides
Histidine	2-Oxohistidine, asparagine, aspartate, HNE-His
Glutamyl	Oxalic acid, pyruvic acid
Proline	Hydroxyproline, pyrrolidone, glutamic semialdehyde
Threonine	2-Amino-3-ketobutyric acid
Arginine	Glutamic semialdehyde, chloramines
Lysine	a-Aminoadipic semialdehyde, chloramines, MDA-Lys, HNE-Lys, acrolein-Lys, carboxymethyllysine, pHA-Lys

HNE, 5-hydroxynonenal; MDA, malondialdehyde; pHA, p-hydroxyphenylacetaldehyde