Real-time investigation of antibiotics-induced oxidative stress and superoxide release in bacteria using an electrochemical biosensor

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Abstract

The involvement of oxidative stress in the mechanism of antibiotics-mediated cell death is unclear and subject to debate. The kinetic profile and a quantitative relationship between the release of reactive oxygen species (ROS), bacteria and antibiotic type remain elusive. Here we report direct measurements and analytical quantification of the release of superoxide radicals (O₂⁻), a major contributor to ROS, in antibiotics-treated bacterial cultures using a cytochrome c electrochemical biosensor. The specificity of electrochemical measurements was established by the addition of superoxide dismutase (SOD) which decreased the O₂⁻ signal. Measurements using a general ROS-specific fluorescence dye and colony forming units (CFU) assays were performed side-by-side to determine the total ROS and establish the relationship between ROS and the degree of lethality. Exposure of Escherichia coli and Listeria monocytogenes cultures to antibiotics increased the release of O₂⁻ radicals in a dose-dependent manner, suggesting that the transmembrane generation of ROS may occur as part of the antibiotic action. The study provides a quantitative methodology and fundamental knowledge to further explore the role of oxidative
stress in antibiotics-mediated bacterial death and to assess physiological changes associated with the complex metabolic events related to oxidative stress and bacterial resistance.

**Keywords:** antibiotic resistance, bacteria, superoxide release, oxidative stress, reactive oxygen species, superoxide radicals, electrochemical cytochrome c biosensor

**Introduction**

The continuous emergence of antibiotic resistant bacteria is of great scientific and practical interest [1]. Exploring the mechanisms of antibiotics action against bacteria is essential to improve the understanding of how antibiotics affect bacterial metabolism and address the issue of multidrug resistance. Several potential mechanisms of action have been proposed including inhibition of cell wall assembly, suppression of protein synthesis and disruption of DNA replication and repair. More recent studies have suggested the involvement of oxidative stress, and the release of reactive oxygen species (ROS) in the activity of antibiotics in bacteria, regardless of their molecular targets [2-4]. However, whether oxidative stress is involved in the mechanism of bactericidal antibiotics is still unclear and subject to debate [5].

The ROS hypothesis indicates that the action of bactericidal antibiotics involves the bacterial tricarboxylic acid cycle, NADH depletion, destabilization of iron-sulfur clusters, and stimulation of the Fenton reaction with formation of hydroxyl radicals in both gram-negative and gram-positive bacteria [6]. Recent work using a panel of biochemical and biophysical assays has further supported this hypothesis and provided additional evidence that antibiotics induce significant redox alterations that contribute to cellular damage and death [2]. Antibiotics were found to induce dynamic changes in cellular respiration and lethal levels of intracellular H$_2$O$_2$ that could be diminished by addition of antioxidants, such as glutathione and ascorbic acid. Other works also identified the presence of ROS in antibiotic-treated bacteria for several quinolone antibiotics, by using the nitroblue tetrazolium reduction (NBT) assay [7]. Excessive release of ROS species, including H$_2$O$_2$, hydroxyl (HO•) and superoxide (O$_2$•−) radicals is harmful to cells and may cause severe damage, including necrosis and apoptosis [8]. Excessive ROS has been considered a sign of oxidative stress. The release of hydroxyl radicals in bacteria was evidenced with a hydroxyl radical specific dye, hydroxyphenyl fluorescein (HPF) [6, 9]. The survival percentage of antibiotic-treated bacteria increased after adding thiourea, a hydroxyl radical...
scavenger, providing further evidence that the observed effect is due to oxidative stress [6, 9, 10]. Other studies reported a relation between antibiotic susceptibility and ROS release, detected by chemiluminescence with lucigenin for $O_2^{-}$ and luminol for other ROS species [11, 12]. An increase in ROS species, including $O_2^{-}$ was observed in chloramphenicol treated *Staphylococcus aureus* and *Escherichia coli* and in ciprofloxacin treated *S. aureus, E. coli* and *Enterococcus faecalis* [12]. Antibiotic-resistant strains showed reduced levels of $O_2^{-}$ production in ciprofloxacin treated *S. aureus* [11]. The involvement of $O_2^{-}$ and $H_2O_2$ was also demonstrated in ciprofloxacin treated *E. coli* [13]. These studies provide cumulative evidence that ROS contribute to antibiotic lethality [6, 13].

While a number of studies have indicated the possible connection between cell death from antibiotics action and ROS production, several recent reports have questioned this connection [14-16]. These studies showed that ampicillin, norfloxacin and kanamycin do not increase $H_2O_2$ formation in *E. coli* [14], and reported that kill does not depend on the presence of oxygen, and is not related to ROS production [14-16]. However, new work by Collins and collaborators, using a more extensive battery of tests have reconfirmed the ROS hypothesis, showing that antibiotic lethality is accompanied by ROS generation, and that environmental variables play a role when performing such measurements [2]. Fang attributes the divergent results to differences in experimental protocols and the limitations of measurement techniques to assess oxidative stress and ROS species [5]. Such limitations include lack of specificity of fluorescence dyes [17, 18] and/or the inhibitors for ROS species [14] and potential competitive redox processes involving the dye (e.g. fluorescence based). These studies indicate that better methodologies are needed to study the complex processes of antibiotic-induced responses and test the hypothesis that ROS is indeed involved in the kill of bacteria by antibiotics [14-16].

Methods commonly used to study ROS release in antibiotics-treated bacteria include: growth inhibition assays, cell viability tests (counts of colony forming units (CFU) on nutrient agars), chemiluminescence, colorimetric and fluorescence spectroscopy methods. Growth inhibition and viability tests are primarily used to determine the bacteriostatic and/or lethal effect of the antibiotics, but limited mechanistic information of the drugs against bacteria can be extracted from the results. Spectrophotometric, spectrofluorometric and chemiluminescence methods can provide evidence of the presence of oxidative stress but these methods involve addition of exogenous reagents that might react with other components in the bacteria culture.
and lack the specificity for individual ROS species. More direct measurements of the markers of oxidative stress would be a useful addition to the arsenal of tests used to study physiological aspects related to redox mechanisms in bacteria.

In this paper, we report dynamic release of $\text{O}_2^-$ anion radicals in antibiotic-exposed bacteria using an electrochemical biosensor that allows continuous measurement of $\text{O}_2^-$ directly in the culture, without addition of exogenous reagents. We use a miniaturized cytochrome c based biosensor developed previously [16]. The method consists of a gold wire electrode modified with cytochrome c, immobilized via a mixed thiol layer [19]. The principle of detection is based on the redox reaction of the immobilized cytochrome c with the released $\text{O}_2^-$ at the electrode surface [19]. The immobilized cytochrome c is reduced by $\text{O}_2^-$ and subsequently oxidized at the electrode, generating a redox current. As compared to other methods, the cytochrome c biosensor has several advantages including: (1) direct measurement in the bacterial culture with minimum perturbation of the environment, (2) reagentless operation, (3) high sensitivity and selectivity and (4) measurements are quantitative (with appropriate calibration in the bacterial growth medium). These sensors are inexpensive to produce and relatively easy to use [20]. Electrochemical cytochrome c based biosensors have been applied to quantify $\text{O}_2^-$ primarily in standards of enzymatically-produced $\text{O}_2^-$ [21]. To our knowledge such studies have not been conducted in bacterial cultures. Therefore, in this study, we first evaluated the sensitivity and specificity of measurements in the bacteria culture medium. The sensor was then used to detect $\text{O}_2^-$ radicals released from antibiotics-treated Gram-negative and Gram-positive bacteria (E. coli and L. monocytogenes, respectively). Amoxicillin (a β-lactam antibiotic), norfloxacin (a quinolone), and kanamycin (an aminoglycoside), were used as model antibiotics possessing different molecular targets. Specificity of the biosensor measurements was established using SOD. The method allowed us to measure the continuous release of $\text{O}_2^-$ in bacterial cells and estimate the rate of $\text{O}_2^-$ production.

CFU assays were used to screen the killing effect of the antibiotics through a wide range of concentrations. Furthermore, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA), a general fluorescent indicator for ROS, was also used alongside electrochemical measurements to determine the presence of ROS in the antibiotic-treated bacteria. Results from this work indicate that antibiotics trigger the release of $\text{O}_2^-$ in E. coli and L. monocytogenes in a dose-dependent manner. This study also provides a methodology for
assessing the dynamics of $O_2^-$ release in bacteria cultures and demonstrates the potential of this approach for studying oxidative stress mechanisms in bacteria. The results provide a link between $O_2^-$ production and oxidative stress, and demonstrate involvement of $O_2^-$ in the bactericidal action of these antibiotics.

**Experimental**

**Materials and reagents**

XOD from bovine milk (EC1.17.3.2), cytochrome $c$ from horse heart, superoxide dismutase (SOD), hypoxanthine (HX), 11-mercapto-1-undecanol (MU), 3-mercapto-1-propionic acid (MPA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma (St Louis, MO) and used as received. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) was obtained from Invitrogen, Life Technology (Carlsbad, CA). Ampicillin sodium salt and kanamycin sulfate were purchased from Amresco (Framingham, MA). Norfloxacin nicotinate was purchased from Enzo Life Science (Farmingdale, NY). Difco™ LB broth and agar, Miller (Luria-Bertani) and BBL™ Brain Heart Infusion broth and agar were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Deionized water from Direct-Q system (Millipore, Billerica, MA) with a resistivity of 18.2 MΩ·cm was used to prepare all solutions.

**Instrumentation**

Gold wires with the diameter of 0.5 mm purchased from Goodfellow Corporation (Coraopolis, PA) were used to prepare the working electrodes. A Mini Shaker Incubator from VWR (Radnor, PA) was used for the bacterial growth. All electrochemical measurements were run on a CHI 1232b electrochemical analyzer (CH Instruments, Inc., Austin, TX). A Cary Eclipse Fluorescence Spectrometer (Agilent Technologies, Santa Clara, CA) was used for the fluorescence measurements.

**Preparation and electrochemical characterization of antibiotic stock solutions**

Ampicillin, norfloxacin and kanamycin were obtained in their salt form to facilitate the dissolution in aqueous medium. The concentration of antibiotics stock solution, preparation procedures and storage conditions followed the protocols described by Cockerill [22]. Since
electrochemical measurements are used to assess the oxidative stress in antibiotic treated samples, it was important to investigate whether these antibiotics have characteristic oxidation or reduction peaks that may interfere with measurements. Control experiments were run with the cytochrome c biosensor placed in an electrochemical cell containing ampicillin, norfloxacin or kanamycin at a final concentration of 512 µg/mL. The solutions were analyzed by cyclic voltammetry at a potential cycled from -0.4 V to 0.4 V, in the same range as that used for \( \text{O}_2^- \) measurements.

**Bacterial strains and growth conditions**

*E. coli* HS(pFamp)R (ATCC 700891) and *L. monocytogenes* NCTC 7973 (ATCC 35152) were purchased from ATCC (Manassas, VA). *E. coli* HS(pFamp)R was chosen as a representative gram-negative bacterium and as one of the most studied in research of oxidative stress responses [23]. *E. Coli* exhibit SOD activity involved in the protection against oxygen radicals [24]. *E. coli* HS(pFamp)R is resistant to ampicillin, streptomycin and nalidixic acid [25]. *L. monocytogenes* NCTC 7973 was chosen as a representative gram-positive bacterium. *E. coli* was cultivated at 37 °C in LB Broth, Miller to late log phase. *L. monocytogenes* was cultivated at 37°C in BHI broth overnight.

**Antibiotics exposure**

The bacterial density of the initial culture used for antibiotics exposure studies and electrochemical measurements was determined by plate counting method before each experiment. Appropriate concentrations of antibiotics solutions were inoculated into log-phase bacterial culture (*E. coli* or *L. monocytogenes*) with final concentrations of 0.5, 1, 4 and 8 µg/mL, respectively. The control culture (in the absence of antibiotics) and the antibiotics-treated cultures were then incubated at 37 °C for 1 hour. After the incubation, a decimal dilution series of the control culture and antibiotics-treated cultures were inoculated onto LB and BHI agar Petri dish plates without antibiotics, respectively. Colony forming units were counted after 24 hours incubation at 37 °C.
Fabrication and calibration of the superoxide biosensor

The cytochrome c biosensor used in this study was fabricated and calibrated using the procedure established by Ganesana et al. [26] with some optimizations (Supplementary information (SI) section, Figure S1). Briefly, a mixture of 3-mercaptopropionic acid and 11-mercaptoundecanol was used to create an oriented self-assembled monolayer (SAM) on an electrochemically cleaned gold wire microelectrode. Cytochrome c was immobilized on the surface thiols by first incubating the SAM gold wire in an aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 200 mM) and N-hydroxysuccinimide (NHS, 50 mM) for 30 min to activate the carboxyl groups of the surface thiols, and then in a cytochrome c solution (5 µM) for 2 hrs. The amperometric response of the biosensor was recorded with the electrode polarized at the potential of 0.15 V vs. Ag/AgCl, which corresponds to the oxidation potential of cytochrome c.

To calibrate the sensor, the amperometric responses generated upon addition of different concentrations of xanthine oxidase (XOD) to the electrochemical cell containing 100 µM hypoxanthine (HX) was measured and correlated with the theoretical \( O_2^- \) concentration. XOD catalyzes the oxidation of HX in the presence of oxygen, with the production of \( O_2^- \) and uric acid (Equation 1 and 2). When the HX/XOD enzymatic system is used to generate \( O_2^- \) and calibrate the biosensor, a steady-state signal is obtained corresponding to the \( O_2^- \) generation and dismutation (Equations 1 and 2). This steady-state signal is proportional to the square root of XOD activity [27]. The theoretical \( O_2^- \) concentration is calculated from Equation 3 [19] in which \( k_1 \) and \( k_2 \) are the reaction rate constants of Equations 1 and 2.

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\begin{align*}
HX + O_2 \xrightarrow{XOD, k_1} \text{Uric Acid} + O_2^- & \quad (1) \\
2O_2^- + 2H^+ \xrightarrow{k_2} O_2 + H_2O_2 & \quad (2) \\
\left[ O_2^- \right]_{\text{steady-state}} = \frac{k_1}{2k_2} \left[ \text{XOD} \right] & \quad (3)
\end{align*}
\]

where \( k_1 \) was 1 s\(^{-1} \) for the enzymatic reaction used in this study [19], \( k_2 \) is the pH-dependent reaction rate constant for the spontaneous dismutation of \( O_2^- \) [28], \( k_2 \) is \( 2.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \) at pH=7.5 [29]. Superoxide measurements using the cytochrome c biosensor is based on the redox reaction between the enzymatically generated \( O_2^- \) and the immobilized cytochrome c with electrochemical recording of the electron transfer from the reduced cytochrome c to the electrode [30]. The calibration of the biosensor was conducted in an electrochemical cell placed in a water bath with continuous water supply maintained at 37 °C from an Isotemp™ 2150 heated bath.
circulator (Fisher Scientific, Waltham, MA). To perform the measurement in growth medium, the biosensor was first calibrated in the LB and BHI media. A scheme of the cytochrome c O$_2^-$ biosensor and the redox reaction of cytochrome c with O$_2^-$ released by antibiotic-stressed bacteria is shown in Figure 1.

Figure 1
Figure S1 in SI

*Electrochemical measurement of superoxide release in bacteria*

Bacterial cultures, grown as described above, were transferred to a conventional electrochemical cell containing an Ag/AgCl reference electrode, a platinum counter electrode and the cytochrome c biosensor, in an incubator set at 37 °C. Constant potential amperometry with the electrode polarized at 0.15 V vs. Ag/AgCl was used to quantify O$_2^-$ production overtime. The current was measured until the signal stabilized (after ca. 600 sec). Antibiotics were then added to the bacterial culture to achieve desired concentrations and the electrochemical signal corresponding to O$_2^-$ released in response to addition of antibiotics was recorded under continuous measurement. Finally, SOD was added to the bacterial culture to establish the specificity of the signal against O$_2^-$ radicals.

*Fluorescent dye based measurement of ROS*

A general ROS indicator, CM-H$_2$DCFDA, was used to determine ROS production in antibiotic-treated bacteria and control samples without antibiotics, in identical conditions as those used in the electrochemical experiments. Bacteria cultures with and without antibiotics were incubated at 37°C for 1 hour in a shaking incubator. Ten micromolar CM-H$_2$DCFDA was added to each sample and incubated for 1 hour in dark. Subsequently, the bacteria were washed and resuspended in 0.1 M PBS (pH 7.5) for fluorescence measurement at a 495 nm excitation wavelength [31]. The emission spectrum was recorded from 500 to 600 nm with a 5 nm slit and slow speed scan. All values are reported as the mean ± standard deviation of three replicate measurements.
Quantitative assessment of the dynamics of antibiotics-induced ROS release in bacteria is provided.