Detecting Reactive Species in Bacteria

Bachelor thesis

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Content

Abstract

Free radicals are short-lived intermediates with at least one unpaired electron which makes them highly reactive. Under normal conditions the cell keeps the radicals at a steady-state level using several defence mechanisms. However, when there is an excessive amount of reactive species, the cell experiences oxidative stress which can cause damage to several cell compounds, such as lipids, protein and DNA and sometimes even result in cell death. Because there is still a lot unknown about free radicals and their roles in cell function and dysfunction they are an interesting subject for research. To study these radicals, detection is necessary, but this is challenging due to their short lifetimes, high reactivity and the low concentrations in the cell. This thesis is intended to give an overview of widely used methods for radical detection in bacteria. The gold standard for radical detection is by Electron Paramagnetic Resonance, however in bacteria the radical concentration often does not reach the detection limit. More commonly used methods for radical detection in bacteria are by using chemiluminescent and fluorescent probes that can be detected upon reaction with radicals. These probes are not very specific and might react with cellular compounds which can influence the measurement. Indirect methods for radical detection is fingerprinting, a method to assess cellular damage. There is not yet a method for specific and direct detection for radicals in bacteria and to get a better understanding of cell function and the role of free radicals, new methods have to be developed.

Introduction

In aerobic bacteria molecular oxygen (O_2) is used for respiration or oxidation of nutrients to obtain energy. During these processes, free radicals are formed in small amounts as a by-product [1]. Most of these species are products from sequential univalent reductions of molecular oxygen catalyzed by respiratory enzymes [2]. In Escherichia coli was seen that single electrons from the respiratory chain leaked at the NADH dehydrogenase site [2].

Free radicals are unstable and highly reactive molecules that contain at least one unpaired electron [3]. To become stable they try to gain an electron by oxidizing molecules they encounter. As a result, the molecule that loses an electron becomes a free radical which causes a chain reaction. Because of their high reactivity these chain reactions can cause damage to biological systems in a short amount of time [4].

There are two main types of reactive species: reactive oxygen species (ROS) derived from oxygen molecules and reactive nitrogen species (RNS), derived from nitrogen molecules. There are also molecules that are radicals but are not ROS or RNS. Commonly detected reactive species in biological systems are Hydrogen peroxide (H_2O_2), Superoxide anions (O_2-), Hydroxyl radicals (OH), peroxynitrite (ONOO-) and nitric oxide (NO-) [5] (see *table 1*). Many of them are either radicals themselves or lead to radical formation.

Table 1. Reactive oxygen species and reactive nitrogen species

ROS	RNS
Superoxide anion (O_2^-)	Nitric Oxide (NO^-)
Hydrogen peroxide (H_2O_2)	Nitric dioxide (NO_2^-)
Hydroxyl radical (OH)	Peroxynitrite ($ONOO^-$)

At physiological levels, ROS play an important role in maintaining cell function but too high amounts can damage several cell compounds such as proteins, lipids and even DNA [6]. To protect themselves from cell damage, bacteria use several scavenging enzymes and repair systems such as catalase and superoxide dismutase (SOD) [7]. These defense mechanisms are regulated by *oxy*R and *sox*R regulons [8]. When the balance between generation and elimination of reactive species is lost, leading to an enhanced level of reactive species, the cell experiences oxidative stress which can result in cell death [9] . Therefore it is essential that ROS are maintained at homeostatic levels.
Because free radicals play major roles in physiological processes and are therefore an interesting subject for research, detection and quantification of free radicals in biological systems is necessary for understanding these reactive species and the pathophysiological processes they are involved in. To achieve this, both direct and indirect methods have been developed, but due to their short lifetime and high reactivity, measurement of reactive species is challenging [10]. This review is intended to provide an overview of commonly used methods for detection of reactive species in bacteria.

Detection of reactive species

There are direct and indirect methods for detection of free radicals. Electron spin resonance (ESR), fluorescence and chemiluminescence are commonly used spectroscopy techniques used to determine the level of reactive species in biological systems [11]. Indirect approaches to see if the cell experiences oxidative stress are by studying the cells response to reactive species. Products of damage to biomolecules (e.g. lipids, proteins and DNA) can be determined by several assays.

Electron Paramagnetic Resonance

Electron Paramagnetic Resonance (EPR) (also known as Electron Spin Resonance, ESR or electron magnetic resonance, EMR) is the ''gold standard'' for detection of radicals in biological systems [13]. It is a spectroscopic technique that can be used to detect unpaired electrons and therefore free radicals.
An unpaired electron has a spin and behaves as a small magnet. When the electrons are exposed to an external magnetic field, they align parallel or anti-parallel to that field, so two states of energies are created. The difference between those states is measured by absorption in the microwave region of the electromagnetic spectrum [14]. Resonance occurs when the magnetic field has been adjusted so that the following equation is obeyed [10, 14] :

$$\Delta E = h\gamma = g\beta H$$

Where ΔE is the gap between the two energy levels of the electron, h is Planck's constant, γ is the applied electromagnetic radiation frequency, g the splitting factor for the electron, β the Bohr magnetron constant and H the applied magnetic field strength [15].

Because free radicals are only directly detectable by EPR when they have long half-lives or when they are amass in high concentrations, this method is insufficiently sensitive to detect free radicals in cells (e.g. O_2- , OH and NO-) [12]. The sensitivity of EPR is $3x10^{-9}$ mol/L (nM), whereas the concentration of free radicals under normal conditions is below 1 nM or 1 pM (10^{-12} mol/L) [21]. To make EPR measurements of short-lived reactive oxygen derived species possible, several techniques have been developed.
There are multiple approaches to increase the lifetimes of the free radicals for EPR detection [16]. One of them is to prevent the radicals from undergoing reaction by rapid freezing [17]. The radicals are rapidly frozen by the use of conventional freeze-quenching systems [16]. Another approach to circumvent the short radical lifetimes is a continuous or stopped-flow system. However, this is limited to systems that can withstand high pressures [16]. For detection of radicals in biological systems; a more common method is spin trapping [14].

Spin trapping

Spin trapping is a technique in which free radical (R•) reacts with a diamagnetic molecule, the spin-trap (S=T), to form a more stable and long-lived free radical, a radical adduct (R-S-T•).

R• + S=T → R-S-T•

By extending the lifetime of the free radical, the concentration increases which results in a higher signal-to-noise ratio of the EPR spectrum [18]. Frequently used spin traps are nitrone and nitroso compounds (see *figure 2*).

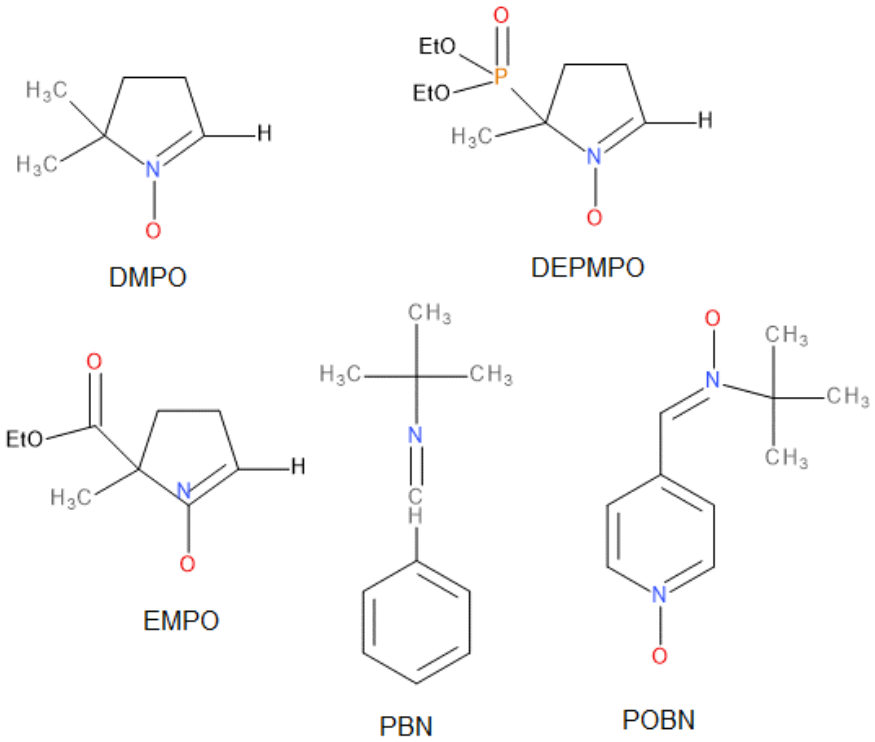

Figure 2. Chemical structures of commonly used spin traps.
5,5-dimethyl-1-pyrroline N*-oxide (DMPO); 5-diethoxyphosphoryl-5-methyl-1-pyrroline* N*-oxide (DEPMPO); 5-(ethoxycarbonyl)-5-methyl-1-pyrroline* N*-oxide (EMPO);* N*-tert-butyl-α-phenylnitrone (PBN); α-(4-pyridyl-1-oxide)*-N*-tert-butylnitrone (POBN)*

There are several aspects that have to be taken into account when a trap is chosen to form a more stable compound. Ideal is a trap that reacts fast and specifically with one radical and has a slow radical adduct decay rate. Also it should not be metabolized in the biological system. Another factor that has to be considered is the polarity of the trap because that can influence the localization of the trap in the cell [14]. Additionally, the purity of the spin trap is an important factor because they can influence the EPR spectrum [13]. To ensure reaction of the radicals with the spin trap, often high concentrations of the trap are used. Usually spin traps of commercial suppliers are more than 99% pure (mM levels) [13]. However, using high concentrations for EPR spin trapping also increases the number of impurities, which can result in artefact signals [19, 20].

The EPR technique is a widely used method for detection and identification of radicals in biological systems. Combined with spin trapping it is a good method for ROS detection, however, antioxidants can compete with the spin trap and decrease the radical adduct concentration which complicates detection using EPR [21]. When it is correctly used it can provide detailed information about the radicals (e.g. identity, structure, concentration, motion and environment)[13]. A problem remains with regard to determination of the radical formation. It often is not clear if radical formation is a major process responsible for the observed damage or if it is minor side reaction [13]. To provide a quantitive assessment of the processes in the cell, other methods have to be used, such as analysis of oxidative products (e.g. products from lipid peroxidation and oxidized proteins) and caused damage (e.g. changes in membrane properties, DNA strand breaks, protein aggregation) [21].

To analyze protein and DNA radicals by EPR or EPR-spin trapping in chemical systems it usually involves exposure of isolated DNA. The time it takes to isolate the DNA from the biological matrix is longer than the time it takes for the parent radicals or radical adducts to decay. Therefore, the EPR technique is not suited for analyzing protein or DNA radicals. A method that can be used for this purpose is immuno-spin trapping.

Immuno spin-trapping

The nitrone spin trap DMPO is also widely used for immuno-spin trapping. This is a technique based on the reaction of a free radical with a spin trap to form a stable nitrone adduct, but instead of using EPR to detect the formed nitrone adduct, analysis is done using antibodies [21] (see *figure 3*). Immuno spin-trapping can be applied to study protein and DNA-centered radicals in any biological system, including bacteria [22].

As mentioned before, oxidants can inflict damage to proteins and DNA when they outnumber antioxidants, which results in formation of protein and DNA radicals. They are formed as a result of one-electron-mediated oxidation of specific residues in a protein [19]. With DMPO, immuno spin-trapping can be used to study the oxidatively inflicted damage to the proteins/DNA in cells [22]. For analysis of the formed nitrone adducts, immunochemistry (e.g. ELISA, Western blot), mass spectrometry (MS), molecular magnetic resonance imaging (mMRI) [18] and NMR [23] can be used.

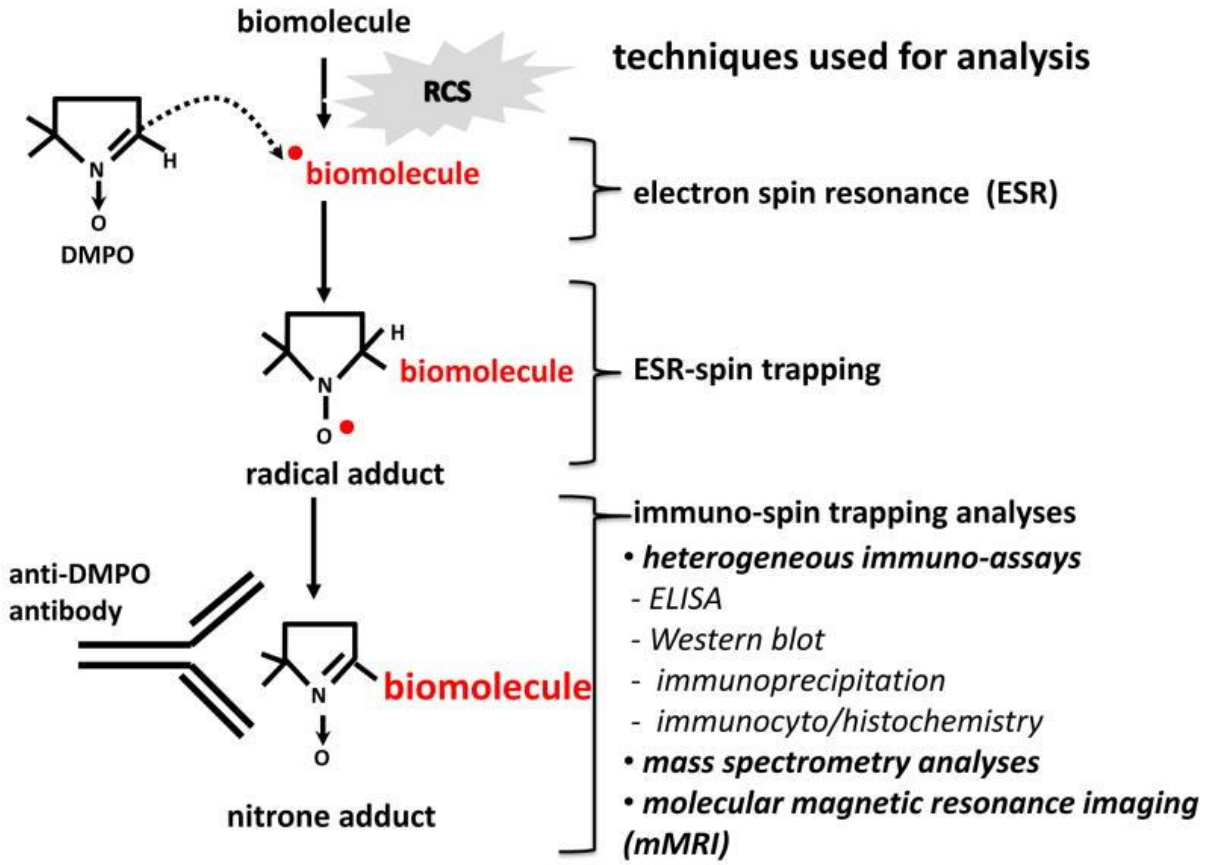

Figure 3. Study of biomolecule-centered radicals via ESR, ESR-spin trapping and immuno-spin trapping analysis. (Adapted from Gomez-Mejiba et al, 2010 [23])

Although EPR is the gold standard and immune-spin trapping can be used for radical detection in biological systems, radicals in cells are often in too low concentrations to be detected. Therefore other methods have been developed for radical detection in cells. In bacteria often chemiluminescent and/or fluorescent probes are used.

Chemiluminescence and Fluorescence

Chemiluminescence (CL) is the emittance of light as a result of a chemical reaction. The reaction can induce transition of an electron from its ground state to an excited electronic state and the molecule experiences rotational and vibrational changes [25]. When the formed molecule decays from the excited state to their ground state, a chemiluminescence is emitted.

To detect ROS release in bacteria with chemiluminescence imaging, a chemiluminescence probe is incubated with the cell and reacts with the reactive species. As a result from the chemical reaction a light is emitted that can be measured by a liquid scintillation counter, a luminosometer [12] or a confocal/platereader.

Another approach is the use of fluorescence probes. Fluorescence differs from chemiluminescence in that the electronically excited state is created by absorption of a photon (see *figure 4*) and not derived from the product of a chemical reaction [12].

Chemical probes such as Lucigenin, bis-*N*-methylacridinium dinitrate (LC^{2+}) and luminol are

commonly used**.** But also other probes (e.g. hydroxylphenyl fluorescein, HPF; dichlorodihydrofluorescein diacetate; DCFH-DA; 2-methyl-6-phenyl- 3,7-dihydroimidazo [1,2-α] pyrazin-3-one, CLA) can indicate presence of reactive species in cells.

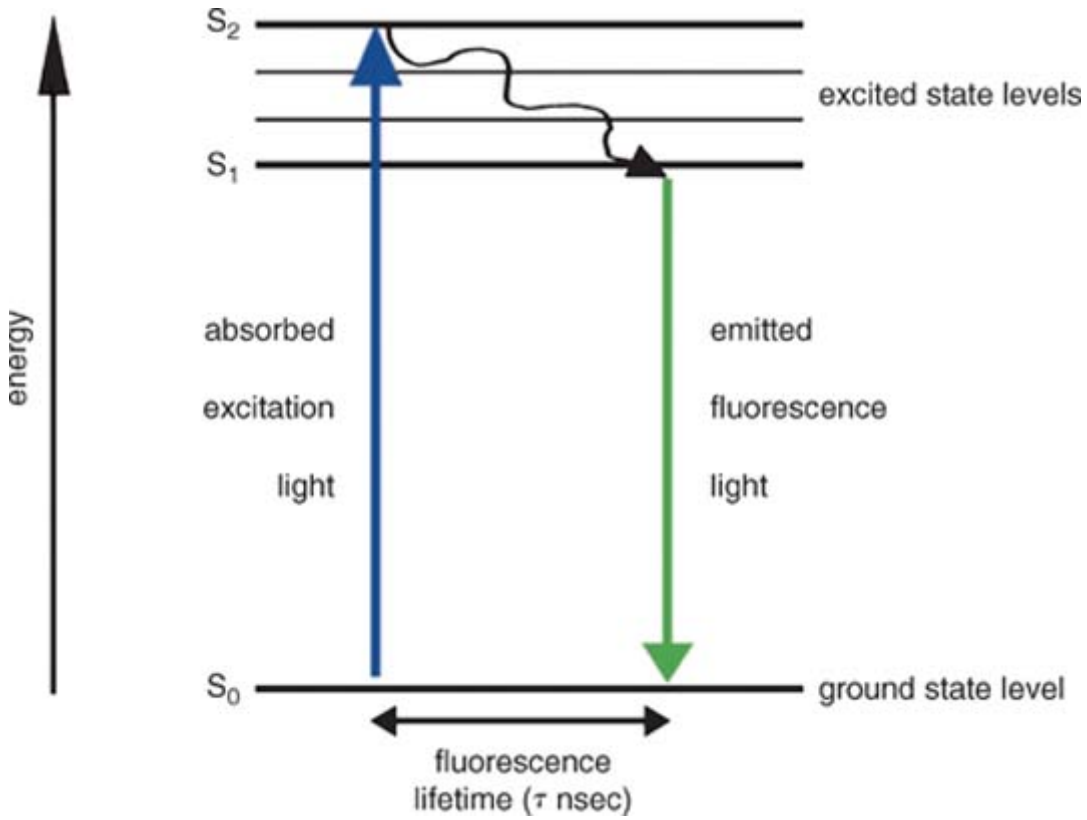

Figure 4 [50]. Jablonski energy diagram of fluorescence. A photon of excitation is absorbed by a molecule which results in an (singlet first electronic S1 or a second electronic S2) excited state. At each energy level, there are multiple vibrational levels. When the energy level gets back to its ground state level S_0, a fluorescence is emitted. (Adapted from Lleres et al, 2007 [50]).

Detection of superoxide

Several approaches for O_2- detection have been developed. A scavenger is used to react with superoxide so a detectable product is formed. The most commonly used scavengers are lucigenin and luminol. Before luminol reacts with superoxide it must be oxidized by other radicals or peroxidases to produce a luminol radical [12]. The resulting luminol radical then reacts with O_2- to generate a light-emitting product. However, luminol can also emit luminescence when it reacts with other ROSs (e.g. H_2O_2, HO and ONOO-) [25]. Furthermore, luminol can reduce oxygen to generate another generation of superoxide so luminol can be the source as well as the detector of O_2- [26].

A more specific probe for O_2- detection is Lucigenin, but before it can decompose to a light-emitting species it needs to be reduced to lucigenin cation radical ($LC^{\bullet+}$). For this conversion other cellular reducing systems, such as xanthine oxidase (an enzyme that can generate reactive species), are required [26]. Just as luminol, $LC^{\bullet+}$ can reduce O_2 to O_2- [25].

Detection of superoxide in bacterial membranes has been successful with the probe coelenterazine [27]. This probe can directly react with O_2- so prior reduction is not necessary. It is a sensitive method for O_2- detection but has limitations because coelenterazine is likely to react with membrane proteins such as detergents and sonicated phospholipids [27].

Another way to measure O_2- is by its ability to reduce cytochrome c or nitroblue tetrazolium (NBT)[26]. However, several cellular substances can reduce cytochrome c, which makes O_2- determination difficult. To make sure production of O_2- is detected, a control assay can be done in the presence of superoxide dismutase (SOD), a scavenger of O_2-. In most cases a quenched signal by SOD indicates production of superoxide. NBT is less often reduced by cellular substances but can generate artefactual O_2- [26].
Although the mentioned probes are widely used for O_2- detection in biological systems they are not specific enough and research for more specific compounds are being studied [25]. Because they all can generate superoxide when they react or are reduced by cellular reductants they are no specific indicators for O_2- [26].

Detection of Singlet Oxygen

Multiple chemiluminescence probes were developed to detect singlet oxygen ($^1O_2$), but commonly used probes are 2-methyl-6-phenyl- 3,7-dihydroimidazo [1,2-α] pyrazin-3-one (CLA), and its derivatives MCLA and FCLA. Because they do not only react with singlet oxygen but also with superoxide anions, they lack selectivity for singlet oxygen [28].

Detection of Hydrogen Peroxide

To be able to visualize H_2O_2 there are several chemical substrates which can serve as a hydrogen donor and when in presence of horseradish peroxidase (HRP) can produce fluorescent products [27]. Luminol is an example of a widely used substrate and can produce a blue light when it reacts with H_2O_2 in the presence of HRP [13]. This is a very sensitive technique for measuring H_2O_2 levels in the cell, but because HRP can react with several cellular substrates it is not very specific for H_2O_2.
Other frequently used probes are homovanillic acid or Amplex red and dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) [29]. When $DCFH_2$-DA enters the cell it will be hydrolyzed to DCFH and after two-electron oxidation the fluorescent product dichlorofluorescein (DCF) is formed. Amplex red can be oxidized when it reacts with hydrogen peroxide and HRP to form the fluorescent product resorufin [30].
HyPer is a specific H_2O_2 detector based on OxyR [29]. The fluorescent probe attaches to the radical sensing oxyR regulon resulting in a fluorescent product. The excitation and emission spectra changes when H_2O_2 is present so HyPer can be a specific detector for H_2O_2. However, the fluorescence can be affected by pH and oxidized Hyper can be reduced by cellular substances [29].

Detection of hydroxyl radicals

Hydroxyl radicals are the most reactive oxygen radicals and have a half-life of approximately 10^{-9} s [25]. For detection of hydroxyl radicals in bacteria a widely used probe is hydroxyphenyl fluorescein (HPF) [31]. Upon reaction with hydroxyl radicals a fluorescent molecule is formed.

However, several studies have indicated that HPF can also be oxidized by redox-active metals present in the cell and therefore may complicate results when used for hydroxyl radicals detection and also, HPF is pH dependent [32].
Another fluorescent probe commonly used is a conjugated nitroxide fluorescent probe specific for hydroxyl groups in dimethyl sulfoxide (DMSOO) [12]. Upon reaction the probe is converted via methyl radicals to a fluorescent product.

Detection of peroxynitrite

HPF is not a specific detector of hydroxyl radicals but also of peroxynitrite[33] . Another probe that can be used for this purpose is Dihydrorhodamine (DHR)[34]. As a result of oxidation of DHR the fluorescent product rhodamine is formed.

An ideal probe for measurements in live cells should meet several conditions [35]. The probe should be used to show evidence for a disturbance in cellular redox state. Also it should be able to detect a specific reactive species and show when altered formation or destruction of the species takes place. Above that it should identify the location of the reactive species and how many is produced. Therefore, the probe should be able to target different sites in the cell. To prevent the radical to diffuse away, the probe should be highly reactive so the radical gets rapidly trapped [34].
The output of these probes can be measured using fluorescence plates. A disadvantage when using this method is that the total fluorescence is measured and there is no distinction between fluorescence from intracellular and extracellular chemical reactions [26]. Another method that can be used is flow cytometry. This can be used to measure intracellular fluorescence in culture media [26]. When cells are loaded with fluorescent dyes, they can be studied with the confocal microscope.
While chemical probes are widely used to detect reactive species in biological systems, they lack specificity [24]. Some probes can even be oxidized by other cellular substrates which can give a false indication for reactive species present. Furthermore, often multiple reactions are needed before detection via chemiluminescence or fluorescence is possible.
This probe based method can be used as an indicator for redox changes in the cell but to identify what specific radical is responsible it has to be combined with other assays.

Electrochemical biosensor

A recent study demonstrated the use of an electrochemical cytochrome c biosensor to measure the continuous release of superoxide radicals in bacterial cells and to estimate the rate of O_2- production [44]. The sensor consists of a gold wire electrode with immobilized cytochrome c. When the cytochrome c reacts with the released O_2^- at the surface of the electrode it generates a redox current that can be measured [45]. Advantages of this method are direct measurement of O_2^- in bacteria cultures in the range of nM [44]. Also the measurements are quantitive when the sensor is appropriately calibrated [44]. However, sensitivity of the sensor is dependent on the culture media. The study showed that the sensor was more sensitive in phosphate buffer solutions than in culture media. Also the sensitivity is dependent on the culture media. The study reported successful use in Lysogeny (LB) and Brain Heart Infusion (BHI) broth.

Fingerprinting (or Footprinting)

To provide a quantitative assessment, indirect measurements of reactive species can be done by measuring products of oxidative damage to biomolecules. This is also known as finger- or footprinting of reactive species [13]. Because radicals can damage several cell compounds, detection of cell damage can be an indicator for present reactive species even if they are not directly measured. Therefore measuring damage done to molecules in a cell (e.g. lipids, proteins and DNA) can be an indirect approach for detection of reactive species.

Quantification of lipid peroxidation

The primary target of ROS in bacteria are believed to be in the cytoplasm [7]. However, when a bacterial cell experiences oxidative stress, the membrane can be damaged by lipid peroxidation. Lipid peroxidation is the oxidative deterioration of any polyunsaturated compound that contains multiple carbon-carbon bonds (e.g. unsaturated fatty acids, phospholipids, glycolipids, cholesterol and polyunsaturated hydrocarbon) [7].

There are several approaches to detect ROS enhanced lipid peroxidation in cells, but widely used is the thiobarbituric acid reactive species (TBARS) assay [37, 38, 41]. This is an assessment for the malondialdehyde (MDA) concentration in physiological systems [36]. MDA is formed during lipid peroxidation reactions and upon reaction with thiobarbituric acid (TBA) a pink-colored dimeric $(TBA)_2$–MDA adduct is formed [37]. This compound can be measured colorimetrically at 532 nm or by fluorescence using a 530 nm excitation wavelength and a 550 nm emission wavelength [27].

Another approach is by the use of liposome-trapped carboxyfluorescein (CF). This can be used as a fluorescent probe as an indicator of membrane disruption and lipid oxidation/degradation in eukaryotic and prokaryotic cells [39]. When the concentration of CF decreases, it is strongly fluorescent. Assessment of membrane damage can be done by measuring the fluorescence intensity of CF released by damaged liposomes [40]. When using a self-quenching CF solution, the membrane damage is proportional to the released CF levels.

Quantification of protein damage

Spectrophotometric measurements of oxidative stress can be done using the ABTS and DPPH test to assess the total protein content. 2, 2-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are free radicals that can be used to asses antioxidant activity in bacteria [41].

The DPPH test is a method based on the ability of DPPH to react with hydrogen donors. DPPH has an absorbance of 515 nm but when it reacts with a free radical or antioxidant it loses this absorption [42]. The ABTS method works the same way. ABTS has an absorbance of 734 nm but its reduction can be measured by the decrease in absorbance during the reaction with antioxidants or radicals[42].

Another method to asses protein damage in bacteria is by the use of electrophoretic analysis by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [38]. With this method proteins can be separated according to their size and therefor be used to determine their relative molecular mass[43].

To measure antioxidant capacity, the Ferric Reducing Antioxidant Power (FRAP) assay can be used. This method is based on the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) to the ferrous tripyridyltriazine (Fe (II)-TPTZ) by electron donating oxidants which occurs at low pH. Fe (II)-TPTZ has a blue color and an absorption of 593 nm [38, 49].

Effect of oxidative stress on DNA

Real-time reverse transcription (qRT) followed by polymerase chain reaction (RT-PCR) can be used to analyze gene expression. As mentioned earlier, bacteria use *oxy*R and *sox*R genes to regulate the response against oxidative stress. The transcriptional profile of for example these oxidative-stress responsive genes can be analyzed using this method.
In bacteria qRT-PCR is commonly used to measure oxidative stress by using a fluorescent dye SYBR Green that binds to the minor groove of the DNA double helix [38, 46]. Total RNA has to be extracted from the cell and isolated prior to performing reverse transcriptase to synthesize cDNA.

For quantification of DNA fragmentation a Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay can be used [38, 47]. When radicals damage DNA, a 3'-OH DNA end is formed which is used as a substrate to synthesize new DNA. The TUNEL assay labels these 3'-OH DNA ends with with dUTP-fluorescein isothiocyanate using TdT and can therefore be an indicator for DNA damage [48]. After labeling, cells can be detected using fluorescence microscopy of flow cytometry.

Discussion

The scientific research in the field of reactive species and associated processes in biological systems is constantly requiring new sensitive and specific methods to get a better understanding of the mechanistic roles of these species in life science. To obtain a definitive picture of oxidative stress in biological cells and the radicals that partake in associated processes, several approaches have been developed and are often being combined. However, the short lifetime of free radicals and the variety of defense mechanisms against free radicals make their detection challenging. To get a good understanding of the reactive species and how they contribute to cell behavior, several aspects have to be studied. It is essential to know what species and when they are being produced in what quantity.
The EPR method is the ''gold standard'' for detection of reactive species in biological systems and can be used to detect radicals present in both physiological and pathological situations. There still are problems concerning the sensitivity of the EPR technique for detecting radicals in cells. This technique is mostly used for detection of radicals in biological systems experiencing stress, because then excessive amounts of radicals are generated, but even then detection is challenging. Radicals formed in physiological processes are generated in too small amounts and therefore do not reach the detection limit. To make detection of free radicals in low concentrations possible, spin trapping is used to create longer lasting and more stable radicals.
A disadvantage of using EPR is that it is difficult to determine whether radicals are major or minor intermediates in biological pathways. A problem remains with regard to detection of

artefacts as a result from reagents and side-reactions. Another aspect that can result in artefact signals are when impurities of the spin trap are detected. Above that, insufficient spatial resolution could be a drawback of EPR.
To develop better EPR techniques several factors have to be taken into account. For quantification of data obtained by spin trapping, more detailed information is needed about rate constants for spin trapping and the spin adduct decay processes. Ideal would be a spin trap that gives a persistent adduct species in a short amount of time but has a slow radical adduct decay rate. Additionally, the spin trap has to be inert and should be situated at a particular site in the cell.
Another commonly used method for detection of reactive species in cells is by the use of chemical probes. A disadvantage of this approach is that it often takes multiple reactions before detection is possible via chemiluminescence or fluorescence. Another problem is that some probes can be oxidized by several cellular substrates and even artefactual generations of free radicals can be formed. Another drawback of this method is that the probes have limited specificity for different reactive species, which makes it difficult to study the role of a reactive species individually.
To measure reactive species in cells, the probe should meet several conditions. It should be able to show when there is a disturbance in the cellular redox state. Above that it should be specific for one reactive species and show when that species generates or destructs. Also it should be able to identify the location of the reactive species and how many are formed.
In bacteria oxidative stress is often measured by assessing cell damage inflicted by the radicals. This fingerprinting technique is an indirect method for radical detection because it does not detect the radicals but the damage they have inflicted to the cells. It is a method to measure oxidative stress in the cell, but is not suited for direct detection of radicals themselves.
There is not a method for radical detection in bacteria that can be used for direct and specific detection of free radicals so to improve knowledge about these species and the processes they are involved in, current methods have to be improved or new methods should be developed.

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