Free Radical Biology and Medicine 91 (2016) 25–33

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

Xiaobo Liu [a], Mouna Marrakchi [a,b,c], Michael Jahne [d], Shane Rogers [d], Silvana Andreescu [a,*]

[a] Department of Chemistry & Biomolecular Science, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5810, USA
[b] University of Carthage, Laboratoire d'Ecologie et Technologie Microbienne (LETMi), Institut National des Sciences Appliquées et de Technologie (INSAT), 1080 Tunis, Tunisia
[c] Tunis El Manar University, Higher Institute of Applied Biological Science of Tunis (ISSBAT), 1006 Tunis, Tunisia
[d] Department of Civil and Environmental Engineering, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5710, USA

ARTICLE INFO

Article history:
Received 25 June 2015
Received in revised form
16 November 2015
Accepted 1 December 2015
Available online 2 December 2015

Keywords:
Antibiotic resistance
Bacteria
Superoxide release
Oxidative stress
Reactive oxygen species
Superoxide radicals
Electrochemical cytochrome *c* biosensor

ABSTRACT

The involvement of oxidative stress in the mechanism of antibiotics-meditated 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 ($O_2^{\bullet -}$), 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 $O_2^{\bullet -}$ 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 $O_2^{\bullet -}$ 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-meditated bacterial death and to assess physiological changes associated with the complex metabolic events related to oxidative stress and bacterial resistance.

1. 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_2O_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_2O_2, hydroxyl ($HO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) 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

* Corresponding author.
E-mail address: eandrees@clarkson.edu (S. Andreescu).

http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.001
0891-5849/

with lucigenin for $O_2^{\bullet-}$ and luminol for other ROS species [11,12]. An increase in ROS species, including $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production in ciprofloxacin treated *S. aureus* [11]. The involvement of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ anion radicals in antibiotic-exposed bacteria using an electrochemical biosensor that allows continuous measurement of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ at the electrode surface [19]. The immobilized cytochrome *c* is reduced by $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ primarily in standards of enzymatically-produced $O_2^{\bullet-}$ [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 $O_2^{\bullet-}$ radicals released from antibiotics-treated Gram-negative and Gram-positive bacteria (*E. coli* and *L. monocytogenes*, respectively). Ampicillin (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 $O_2^{\bullet-}$ in bacterial cells and estimate the rate of $O_2^{\bullet-}$ 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_2DCFDA), 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 $O_2^{\bullet-}$ in *E. coli* and *L. monocytogenes* in a dose-dependent manner. This study also provides a methodology for assessing the dynamics of $O_2^{\bullet-}$ release in bacteria cultures and demonstrates the potential of this approach for studying oxidative stress mechanisms in bacteria.

2. Experimental

2.1. 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_2DCFDA) 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-Qsystem (Millipore, Billerica, MA) with a resistivity of 18.2 MΩ cm was used to prepare all solutions.

2.2. 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.

2.3. 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 $O_2^{\bullet-}$ measurements.

2.4. 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.

2.5. 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 h. 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 h incubation at 37 °C.

2.6. 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, Fig. 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 h. 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 $O_2^{\bullet-}$ concentration. XOD catalyzes the oxidation of HX in the presence of oxygen, with the production of $O_2^{\bullet-}$ and uric acid (Eqs. 1 and 2). When the HX/XOD enzymatic system is used to generate $O_2^{\bullet-}$ and calibrate the biosensor, a steady-state signal is obtained corresponding to the $O_2^{\bullet-}$ generation and dismutation (Eqs. 1 and 2). This steady-state signal is proportional to the square root of XOD activity [27]. The theoretical $O_2^{\bullet-}$ concentration is calculated from Eq. (3) [19] in which k_1 and k_2 are the reaction rate constants of Eqs. (1) and (2).

$$HX + O_2 \xrightarrow{XOD,k_1} Uric\ Acid + O_2^{\bullet-} \quad (1)$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{k_2} O_2 + H_2O_2 \quad (2)$$

$$[O_2^{\bullet-}]_{Steady-state} = \sqrt{\frac{k_1}{2k_2}[XOD]} \quad (3)$$

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 $O_2^{\bullet-}$ [28]. k_2 is $2.3 \times 10^5\ M^{-1}s^{-1}$ at pH = 7.5 [29]. Superoxide measurements using the cytochrome *c* biosensor is based on the redox reaction between the enzymatically generated $O_2^{\bullet-}$ 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* $O_2^{\bullet-}$ biosensor and the redox reaction of cytochrome *c* with $O_2^{\bullet-}$ released by antibiotic-stressed bacteria is shown in Fig. 1.

2.7. 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 $O_2^{\bullet-}$ production overtime. The current was measured until the signal stabilized (after ca. 600 s). Antibiotics were then added to the bacterial culture to achieve desired concentrations and the electrochemical signal corresponding to $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ radicals.

2.8. Fluorescent dye based measurement of ROS

A general ROS indicator, CM-H_2DCFDA, 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 h in a shaking incubator. Ten micromolar CM-H_2DCFDA was added to each sample and incubated for 1 hour in dark. Subsequently, the bacteria were washed and re-suspended 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.

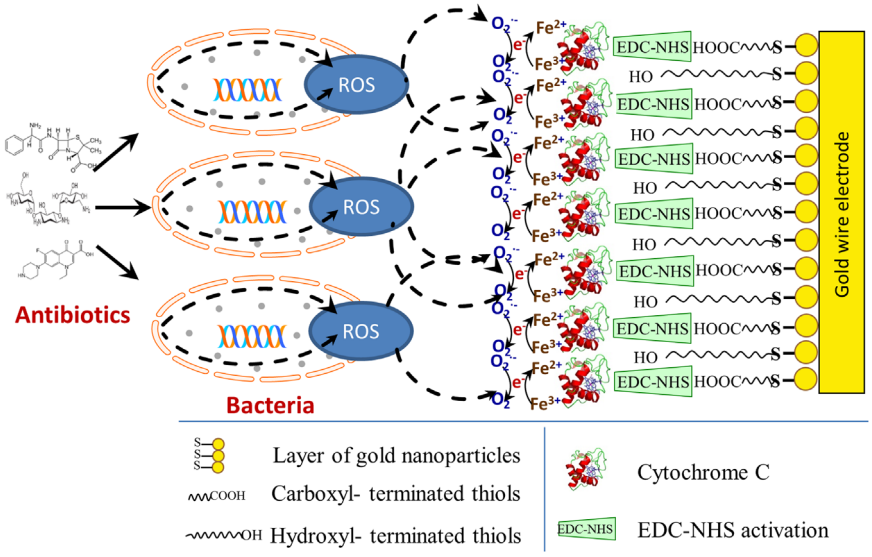

Fig. 1. Schematic of the electrochemical cytochrome *c* biosensor showing the redox reaction of cytochrome *c* with $O_2^{\bullet-}$ radicals at the surface of a gold electrode released by antibiotic-stressed bacteria.

3. Results

3.1. Effect of antibiotics exposure on bacteria

Initial experiments were performed to examine the effect of antibiotics on bacteria. The bacterial density of the initial culture was 4.9×10^8 CFU/mL for *E. coli* HS(pFamp)R and 2.2×10^9 CFU/mL for *L. monocytogenes* NCTC 7973, as determined by the CFU method. Fig. 2 shows the survival rate of *E. coli* and *L. monocytogenes* treated with various concentrations of ampicillin, norfloxacin and kanamycin. *E. coli* HS(pFamp)R is resistant to ampicillin. Norfloxacin decreased the survival percentage at 4 μg/mL and greater. Kanamycin showed a stronger killing effect as compared to norfloxacin. Exposure of *L. monocytogenes* to low concentrations of the three antibiotics showed no significant bactericidal effect. A decrease of survival percentages was observed with ampicillin and kanamycin at concentrations of 4 and 8 μg/mL. These four antibiotic concentrations, 0.5, 1, 4 and 8 μg/mL, were selected in exposure experiments to examine the $O_2^{\bullet-}$ release.

3.2. Biosensor functionality in antibiotic-treated bacteria: calibration and selectivity measurements

To quantify $O_2^{\bullet-}$ radical release from bacterial cultures, we first established the functionality of the biosensor in the growth media. The cytochrome *c* biosensor shows amperometric responses to enzymatically produced $O_2^{\bullet-}$ in a concentration dependent manner. Prior to each exposure experiment, the biosensor was calibrated in the LB and BHI broth using enzymatically generated $O_2^{\bullet-}$ by HX (100 μM) and XOD at variable concentrations, as shown in Fig. 1. Fig. 3 shows the amperometric current–time response and the corresponding calibration curves of the cytochrome *c* biosensor in LB and BHI media in comparison with PBS. Within 1 s after addition of XOD to the medium containing HX, an increase of the current resulting from the redox reaction of $O_2^{\bullet-}$ and the immobilized cytochrome *c* was observed. Approximately 3 s after the injection of XOD, the amperometric signal reached a plateau. The current difference between the baseline and the maximum recorded value at the plateau was used to build the calibration curve. The sensor signal was linearly dependent on the XOD activity and the theoretically estimated steady-state $O_2^{\bullet-}$ concentration. The linear ranges of the detection of $O_2^{\bullet-}$ from the calibration curves were 0.14–0.59 μM in 0.1 M PBS. In the LB broth, the linear range was 0.57–0.93 μM, and in the BHI, 0.14–0.59 μM. The limit of detection (LOD) of the biosensor was calculated according to the $3\sigma/R$ criteria, where R is the slope of the linear calibration curve in terms of square root of XOD concentration and σ is the standard deviation of the amperometric signal from the blank buffer [32]. The LOD of the biosensor were: 6.5 nM in 0.1 M PBS pH = 7.5, 22.98 nM in BHI broth and 32.14 nM in LB broth. We

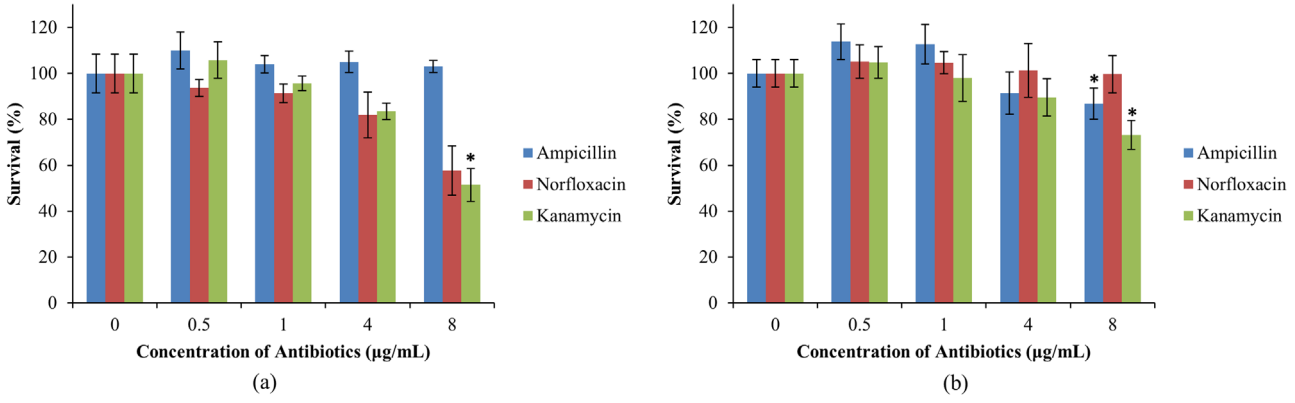

Fig. 2. Survival percentages of (a) *E. coli* and (b) *L. monocytogenes* treated with selected concentrations of ampicillin (■), norfloxacin (■) and kanamycin (■). Asterisk indicates $p < 0.05$.

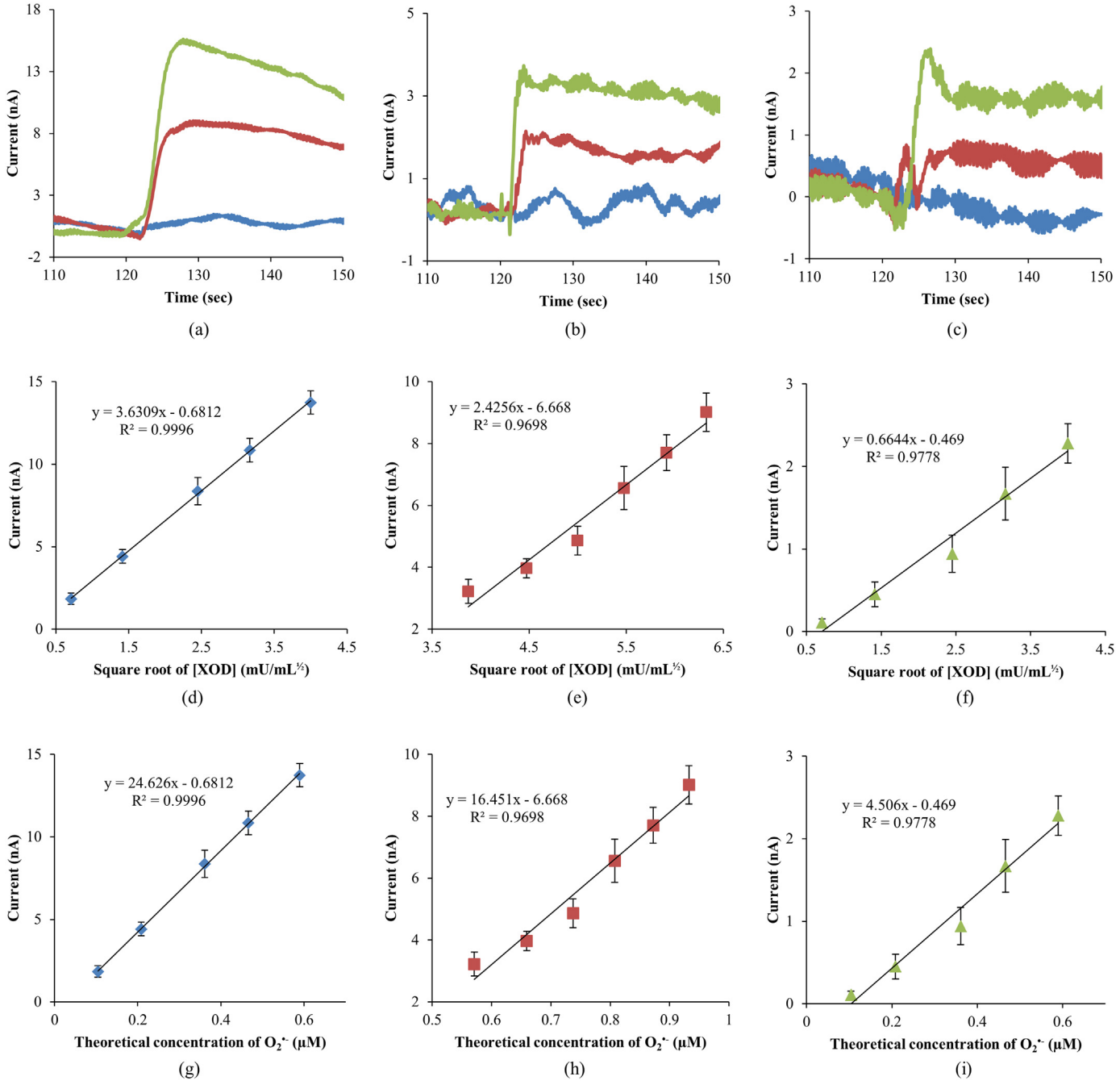

Fig. 3. Top line: Typical amperometric current–time response of the $O_2^{\bullet-}$ biosensor upon addition of 0 (Blue line), 6 (Red line) and 16 mU/mL (Green line) XOD in the presence of 100 μM HX after normalization in (a) 0.1 M PBS, pH=7.5, (b) LB broth and (c) BHI broth. Calibration curves of the biosensor in (d) 0.1 M PBS, pH=7.5 (♦), (e) LB broth (■) and (f) BHI broth (▲) as a function of square root of XOD concentration, and calibration curves of the biosensor in (g) 0.1 M PBS, pH=7.5 (♦), (h) LB broth (■) and (i) BHI broth (▲) as a function of the theoretical $O_2^{\bullet-}$ concentration.

note that the biosensor response in the two broth media showed higher noise and decreased sensitivity as compared to the response in 0.1 M PBS (Fig. 3); this may be due to surface passivation effects by proteins and other charged molecules (e.g. amino acids) present in the growth media that increase the background noise, and/or to a decreased conductivity of the growth media. The biosensor can quantitatively measure $O_2^{\bullet-}$ in the two media with good reproducibility, but requires appropriate calibration in the specific growth medium. For example, in LB broth, the response of the sensor to the addition of 40 mU/mL XOD was 9.01 ± 0.61 nA ($n=5$ replicate experiments). In BHI broth, the response of the sensor to the addition of 16 mU/mL XOD was 2.28 ± 0.24 nA ($n=5$).

Fig. 4 shows an example of amperometric current–time response of the $O_2^{\bullet-}$ biosensor in *L. monocytogenes* NCTC 7973 culture upon addition of norflaxicin (4 μg/mL). The antibiotic was added to the reaction cell after the sensor reached a steady state current in the bacteria culture (about 10 min). Immediately following addition of the antibiotic (~ 1 s), a gradual increase in current was observed, which we attribute to a steady release of $O_2^{\bullet-}$. By comparison, a control experiment without norfloxacin showed no increase. To further confirm that the signal was associated with the release of $O_2^{\bullet-}$, we used SOD, which is known to catalyze the dismutation of $O_2^{\bullet-}$ radicals. A decrease of the current was observed immediately after addition of SOD (10 U/ml), until it reached the baseline value, indicating that the released

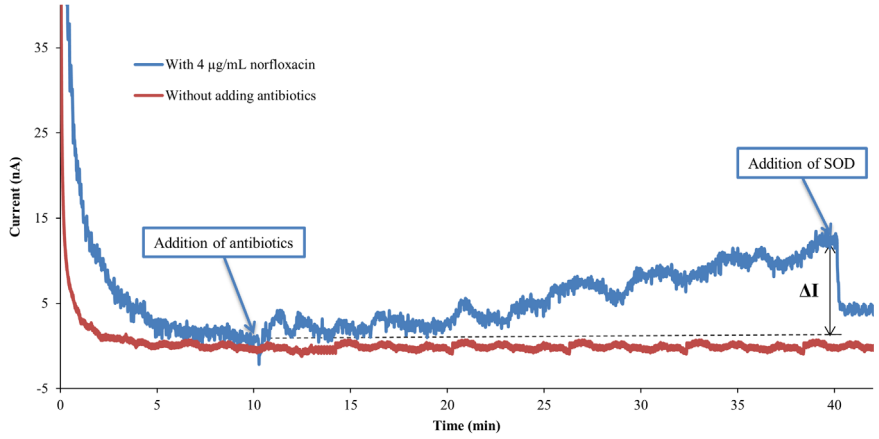

Fig. 4. Amperometric–time response of the $O_2^{\bullet-}$ biosensor upon the addition of norfloxacin (4 μg/mL final concentration) in *L. monocytogenes* NCTC 7973 culture, and control in the absence of antibiotic. Arrows indicate addition of norfloxacin and SOD to confirm specificity of the signal.

$O_2^{\bullet-}$ was inactivated entirely. The low applied potential and the densely packed thiols in the biosensor design provided selectivity against H_2O_2 and uric acid in concentrations up to 50 μM [26]. CV experiments of the three antibiotics showed no oxidation/reduction waves overlapping to that of the immobilized cytochrome *c* (Fig. S2). These results demonstrate the ability of the cytochrome *c* biosensor to measure the continuous release of $O_2^{\bullet-}$ in bacteria cultures selectively. The sensor was further used to quantitatively measure the amount of $O_2^{\bullet-}$ released by bacteria in response to various concentrations of antibiotics.

3.3. Quantitative measurements of $O_2^{\bullet-}$ in antibiotic-treated bacteria: effect of incubation time, type and dose of antibiotics

To examine whether the steady increase in $O_2^{\bullet-}$ release was a common feature among the three antibiotics, we exposed bacteria to two different concentrations of antibiotics and calculated the $O_2^{\bullet-}$ concentration (using the calibration curve in their appropriate medium) at different incubation times. Fig. 5 shows the $O_2^{\bullet-}$ concentrations measured by the sensor for the three antibiotics. All three antibiotics induced the release of $O_2^{\bullet-}$ in a time- and concentration-dependent manner. In all cases, the levels of $O_2^{\bullet-}$ per well started at ~0.2 to 0.5 μM and reached maximum values between 1 and 2 μM following 40 min exposure. $O_2^{\bullet-}$ levels for *L. monocytogenes* NCTC 7973 were higher than those recorded for *E. coli* HS(pFamp)R. We also note that *E. coli* exposed to ampicillin showed $O_2^{\bullet-}$ release in spite of its resistance to this antibiotic.

3.4. Correlation of biosensor responses with a fluorescent indicator dye for ROS

To further confirm the occurrence of an oxidative stress response, we performed side-by-side experiments to determine the general ROS release using a ROS-specific fluorescent dye. Fig. 6a–c shows the correlation between the $O_2^{\bullet-}$ concentrations and the fluorescence intensities measured in *E. coli* HS(pFamp)R exposed to various concentrations of antibiotics for 30 min. Both the biosensor and the fluorescence assay indicated an increase in the oxidative stress level for antibiotic exposure levels between 0.5 and 8 μg/mL. In general, the $O_2^{\bullet-}$ level recorded by the sensor matched the trend recorded by the fluorescence dye method. Although the *E. coli* strain used in this study is resistant to ampicillin, ROS and $O_2^{\bullet-}$ release is still observed by both methods. This increase is most notable for ampicillin concentrations up to 1 μg/mL, after which no further increase was observed. There was, however, no significant difference in terms of $O_2^{\bullet-}$ release by the three types of antibiotics tested. Kanamycin triggered the highest ROS level as compared to ampicillin and norfloxacin. A similar trend was observed for *L. monocytogenes* NCTC 7973 exposed to the three antibiotics, with a slightly higher increase in the electrochemically measured $O_2^{\bullet-}$ in the ampicillin-treated bacteria at exposure concentrations greater than 1 μg/mL (Fig. 6d–f). Under similar antibiotics stress, *L. monocytogenes* showed greater $O_2^{\bullet-}$ and ROS release than *E. coli*. For instance, the theoretical $O_2^{\bullet-}$ concentration from *E. coli* HS(pFamp)R treated with 8 μg/mL ampicillin is smaller than the theoretical $O_2^{\bullet-}$ concentration from

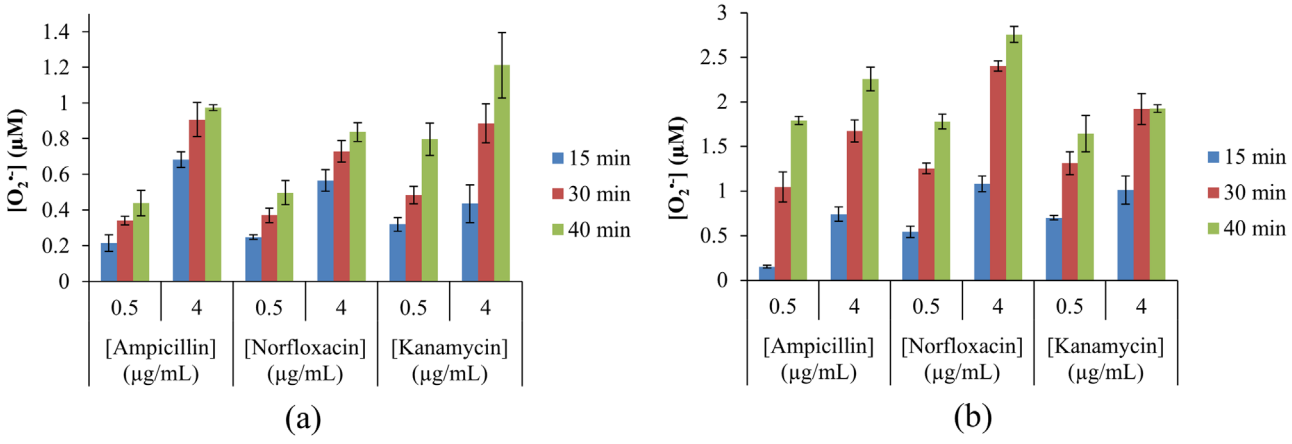

Fig. 5. Theoretically calculated $O_2^{\bullet-}$ concentrations following 15, 30, and 40 min exposure of (a) 4.8×10^8 CFU/mL *E. coli* HS(pFamp)R in LB broth, Miller and (b) 2.0×10^9 CFU/mL *L. monocytogenes* NCTC 7973 in BHI broth to ampicillin, norfloxacin, or kanamycin at 0.5 and 4 μg/mL final concentration.

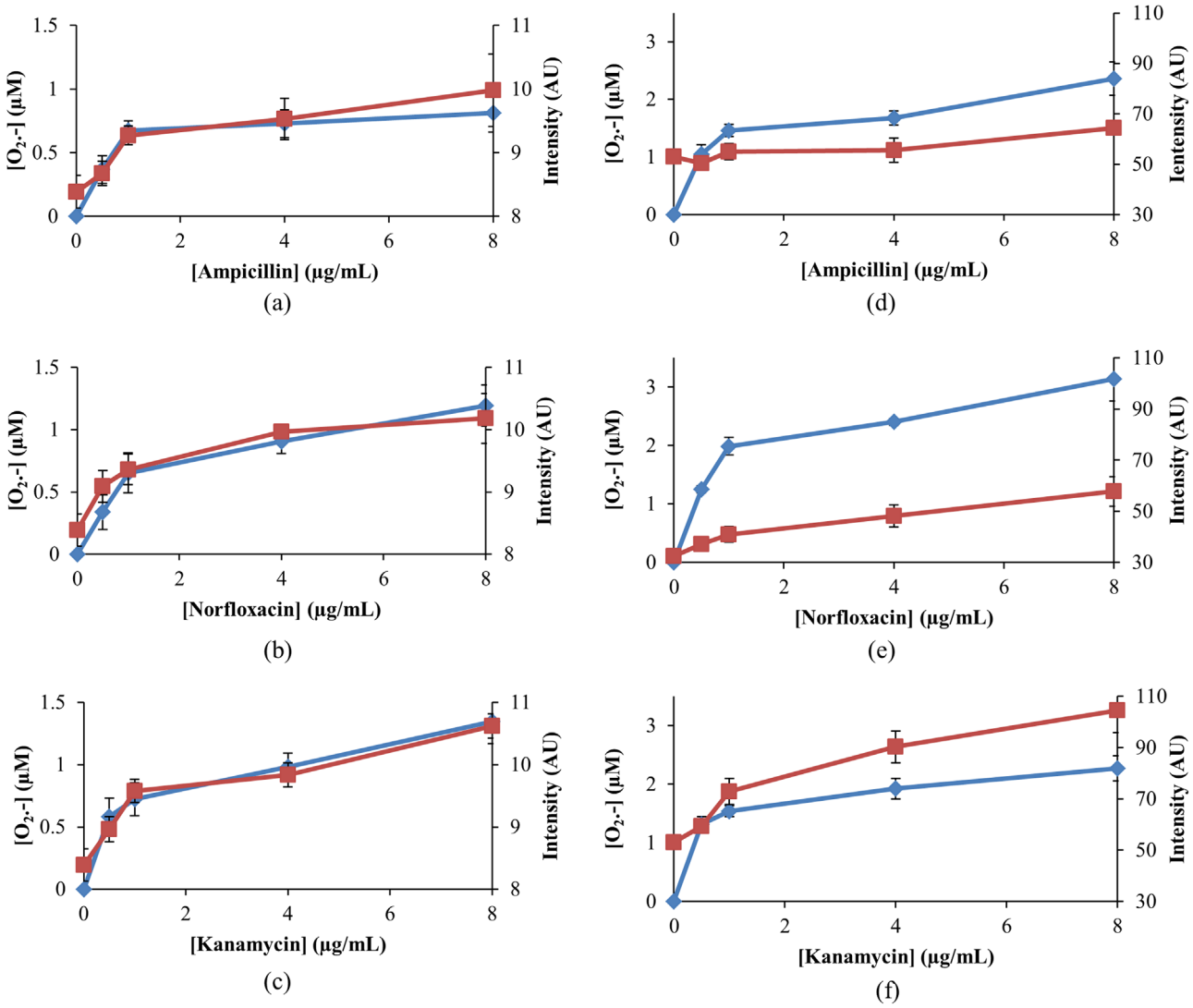

Fig. 6. Theoretically calculated $O_2^{\bullet -}$ concentrations (♦) and fluorescence intensities (■) of *E. coli* HS(pFamp)R (a, b, and c; starting concentration = 4.9×10^8 CFU/mL in LB broth, Miller) and *L. monocytogenes* NCTC 7973 (d, e, and f; starting concentration = 2.2×10^9 CFU/mLin BHI broth) 30 min following exposure to antibiotics at concentrations ranging from 0 μg/mL to 8 μg/mL.

8 μg/mL ampicillin-treated *L. monocytogenes* NCTC 7973 ($p < 0.05$). Under the same ampicillin concentration, the fluorescence intensities from ampicillin-treated *E. coli* are smaller than ampicillin-treated *L. monocytogenes* ($p < 0.001$).

4. Discussion

The goals of this study were to monitor quantitatively the real time release of $O_2^{\bullet -}$ by bacteria using electrochemical biosensors and to evaluate the relationship between antibiotic exposure (e.g. type, amount and exposure time of antibiotics) and ROS release. Previous studies that addressed this issue have reported contradictory views [2,5,14]. Methods used in previous works included fluorescence spectroscopy and conventional bacterial culture assays, which are time consuming and lack selectivity and sensitivity for ROS measurements [5]. This study demonstrated a new methodology that has the ability to provide real time quantitative analysis of ROS directly in bacterial culture without addition of exogenous reagents, and without sample preparation. Electrochemical biosensors have been previously reported to measure $O_2^{\bullet -}$ but few have been used for direct measurements in biological systems. The results of this study show that cytochrome *c* biosensors are robust and sensitive tools that can be used to assess oxidative stress in bacterial cultures. However, we observed that the biosensor is less sensitive in the culture media than in a standard phosphate buffer solution. Under identical experimental conditions, different bacterial growth broth showed variable current intensities to enzymatically generated $O_2^{\bullet -}$ indicating that biosensor sensitivity also varies with the media composition. For accuracy, the sensors were calibrated in the appropriate growth media prior to measurements. Our results showed that the biosensor can be used in LB and BHI media, and can detect $O_2^{\bullet -}$ levels with LOD in the nM range. The sensor was more sensitive in the LB broth as compared to the BHI broth. The selectivity of the biosensor against $O_2^{\bullet -}$ was established by addition of exogenous SOD to the bacterial culture, which reduced the $O_2^{\bullet -}$ signal demonstrating selectivity of measurements.

The results from the real time electrochemical recordings demonstrated the release of $O_2^{\bullet -}$ from antibiotic-treated bacterial culture in both gram-positive and gram-negative bacteria. A similar trend was observed for three antibiotics, representative of three major classes (β-lactams, quinolones, and aminoglycosides), suggesting that oxidative stress might be a common feature of

antibiotic stress in bacteria. Fluorescence measurements with a general ROS dye, carried out in parallel with the biosensing experiments, also showed a similar ROS profile. ROS release was time and concentration dependent. The electrochemical method allowed quantitative estimation of the $O_2^{\bullet-}$ concentration released by bacteria using the calibration curve in the culture medium. Theoretically estimated $O_2^{\bullet-}$ levels were in the μM range, varying with the antibiotic and the bacterial strain. An interesting observation was that the ampicillin-treated *E. coli* containing the Famp plasmid also showed $O_2^{\bullet-}$ release at levels up to 0.6 μM/well, in spite of its resistance to ampicillin. It is possible that ROS at such concentration may not lead to cell death. According to the dual-function stress responses model, low-level oxidative stress might provide beneficial mutations for bacteria, and induce lethality only when the stress is high [33,34]. It should be noted that although the theoretical $O_2^{\bullet-}$ concentration from ampicillin-treated *E. coli* is lower than that obtained with norfloxacin and kanamycin, the difference between the three antibiotics is relatively small. A higher level of $O_2^{\bullet-}$ was observed in *L. monocytogenes* as compared to *E. coli*. The different membrane structure of the two bacteria and the intracellular SOD in *E. coli* may provide some antioxidant defense effects [35], which might explain the differential levels observed. Fig. S3 shows the correlation between the extent of killing by antibiotics and the $O_2^{\bullet-}$ release for the two bacteria and the three antibiotics tested. In general, with the exception of ampicillin treated *E. coli*, the level of $O_2^{\bullet-}$ released increases with the decrease in the survival rate (e.g. killing efficiency). $O_2^{\bullet-}$ increases more rapidly at low antibiotic concentrations (≤ 4 μg/ml) and seems to reach a plateau at higher exposure levels. The results indicate that antibiotics might induce a redox disequilibrium state in bacteria. The activation of *soxR* in bacteria, a genetic locus that operates as an inducible defense to the superoxide stress, might lead to this phenomenon [36,37]. In sum, our measurements confirm the release of $O_2^{\bullet-}$ in antibiotics-treated *E. coli* and *L. monocytogenes*. However, higher antibiotic levels do not generate significantly greater $O_2^{\bullet-}$ indicating that oxidative stress may not be solely responsible for the antibiotic-induced bacterial killing.

Although the understanding of the antibiotics-induced oxidative stress remains limited, researchers have begun to utilize this knowledge to increase the efficiency of antibiotics therapy [4]. One proposed pathway is to suppress the oxidative defensive system of the bacteria, and subsequently increase the sensitivity of bacteria to ROS [38]. Another method is to amplify the ROS generation to enhance bacterial death [39]. Understanding the contribution of ROS to cell death in antibiotic exposure scenarios is important for advancing antibiotics therapy. The natural antioxidants for mammalian systems, such as glutathione, ascorbate and vitamin E, are not able to scavenge free radicals, such as $O_2^{\bullet-}$ and H_2O_2 at an efficient rate, or suppress lipid peroxidation in bacteria [40–43]. Therefore, several new antioxidant systems that have demonstrated potent ROS scavenging activity could be used in future therapies. Recent examples include the newly designed class of SOD-mimetic nanoparticles such as cerium oxide [44]. Future studies are needed to relate ROS release with antioxidant treatment and correlate these findings with genomic and proteomic analysis to understand the origin of ROS release in both antibiotic resistant and sensitive bacterial strains.

5. Conclusion

In summary, this study demonstrated that electrochemical biosensors provide a convenient way to measure real-time $O_2^{\bullet-}$ release in bacteria cultures, as an alternative method for assessing oxidative stress release. This biosensor can be a useful tool in investigations requiring real time monitoring and quantitative assessment of $O_2^{\bullet-}$ in bacterial cultures, and potentially in other biological environments. Fundamentally, the results demonstrate the presence of ROS induced by the presence of different types of antibiotics, indicating a general antibiotic-induced oxidative stress. ROS release was dependent on incubation time, antibiotic type and concentration, and bacterial species. $O_2^{\bullet-}$ increase was observed for bacteria treated with low antibiotic concentrations, but no further increase was observed for higher antibiotic exposure levels. Oxidative stress was also observed with antibiotic resistant bacteria, even though lethality by this antibiotic was not present. This shows that a low level of oxidative stress is present in all antibiotic-treated bacteria, and that this mechanism may not be uniquely responsible for the lethal effects of antibiotics on bacteria. Electrochemical testing can be useful in future work to study the complex redox related mechanisms and metabolic events involved in bacterial pathogenicity and antibiotic resistance, and to advance our knowledge of the relationship between oxidative stress and antibiotics action.

Acknowledgments

The authors thank Dr. Rıfat Emrah Özel for his generous help and discussions at the initial stages of this work. Mouna Marrakchi gratefully acknowledges the Fulbright Foundation for the research fellowship at Clarkson University.

This work was supported by grant NSF- 1336493 to SA. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.001.

References

[1] A. Rodríguez-Rojas, et al., Antibiotics and antibiotic resistance: a bitter fight against evolution, Int. J. Med. Microbiol. 303 (6–7) (2013) 293–297.
[2] D.J. Dwyer, et al., Antibiotics induce redox-related physiological alterations as part of their lethality, Proc. Natl. Acad. Sci. 111 (20) (2014) E2100–E2109.
[3] M. Marrakchi, X. Liu, S. Andreescu, Oxidative stress and antibiotic resistance in bacterial pathogens: state of the art, methodologies, and future trends, in: A. G. Woods, C.C. Darie (Eds.), Advancements of Mass Spectrometry in Biomedical Research, Springer International Publishing, 2014, pp. 483–498.
[4] D.J. Dwyer, J.J. Collins, G.C. Walker, Unraveling the physiological complexities of antibiotic lethality, Annu. Rev. Pharmacol. Toxicol. 55 (1) (2015) 313–332.
[5] F.C. Fang, Antibiotic and ROS linkage questioned, Nat. Biotechnol. 31 (5) (2013) 415–416.
[6] M.A. Kohanski, et al., A common mechanism of cellular death induced by bactericidal antibiotics, Cell 130 (5) (2007) 797–810.
[7] N. Umezawa, et al., Participation of reactive oxygen species in phototoxicity induced by quinolone antibacterial agents, Arch. Biochem. Biophys. 342 (2) (1997) 275–281.
[8] T. Xia, et al., Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm, Nano Lett. 6 (8) (2006) 1794–1807.
[9] S.S. Grant, et al., Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals, Proc. Natl. Acad. Sci. 109 (30) (2012) 12147–12152.
[10] X. Wang, et al., Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death, J. Antimicrob. Chemother. 65 (3) (2010) 520–524.
[11] M.C. Becerra, I. Albesa, Oxidative stress induced by ciprofloxacin in *Staphylococcus aureus*, Biochem. Biophys. Res. Commun. 297 (4) (2002) 1003–1007.
[12] I. Albesa, et al., Oxidative stress involved in the antibacterial action of different antibiotics, Biochem. Biophys. Res. Commun. 317 (2) (2004) 605–609.
[13] M. Goswami, S.H. Mangoli, N. Jawali, Involvement of reactive oxygen species in the action of ciprofloxacin against *Escherichia coli*, Antimicrob. Agents Chemother. 50 (3) (2006) 949–954.

[14] K.-T. Yong, et al., Nanotoxicity assessment of quantum dots: from cellular to primate studies, Chem. Soc. Rev. 42 (3) (2013) 1236–1250.
[15] I. Keren, et al., Killing by bactericidal antibiotics does not depend on reactive oxygen species, Science 339 (6124) (2013) 1213–1216.
[16] D.J. Hassett, J.A. Imlay, Bactericidal antibiotics and oxidative stress: a radical proposal, ACS Chem. Biol. 2 (11) (2007) 708–710.
[17] P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects, Free Radic. Biol. Med. 43 (7) (2007) 995–1022.
[18] Michael P. Murphy, et al., Unraveling the biological roles of reactive oxygen species, Cell Metab. 13 (4) (2011) 361–366.
[19] B. Ge, F. Lisdat, Superoxide sensor based on cytochrome c immobilized on mixed-thiol SAM with a new calibration method, Anal. Chim. Acta 454 (1) (2002) 53–64.
[20] P.T. Kissinger, Biosensors—a perspective, Biosens. Bioelectron. 20 (12) (2005) 2512–2516.
[21] C. Calas-Blanchard, G. Catanante, T. Noguer, Electrochemical sensor and biosensor strategies for ROS/RNS detection in biological systems, Electroanalysis 26 (6) (2014) 1277–1286.
[22] F. Cockerill, Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement, Clinical and Laboratory Standards Institute, 2013.
[23] G. Storz, J.A. Imlay, Oxidative stress, Curr. Opin. Microbiol. 2 (2) (1999) 188–194.
[24] A. Battistoni, et al., Overexpression of a hydrogen peroxide-resistant periplasmic Cu,Zn superoxide dismutase protects *Escherichia coli* from macrophage killing, Biochem. Biophys. Res. Commun. 243 (3) (1998) 804–807.
[25] J. Debartolomeis, V.J. Cabelli, Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages, Appl. Environ. Microbiol. 57 (5) (1991) 1301–1305.
[26] M. Ganesana, J.S. Erlichman, S. Andreescu, Real-time monitoring of superoxide accumulation and antioxidant activity in a brain slice model using an electrochemical cytochrome c biosensor, Free Radic. Biol. Med. 53 (12) (2012) 2240–2249.
[27] J.M. McCord, I. Fridovich, The reduction of cytochrome c by milk xanthine oxidase, J. Biol. Chem. 243 (21) (1968) 5753–5760.
[28] S. Marklund, Spectrophotometric study of spontaneous disproportionation of superoxide anion radical and sensitive direct assay for superoxide dismutase, J. Biol. Chem. 251 (23) (1976) 7504–7507.
[29] D. Behar, et al., Acid dissociation constant and decay kinetics of the perhydroxyl radical, J. Phys. Chem. 74 (17) (1970) 3209–3213.
[30] K. Tammeveski, et al., Superoxide electrode based on covalently immobilized cytochrome c: modelling studies, Free Radic. Biol. Med. 25 (8) (1998) 973–978.
[31] T. Ohashi, et al., Rapid oxidation of dichlorodihydrofluorescin with heme and hemoproteins: formation of the fluorescein is independent of the generation of reactive oxygen species, FEBS Lett. 511 (1–3) (2002) 21–27.
[32] G. Currell, Analytical Instrumentation: Performance Characteristics and Quality, vol. 27, John Wiley & Sons, 2008.
[33] M.A. Kohanski, M.A. DePristo, J.J. Collins, Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis, Mol. Cell 37 (3) (2010) 311–320.
[34] A. Dorsey-Oresto, et al., YihE kinase is a central regulator of programmed cell death in bacteria, Cell. Rep. 3 (2) (2013) 528–537.
[35] J.A. Imlay, I. Fridovich, Assay of metabolic superoxide production in Escherichia coli, J. Biol. Chem. 266 (11) (1991) 6957–6965.
[36] J.T. Greenberg, et al., Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*, Proc. Natl. Acad. Sci. 87 (16) (1990) 6181–6185.
[37] P.J. Pomposiello, B. Demple, Redox-operated genetic switches: the SoxR and OxyR transcription factors, Trends Biotechnol. 19 (3) (2001) 109–114.
[38] M.P. Brynildsen, et al., Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production, Nat. Biotechnol. 31 (2) (2013) 160–165.
[39] I.-s Hwang, et al., Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved, J. Med. Microbiol. 61 (12) (2012) 1719–1726.
[40] C.C. Winterbourn, D. Metodiewa, Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide, Free Radic. Biol. Med. 27 (3–4) (1999) 322–328.
[41] D.S. Nichols, T.A. McMeekin, Biomarker techniques to screen for bacteria that produce polyunsaturated fatty acids, J. Microbiol. Methods 48 (2–3) (2002) 161–170.
[42] B.H. Bielski, R.L. Arudi, M.W. Sutherland, A study of the reactivity of HO2/O2- with unsaturated fatty acids, J. Biol. Chem. 258 (8) (1983) 4759–4761.
[43] J.A. Imlay, Diagnosing oxidative stress in bacteria: not as easy as you might think, Curr. Opin. Microbiol. 24 (2015) 124–131.
[44] V. Shah, et al., Antibacterial activity of polymer coated cerium oxide nanoparticles, Plos One 7 (10) (2012).