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Advanced Amperometric Respiration Assay for Antimicrobial Susceptibility Testing

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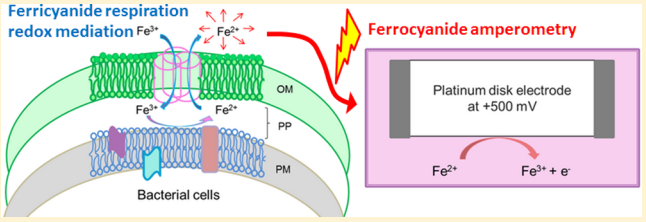
Supporting Information

ABSTRACT: A ferricyanide-based electrochemical cell respiration assay was adapted for use in broad-spectrum antimicrobial susceptibility testing (AST). Total bacterial respiration was converted into faradaic current by electrooxidation of ferrocyanide, produced when ferricyanide is reduced by bacterial electron-transport. For *Escherichia coli* (*E. coli*), the signal was linear with 5−13 × 10^8 colony-forming units in measuring buffer. For AST, test cells were treated with drugs before ferricyanide addition; cell counts from the amperometric assay provided a measure of drug-induced cell death. Initial trials with six antimicrobial agents produced incorrect susceptibility classifications for drugs that were electroactive at the potential used to detect ferrocyanide or which affected cellular respiration rates. We therefore changed the procedure from drug-treatment and assay in the same buffer to sequential drug exposure in treatment buffer, centrifugal separation of surviving cells, cell resuspension, incubation in the presence of ferricyanide and finally ferrocyanide amperometry in drug-free buffer. Data analysis with *E. coli* led to an activity classification that agreed with cell culture-based ASTs, obtained by a quicker, more convenient procedure. The potential of this approach was confirmed by trials with the highly virulent bacterium *Burkholderia pseudomallei*, a particularly antimicrobial-resistant pathogen that is the cause of lethal melioidosis in tropical climates and is currently of concern as a potential bioterrorism agent.



The growth of bacterial antibiotic resistance is a critical threat to public health for the foreseeable future.[1−3] Several infections that previously were treatable have already become deadly because of the failure of antimicrobial treatments, particularly in countries with weak health-care systems. Both Gram-negative and Gram-positive microorganisms have acquired resistance to many available antimicrobial agents.[4] *Burkholderia pseudomallei* (*B. pseudomallei*), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter* species, *Acinetobacter baumannii*, *Escherichia coli* (*E. coli*) and *Klebsiella pneumonia* are prominent examples of (multi-) drug resistant bacteria and there are serious concerns about their potential use as bioterror agents that harm or even kill through untreatable bacteremia.[5−10] The rapid spread of antimicrobial resistance and the consequent problems for physicians and risks for patients requires the rational prescription of antibiotic drugs, prevention of their excessive or inappropriate use and also efficient antimicrobial susceptibility tests (ASTs) for clinical analysis and pharmaceutical R&D. Currently, disk diffusion, broth dilution and antimicrobial gradient tests are routinely used as ASTs.[11] All three assays can provide vital information on the effectiveness of an antimicrobial agent's action on bacterial cells and hence valuable guidance in choosing first- and second-line rescue therapy, but they are quite laborious and time-consuming. There is a need for alternative approaches that allow prompt selection of an antimicrobial agent for a particular bacterial infection, facilitate detection of novel resistance patterns and additionally may reduce costs, through a reduced demand for reagents and labor. Proposed options for drug susceptibility trials include the assessment of bacterial cell wall integrity through fluorescent nucleic acid staining,[12] a plug-based microfluidic technology that works with the confinement of individual target bacteria into nanoliter volumes,[13] a cell screen based on electrochemical DNA biosensing[14] and a plaque assay.[15] Susan Mikkelsen and co-workers pioneered a potentially rapid redox mediator-assisted electrochemical AST assay that assesses the effects of antimicrobials on bacterial cells through the decline of their metabolic respiratory activity (Scheme 1).[16,17]

Redox active ferricyanide, $[Fe^{III}(CN)_6]^{3-}$, is added to test media as a diffusible molecular probe that can diffuse through bacterial outer membrane protein channels ("porins") into periplasm and access the terminal complexes of the electron transport chain, which are exposed on the cytoplasmic membrane. Cellular electron transport to $Fe^{III}(CN)_6]^{3-}$ creates $[Fe^{II}(CN)_6]^{3-}$, which diffuses through the porins into the assay

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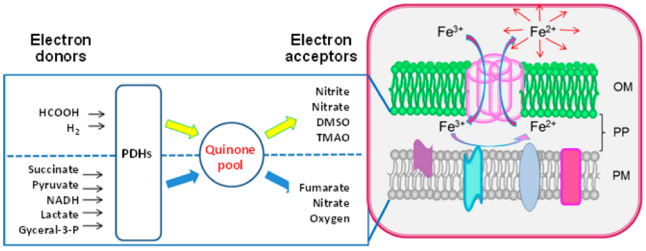
Scheme 1. Graphical Illustration of the Utilization of the Bacterial Electron Transport Chain with a Dissolved Redox Mediator (Here: Ferricyanide) in the Electrochemical Antimicrobial Susceptibility Assay[a]



[a] $[Fe(CN)_6]^{3-}$ moves from the extracellular milieu through outer membrane protein channels into the periplasm, and there make electron-exchange contact with terminal metabolic redox enzymes. Mediator/protein interaction produces $[Fe(CN)_6]^{4-}$, which leaves the periplasm and enters the surrounding medium, where it is detected electrochemically with a Pt disk electrode at anodic Fe(II) oxidation potential.

buffer, where a rotating platinum working electrode enables amperometric or chronocoulometric detection. Electrochemical AST depends upon the level of the Fe(II) oxidation current being proportional to the number of respiring cells in the assay buffer. Hence, the presence of an effective antimicrobial agent appears as a decrease in current, compared to that for the drug-free control or for drug-resistant cells. In the reported study, a known number of healthy, exponential- or stationary-phase *E. coli* JM105 cells was incubated for 15 (amperometry) or 10 (chronocoulometry) min in nutrient-reduced growth buffer containing the drug. In the measuring step, the incubated cell suspensions were mixed with suitable aliquots of ferricyanide-containing test buffer and respiratory ferrocyanide generation was traced through real-time current recordings in the amperometry or chronocoulometry mode. The drugs tested were cell wall synthesis inhibitors (penicillin G, D-cycloserine, vancomycin, bacitracin and celphalosporin C), protein synthesis inhibitors (tetracycline, erythromycin, chloramphenicol and streptomycin), nucleic acid synthesis inhibitors (nalidixic acid, rifampicin and trimethoprim) and a cell membrane permeation modulator (nystatin). For most of the drugs tested, the AST data confirmed the sensitive/resistant classification suggested by control overnight disk diffusion tests, but some exceptions were observed; for example, *E. coli* cells were classified as resistant to bacitracin by microbiology testing, but in the direct electrochemical assay, markedly increased rates of ferricyanide reduction, rather than the expected unchanged, were observed. Nalidixic acid, on the other hand, was effective in the disk diffusion assay but not in the electrochemical AST assay, which showed enlarged currents. These experimental inconsistencies were explained by stimulatory side effects of the drugs on microbial respiration and/or energy-consuming active efflux for bacitracin and nalidixic acid. Clearly, electrochemical ferri-/ferrocyanide readout of susceptibility worked well with antimicrobial agents that simply killed bacterial cells, but not with those that altered their respiratory rates. Knowledge of the redox and biochemical properties of a particular antibiotic is obviously a prerequisite for successful application of the ferrocyanide/ferricyanide-based electrochemical susceptibility screen.

To further explore the capability and limits of an amperometric Fe(III)/Fe(II) AST assay, this methodology was used in this study to examine the effect of six previously untested antimicrobial agents on *E. coli*. The $[Fe(CN)_6]^{3-/4-}$ system was used as a cell respiration redox reporter because the two hydrophilic ions of the couple can cross with ease the outer membrane of Gram-negative and -positive bacterial cells through the existing water-filled porins and they are readily available at low price, easy to handle, stable and associated with a well-pronounced reversibility. The observed susceptibility to three of the tested drugs, ciprofloxacin, co-trimoxazole and dicloxacillin, was in good accordance with the outcome of control microbiological tests. However, the same assay produced inconclusive results with gentamicin, cefepime and ceftazidime, either because of intrinsic anodic drug electroactivity at the working potential for amperometric Fe(II) detection (gentamycin) or through drug-induced up-regulation of cell metabolism. For the problematic compounds, an adaptation of the original Mikkelsen assay was made and the details of the successful novel protocol are reported here, along with data analysis and discussion in terms of susceptibility classification and calculation of apparent IC_{50} values. Finally, we report a comparative trial with *B. pseudomallei*, a multidrug-resistant, potentially lethal microorganism that is classified by the U.S. Center for Disease Control and Prevention as a Category B Health Hazard and possible bioterror agent.[18]

EXPERIMENTAL SECTION

Reagents and Materials. Potassium ferricyanide was obtained from Acros Organics (New Jersey, USA). D-(+)-glucose monohydrate, dipotassium hydrogen phosphate trihydrate, potassium dihydrogen phosphate, trisodium citrate dihydrate, calcium chloride dihydrate, ammonium chloride, ammonium sulfate and ammonium formate were provided by Carlo Erba (MI, Italy). Magnesium sulfate (anhydrous) was from Fluka (Buchs, Switzerland). Extract of yeast powder and tryptone type-1 powder were provided by Himedia (Mumbai, India) and Muller Hinton broth by Criterion (CA, USA).

E. coli DH5α cells were obtained from Invitrogen (Gibco, CA, USA). *B. pseudomallei* cells were a clinical sample, kindly provided by Thammasat University Hospital, Bangkok, Thailand. Both strains were maintained at 37 °C in Luria Broth (LB) medium (tryptone, 10 g/L, yeast extract, 5 g/L, NaCl, 5 g/L). Ciprofloxacin was from Millimed Co., Ltd. (Samurtprakan, Thailand), cefepime from Kaspa Pharmaceutical (Bangkok, Thailand), ceftazidime from Utopian (Samurtprakan, Thailand), dicloxacillin from Fluka, gentamicin from T.P Drug Laboratories (Bangkok, Thailand) and co-trimoxazole, (a drug combination of trimethoprim and sulfamethoxazole, from Unison (Bangkok, Thailand). Primary antimicrobial standards were prepared at a concentration of 40 mg/mL in a sterile phosphate buffer solution (PBS) pH 6.8 containing KH_2PO_4 (2.88 g/L), $K_2HPO_4{\cdot}3H_2O$ (5.76 g/L), trisodium citrate dihydrate (1.2 g/L), $MgSO_4{\cdot}7H_2O$ (0.48 g/L), $CaCl_2{\cdot}2H_2O$ (0.048 g/L), $(NH_4)_2SO_4$ (1.63 g/L), NH_4Cl (1.34 g/L). Exceptions were co-trimoxazole and ciprofloxacin, which were made up at 10 mg/mL in 10% dimethyl sulfoxide (DMSO) and 5 mg/mL in distilled water, respectively. The drug standards were stored in appropriate aliquots at −20 °C.

Cultivation of Microorganisms. Growth medium for bacterial cell culture was PBS (defined above) containing tryptone (10 g/L) and a yeast extract (5 g/L). The *E. coli* DH5α and *B. pseudomallei* cells were cultured aerobically at 37 °C and 180 rpm for 16 h in 25 mL of a growth medium that was supplemented with glucose at 2 g/L just prior to

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Table 1. Properties of the Antimicrobial Compounds Inspected in This Study and Results of Conventional Disk Diffusion Susceptibility Tests (n = 3) with *E. coli* DH5α

			disk diffusion test	zone diameter breakpoint (mm)	
antibiotic	mode of action	mean diameter of inhibition (mm)	classification	$S\geq$	$R\leq$
cefepime	cell wall	38.33 ± 0.58	sensitive	24	21
ceftazidime	cell wall	34.33 ± 0.58	sensitive	22	19
ciprofloxacin	DNA	24.66 ± 0.58	sensitive	22	19
co-trimoxazole	folate synthesis	34.33 ± 0.58	sensitive	16	13
dicloxacillin	cell wall	NI[a]	resistant	NA[b]	NA[b]
gentamycin	protein synthesis	19.33 ± 0.58	sensitive	17	14

[a]NI: no inhibition zone. [b]NA: not available.

inoculation. The optical density at 600 nm (OD_{600}) of bacterial cell suspensions at different stages was measured with an xMark microplate absorbance spectrophotometer (Biorad, USA). Colony counts were performed by serial 10-fold dilution in 0.9% NaCl, after adjustment of the concentration based on OD_{600}. Aliquots of 100 μL of each sample were plated onto LB agar and incubated for 18 h. Colony forming unit (CFU) counts were from plates containing less than 300 colonies.

Agar Disk Diffusion and Microbroth Dilution Tests. The two standard ASTs were performed following the guidelines from the Clinical and Laboratory Standard Institute (CLSI).[19] For agar disk diffusion tests, Mueller Hinton agar plates were inoculated with bacterial cell suspensions with an OD_{600} of 0.1. Five millimeter diameter disks of filter paper were then uniformly spread around the agar plates at suitable distances and after placement were soaked with aliquots of solutions of the antibiotics under test. The cell plates were developed by overnight incubation at 37 °C, and drug action visualized through the appearance of growth inhibition zones. Drug loadings in the tests were 30 μg (cefepime, ceftazidime and dicloxacillin), 10 μg (gentamicin), 5 μg (ciprofloxacin) and 1.25/23.75 μg (trimethoprim-sulfamethoxazole (co-trimoxazole)) per disk. After incubation, the diameters of the zones of growth inhibition were measured and these values interpreted as "sensitive" or "resistant" according to CLSI recommendations. For the microbroth dilution assay, cells were adjusted to the same concentration as were later also used in the electrochemical assay. After incubation with serial 2-fold dilutions of antibiotics for 16 h at 37 °C, the OD_{600} values of each culture were measured. Positive (without antimicrobial agents) and negative (no cells) controls were carried out in parallel to check the validity of the tests.

Amperometric Fe(III)/Fe(II) Respiration Screening. Tryptone-, yeast- and glucose deficient PBS growth medium was used as treatment buffer in all trials. Using OD_{600} values from cell count calibration measurements, stationary phase *E. coli* cells were adjusted in the medium to desired concentrations and kept on ice at least 15 min before further processing. Usually, a 1 mL aliquot of the cooled cell suspension was then centrifuged for 5 min at 14000 rpm and the pellet resuspended in 1 mL medium with or without antibiotic. Drugs were allowed to interact with the microorganisms for 15 min at 37 °C. To measure the respiratory activity of the cell population electrochemically, the 1 mL cell/drug suspension was added to 1 mL of medium containing 100 mM $K_3Fe(CN)_6$ and 20 mM ammonium formate as supporting respiratory substrate[16] (assay buffer). In the resulting 2 mL mixture, the cells and the redox mediator were allowed to react for 3 h at 37 °C, 100 rpm in the dark. The assay solution was then transferred to a small beaker-type electrochemical cell equipped with a stationary 3 mm Pt disk working electrode, a Ag/AgCl pseudoreference and a Pt wire counter-electrode. Before use, the working electrode surface was thoroughly polished with 0.05 μm alumina slurry (Buehler, Duesseldorf, Germany) and cleaned by 30 min ultrasonication in distilled water.

Respiratory ferricyanide reduction was measured with a 910 PSTAT minipotentiostat (Metrohm AG, Switzerland) and appropriate software, by anodic Fe(II) oxidation at a constant potential. The amperometric protocol used a detection potential of +0.5 V and current recordings over a period of 10 min. Assessment of the susceptibility of bacteria to a particular drug was based on the ratio of the background-corrected amperometric currents in the presence and absence of drug, which is a measurement of the respiratory activity (% RA) remaining after drug action (a value of 100% indicating no drug effect on cell growth, whereas 0% would indicate complete cell death, under the conditions of the assay). From the acquired amperometric traces, % RA values were calculated using the following equation:

$$\%\mathrm{RA} = \left(\frac{I_{(+\mathrm{drug})} - I_0}{I_{(-\mathrm{drug})} - I_0}\right) \times 100$$

where $I_{(+drug)}$ is the current value in the assay buffer with bacterial cells and antibiotic, $I_{(-drug)}$ is the current in the assay buffer with bacterial cells but no antimicrobial agent and I_0 is the current in control assay buffer containing antimicrobial agent but no respiring bacterial cells (background).

A modification of the above standard amperometric AST procedure used antimicrobial agent removal and subsequent amperometric Fe(II) current measurement in drug-free solution. In this protocol, drug/cell suspensions were incubated for 15−180 min and then centrifuged at 14000 rpm for 5 min. The resultant supernatants were discarded and the cell pellets resuspended in 1 mL medium and added to 1 mL of medium, supplemented with 100 mM $K_3Fe(CN)_6$/20 mM ammonium formate. Incubation at 37 °C and 100 rpm for 3 h in the dark allowed respiratory generation of Fe(II), which was finally quantified by amperometry under the conditions used in the standard protocol.

IC_{50} Determination. IC_{50} values were determined by microbroth dilution and by the amperometric assay, using various concentrations of the antimicrobial agents of choice.

RESULTS AND DISCUSSION

Table 1 shows the results of triplicate standard agar disk diffusion tests of the effects of six antibiotics on *E. coli* strain DH5α, a strain used widely in biochemical and microbiological laboratory applications. The bacterium was sensitive to ciprofloxacin, co-trimoxazole, cefepime, ceftazidime and

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gentamicin and within the drug collection resistant only to the cell wall synthesis inhibitor dicloxacillin. This susceptibility classification was requisite starting information for subsequent electrochemical trials, using the faster technique of amperometric respiration screening for the valuation of susceptibility.

A stationary Pt disk electrode in unstirred solution was used here to detect the Fe(II) species generated by the bacterial electron-transport chain, rather than the rotating Pt disk electrode used in the Mikkelsen version of the constant-potential redox assay. Despite this experimental simplification, the expected increase of the anodic Fe(II) oxidation current with growing number of respiring stationary phase *E. coli* DH5α cells was observed. Calibration measurements showed reproducible linearity with R^2 values >0.99 for 5−13 × 10^8 suspended CFUs (Figure 1).

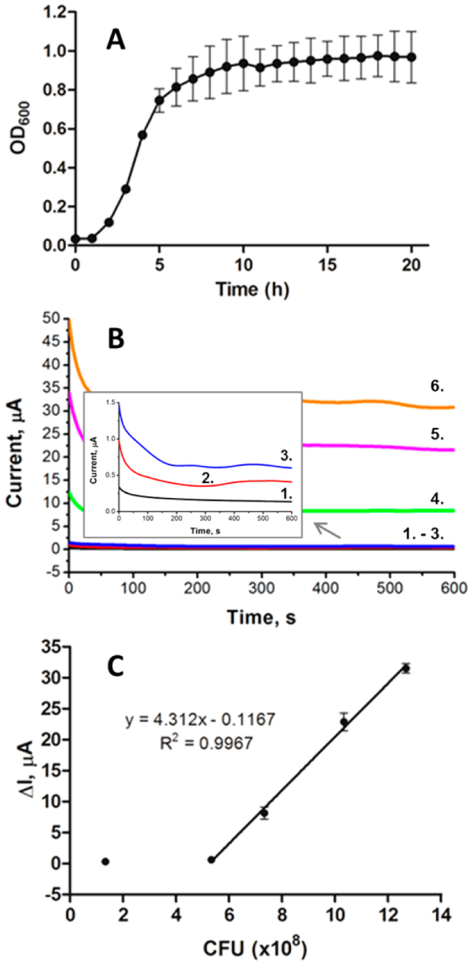

Figure 1. (A) Typical growth curve of *E. coli* as plot of the optical density (OD_{600}) of bacterial cell suspensions at different development stage vs time. (B) Amperometric traces from the electrochemical Fe(III)/Fe(II) cell respiration assay for (1.) 0, (2.) 1.33, (3.) 5.33, (4.) 7.33, (5.) 10.33 and (6.) 12.63 × 10^{08} CFU concentrations of stationary phase *E. coli* cells in assay buffer with 100 mM $K_3Fe(CN)_6$ and 20 mM formate. One milliliter cell suspension of a particular OD_{600} was added to 1 mL of assay buffer. Following incubation to allow interaction of redox proteins with the mediator (3 h, 37 °C, 100 rpm, darkness) a stationary Pt disk electrode (Ø = 3 mm) performed at +0.5 V vs Ag/AgCl the current measurement. (C) Calibration of the electrochemical cellular respiration assay. The equation for the linear regression is the inset. Plotted data averaged values of triplicate samples with their standard deviations.

In our initial amperometric drug susceptibility tests, stationary phase *E. coli* DH5α cells were incubated for 15 min at 37 °C in assay buffer supplemented with ciprofloxacin, co-trimoxazole or dicloxacillin, or omitted in controls. To complete the assay, treated cell suspensions were spiked with 50 mM ferricyanide and 10 mM formate and then further incubated for 3 h at 37°. Finally, the ferrocyanide oxidation current was determined at room temperature (approximately 25°) for a period of 600 s. Representative current−time traces from the trials with ciprofloxacin and co-trimoxazole are shown in Figure 2A,D, respectively. Because both drugs were known to be effective against *E. coli* DH5α (see Table 1), the largest plateau currents were obtained under control conditions, whereas antimicrobial agents produced a concentration-dependent reduction in current, through drug-induced loss of respiring cells. Dose−response curves were generated by analysis of complete sets of amperometric traces (Figure 2B,E) and produced IC_{50} values of 1.02 ± 0.18 mM (n = 3) for ciprofloxacin and 8.52 ± 0.79 mM (n = 3) for co-trimoxazole. Control measurements with the conventional microbroth dilution assay (Figure 2C,F) delivered much smaller IC_{50} values, 0.17 ± 0.01 μM (n = 3) for ciprofloxacin and 0.60 ± 0.06 μM (n = 3) for co-trimoxazole. Control measurements with the conventional microbroth dilution assay (Figure 2C,F) delivered much smaller IC_{50} values, 0.17 ± 0.01 μM (n = 3) for ciprofloxacin and 0.60 ± 0.06 μM (n = 3) for co-trimoxazole. Large differences between microbiologically and electrochemically determined IC_{50} values were also observed in the earlier proof-of-principle study,[16] with different antimicrobial agents and cell strains.

The probable explanation of the discrepancy is the reduction of incubation times from several hours to a few minutes, a change of experimental condition that apparently requires a higher concentration of an applied antimicrobial agent to produce measurable effects. The third drug screened in the initial series of electrochemical ASTs was dicloxacillin, against which *E. coli* DH5α cells were shown to be resistant in the agar disk diffusion prescreen (see Table 1). The outcome of the amperometric trial was the same, since the respiration-dependent electrode current did not change significantly even though the cells were exposed to a drug concentration that with ciprofloxacin and co-trimoxazole produced a 50% decrease (Figure 3).

Although amperometric cell respiration screening of ciprofloxacin, co-trimoxazole and dicloxacillin agreed with conventional methods, the simple protocol described above was not satisfactory in tests of *E. coli* DH5α susceptibility to cefepime, ceftazidime or gentamicin. In the microbiological AST assay, these were identified as effective antibacterial compounds, but in amperometric recordings, we observed an increase in the plateau values of ferrocyanide oxidation currents, instead of the expected decrease; representative traces for cefepime are shown in Figure 4A. The misleading results of electrochemical AST could arise either through drug electroactivity at the working potential used for ferrocyanide detection or, as suggested in the Mikkelsen study for other antimicrobial agents, through drug-induced stimulation of metabolic processes. Potential scanning over the range 0 to +0.8 V, versus the Ag/AgCl reference electrode, was used to characterize the electrochemical behavior of the six antimicrobial agents studied. As shown in the set of voltammograms in Figure 4B, only gentamicin interacted with the charged platinum disk sensor, with an anodic current peak at about +0.4 V. For all

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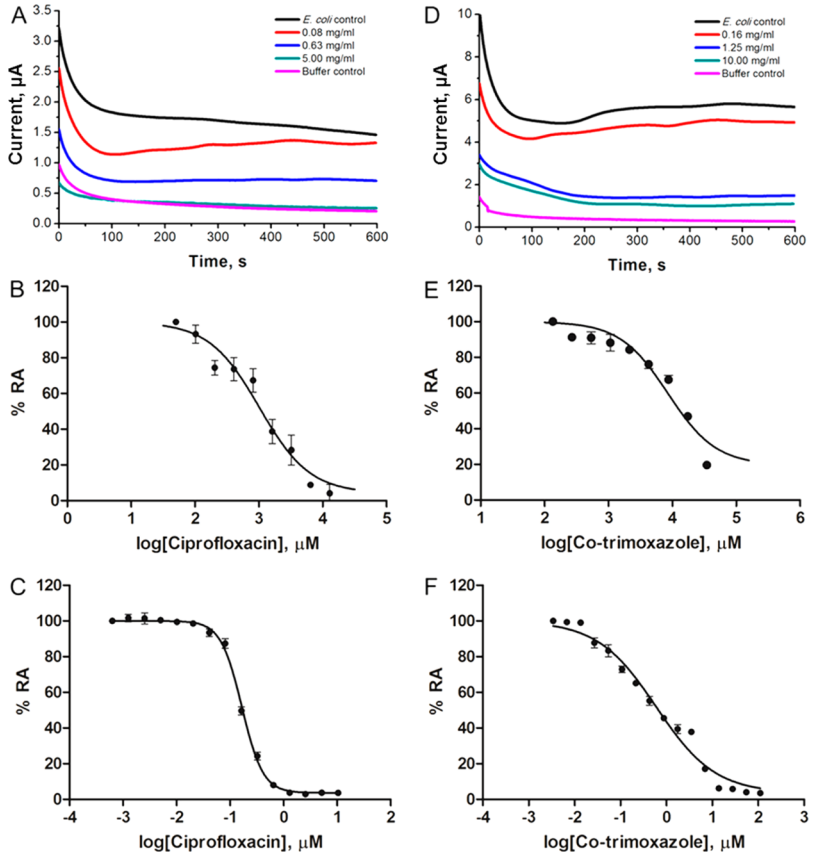

Figure 2. Amperometric testing of *E. coli* antimicrobial susceptibility to ciprofloxacin and co-trimoxazole. Typical current traces from the Fe(III)/Fe(II) redox assay with bacteria exposed to (A) ciprofloxacin and (D) co-trimoxazole. The assay protocol involved 15 min exposure to the drug in treatment buffer followed by 3 h of incubation in assay buffer supplemented with 100 mM $K_3Fe(CN)_6$ and 20 mM formate. At the end of this incubation period, amperometry was carried out with a 3 mm Pt disk working electrode (0.5 V vs Ag/AgCl). IC_{50} plots were derived from the electrochemical recordings for ciprofloxacin (B) and co-trimoxazole (E). IC_{50} plots derived from a microbroth dilution assay are shown for ciprofloxacin (C) and co-trimoxazole (F).

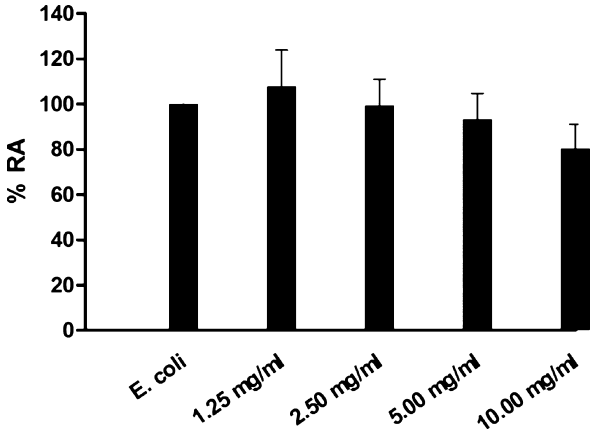

Figure 3. Amperometric testing of the susceptibility of *E. coli* to dicloxacillin. The assay protocol was identical to that detailed in Figure 2. The electrochemically assessed relative respiratory activity (RA) of surviving cells against is plotted against the concentration of dicloxacillin.

other antibiotics tested, I/E traces were virtually flat beyond the Fe(II) detection potential. Thus, the incongruity between the results from the microbiological and electrochemical ASTs was likely to be related to the electroactivity of the drug, in the case of gentamicin, whereas for cefepime and ceftazidime, an indirect effect on the rate of cellular respiration is probably responsible; elucidating this biochemical effect was not within the scope of this study and was not pursued further. However, we adapted the assay protocol so that it functioned despite these two types of interference. In the modified optimal procedure, which was applicable to cefepime, ceftazidime and gentamicin, bacterial cells were incubated for 3 h in the antibiotic treatment buffer. The cell suspension was then centrifuged and the supernatant containing the drug was removed. The cell pellets were gently redispersed in assay buffer containing $[Fe^{III}(CN)_6]^{3-}$ as a redox marker, and respiration was measured as $[Fe^{II}(CN)_6]^{4-}$ formation.

For a reliable assessment of cell death, it was important to allow sufficient time for interaction of $[Fe^{III}(CN)_6]^{3-}$ with the bacterial electron-transport chain. Three hours of incubation was found to result in significant conversion of Fe(III) into Fe(II). The success of the modified protocol is evident in Figure 5A, which displays the data from a typical trial with cefepime as representative case. In contrast to the observation with the original assay protocol, without drug removal after the initial 3 h of preincubation (Figure 4A), the Fe(II) signal in the modified assay decreased in response to cefepime treatment, as expected for a known cytotoxic drug. The modified procedure worked also with ceftazidime and gentamicin and in all cases, the induced cell death was concentration-dependent (Figure S1, Supporting Information). Cefepime or ceftazidime at 40 mg/mL efficiently killed *E. coli* DH5α and the related % RA values for this condition were 10.39 ± 2.66 and 14.21 ± 0.65 (*n*

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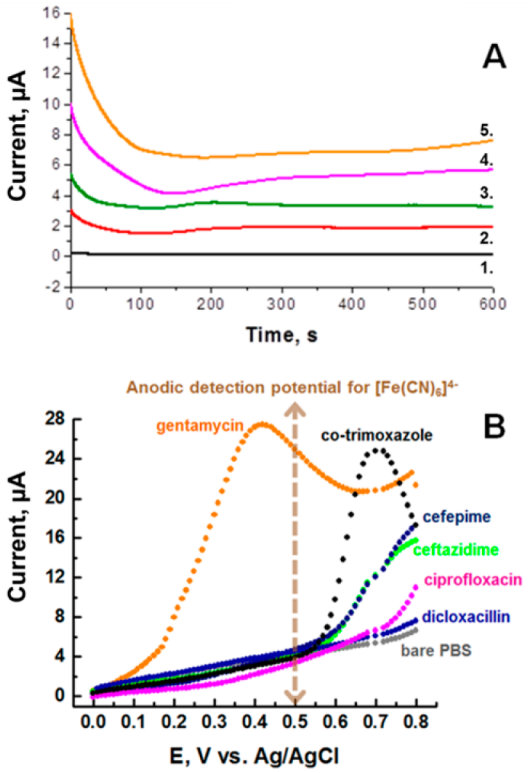

Figure 4. (A) Attempted amperometric testing of the cefepime susceptibility of *E. coli*. Amperometric recordings were with the Fe(III)/Fe(II) redox assay for (3.) 2.5, (4.) 5 and (5.) 10 mg/mL drug, respectively; (1.) is a cell and drug-free buffer and (2.) a drug free cell control. The protocol involved 15 min exposure to the antibiotic in treatment buffer, then addition of 100 mM $K_3Fe(CN)_6$ and 20 mM ammonium formate followed by 3 h of incubation. Incubation was followed by amperometry (3 mm Pt disk electrode, 0.5 V vs Ag/AgCl). (B) Linear sweep voltammograms of 10 mg/mL of the drugs of this study in 100 mM phosphate buffer (pH 6.8). The working, pseudoreference and counter electrodes were a 3 mm Pt disk, an Ag/AgCl wire and a bare Pt wire.

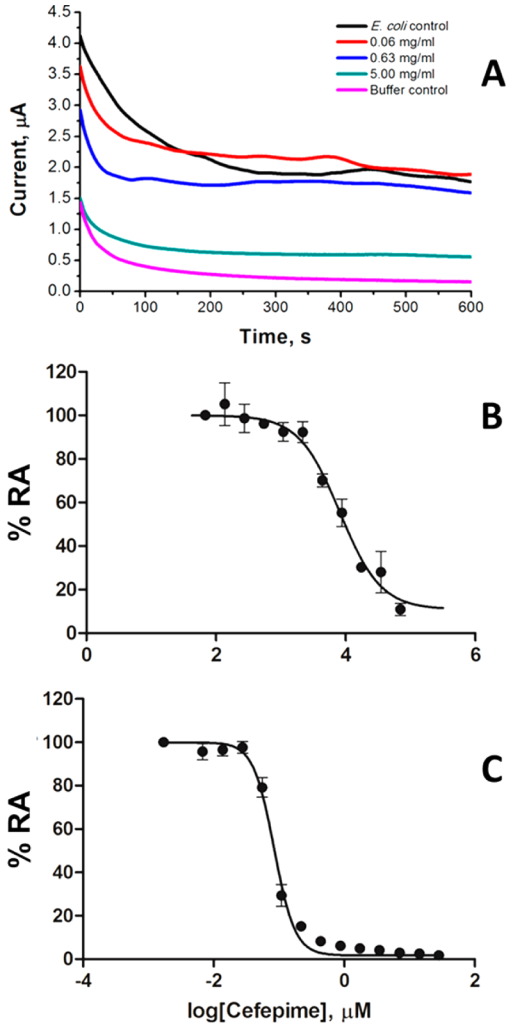

Figure 5. (A) Amperometric testing of the susceptibility of *E. coli* to cefepime. A set of amperometric recordings obtained in the Fe(III)/Fe(II) redox assay for increasing drug concentrations (0, 0.06, 0.63 and 5 mg/mL in the black, red, blue and green traces, respectively; purple is the cell-free control). The assay protocol involved 3 h of drug exposure to the antibiotic in treatment buffer, drug removal by cell centrifugation resuspension and finally 3 h of incubation in assay buffer supplemented with 100 mM $K_3Fe(CN)_6$ and 20 mM formate. After this incubation, amperometry was carried out with a 3 mm Pt disk working electrode (0.5 V vs Ag/AgCl). (B) IC_{50} plot derived from the electrochemical recordings for cefepime. (C) IC_{50} plot for cefepime from a microbroth dilution assay.

= 3), respectively. Gentamicin, on the other hand, was shown with the electrochemical assay to be almost fully toxic to the *E. coli* cells at 20 mg/mL, where the measured % RA was 5.42 ± 0.34 (n = 3).

Dose response curves could be constructed with the data from the adapted cell respiration screen and Figure 5B shows that for cefepime, together with equivalent data from microbroth dilution in Figure 5C. As observed when using the unmodified protocol for ciprofloxacin and co-trimoxazole, IC_{50} values for cefepime derived from electrochemical (8.3 ± 0.96 mM (n = 3)) and microbiological (0.08 ± 0.01 μM (n = 3)) measurements were widely different, presumably because of differences in the incubation times.

Finally, the modified electrochemical AST procedure was evaluated for its capability to report the antibiotic susceptibility of *B. pseudomallei*. This pathogen lives in tropical and subtropical soil and groundwater and is well documented as the etiologic agent of mammalian melioidosis, a severe disease that is endemic in Southeast Asia and Northern Australia and fatal to humans if untreated.[20] The extraordinary intrinsic resistance of *B. pseudomallei* to many common antimicrobials makes its eradication after infection difficult, resulting in high mortality rates from melioidosis, even with early diagnosis and immediate treatment. In affected areas of Thailand, for example, virtually half of melioidosis patients do not survive their infection because of inefficient medication. Our recent study on the involvement of the outer membrane protein channel (porin) in the antimicrobial resistance of *B. pseudomallei* confirmed that the strain under study was susceptible to ceftazidime, the usual first-line antimicrobial agent in the treatment of melioidosis, but resistant to cefepime.[21] These two compounds were therefore chosen here as the model drugs used in the modified amperometric susceptibility screening of *B. pseudomallei*.

Raw amperometric traces similar to those shown in Figures 2A,D and 5A for *E. coli* trials were converted to relative respiratory activity (% RA) and plotted against antibiotic treatment time and antibiotic concentration (Figures 6A,B and S2, Supporting Information). In contrast to the results with *E.*

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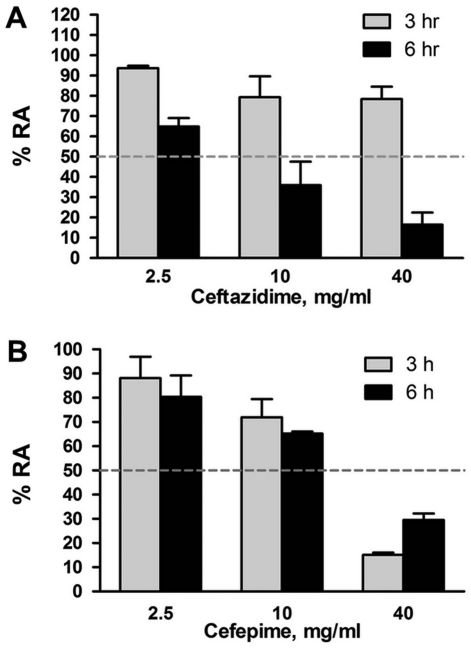

Figure 6. Amperometric testing of the susceptibility of *B. pseudomallei* to (A) ceftazidime and (B) cefepime. The assay protocol was identical to that in Figure 5; however, the antibiotic treatment time was either 3 or 6 h. The bar graph shows the electrochemically assessed relative respiratory activity (RA) of surviving cells with different times and antimicrobial agents concentrations.

coli, a 3 h drug pretreatment of *B. pseudomallei*, before the final (Fe(II)/Fe(III) respiration signal development step, was insufficient to produce an effect on metabolic activity that reflected the known potency of the drug. For 2.5, 10 and 40 mg of ceftazidime per mL treatment buffer, for instance, the corresponding respiratory activities of *B. pseudomallei* were 93.6 ± 1.2%, 79.3 ± 10.3% and 78.5 ± 6.1%, respectively (Figure 6A). These high % RA levels suggested resistance of *B. pseudomallei* to ceftazidime, in contrast with the drug's effectiveness against the microorganism in microbiological AST trials and with its success in clinical melioidosis treatment. However, a longer, 6 h exposure to the drug before the ferricyanide-based respiration screening improved the agreement: treatment with ceftazidime for 6 h at 2.5, 10 and 40 mg/mL produced % RA values of 64.9 ± 4.2%, 36.0 ± 11.6% and 16.4 ± 6.1%, respectively (Figure 6A). For cefepime, a change from 3 to 6 h incubation time did not decrease the % RA values as much as for ceftazidime, and only with 40 mg/mL was the RA less than 50% (Figure 6B). The observed pattern is an adequate reflection of the resistance of *B. pseudomallei* to this drug at low and medium concentrations. Cytotoxicity at the highest concentration may reflect the antimicrobial agent uptake mechanism, which involves diffusion through outer membrane protein channels.[21] Apparently, the development of drug resistance by *B. pseudomallei* resulted in decreased permeability of the porins to antimicrobial drugs such as cefepime, but this is overcome by the very large concentration difference with 40 mg/mL extracellular cefepime. Possibly the difference in treatment time required by *E. coli* and *B. pseudomallei* for successful electrochemical AST is a consequence of differences in their growth rates, their cell doubling times being about 18 and 40 min, respectively.[22,23] Faster cell growth suggests a greater rate of metabolism and therefore of Fe(III) reduction. Respiratory Fe(III)/Fe(II) redox conversion at the inner membrane of cells that survived antimicrobial treatment is, on the other hand, the foundation of the signal generation of the electrochemical screen and slower growing bacteria as *B. pseudomallei*, thus, are expected to need longer assay time for true extraction of their effectiveness. The two microorganisms may also differ in the density and type of the porin channels in the outer membrane, through which the redox mediators pass during the assay, and/or in the properties of the redox centers in the inner membrane. Both would influence the magnitude of signal generation and thus would affect the minimum assay time.

CONCLUSION

We describe here the development of an amperometric method for evaluating the antimicrobial susceptibility of bacteria. In this approach, ferricyanide in the culture medium was reduced by the terminal redox complexes of the electron-transport chain, in the bacterial inner membrane. Amperometric assay of ferrocyanide provided a measure of cellular respiration, which could be calibrated as a cell count and, after exposure of bacteria to antibiotics, used to assess drug-induced cell death. The work of others with a ferricyanide-based electrochemical respiration screen had used it to evaluate the effect of a range of antibiotics on *E. coli* cells as a model system and demonstrated its potential in drug susceptibility testing, offering shorter handling times than is feasible with microbiological assays. A drawback of the suggested protocol of the electrochemical AST, however, was that it did not report correctly on the antimicrobial effectiveness of all drugs tested, and deviations from the expected susceptibility classification were ascribed to the stimulation of bacterial respiration by some antimicrobial agents. This drawback was confirmed in the present work, and an additional problem was identified, namely the electroactivity of some antibiotics at the potential used for ferrocyanide detection.

To overcome these problems, we evolved a modified protocol for ferricyanide-based electrochemical cell respiration screening that involved four steps: (1) exposure of bacterial test cells in to the antimicrobial treatment buffer, (2) cell centrifugation and resuspension to separate surviving cells from the drug, (3) incubation in the presence of $[Fe^{III}(CN)_6]^{3-}$ and (4) $[Fe^{II}(CN)_6]^{4-}$ amperometry in the antibiotic-free buffer.

When applied to *E. coli*, the novel strategy yielded electrochemically derived dose response curves for previously nonaddressable drugs, allowed the assessment of minimum inhibitory concentrations and provided a susceptibility classification that was in agreement with that derived from cell culture-based microbiological ASTs. In addition to the proof-of-principle trials with *E. coli*, the developed assay was applied to a drug susceptibility testing of the clinically important bacterium *B. pseudomallei*, which in tropical climates, is a cause of lethal melioidosis, in particular because of its extreme antimicrobial resistance. Correct susceptibility identification for *B. pseudomallei* required 6 h of drug incubation instead of the 3 h that were sufficient for *E. coli*. Removal of antimicrobial agent after the antimicrobial treatment phase and optimal duration of $Fe^{III}(CN)_6]^{3-}$/cell interaction were required to reach a configuration of the amperometric cell respiration assay that functions as convenient broad-spectrum AST. Although electroanalysis is not yet routine in AST detection, its convenience and suitability for automation suggest that further development and refinement of this

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approach are well worthwhile. Potential options include, for instance, the move from the operation of the scheme on a macroelectrode platform to microelectrode versions in order to profit from stationary instead of gradually decaying Fe(II) oxidation current profiles and the explorations of redox reporters others than the Fe(III)/Fe(II) couple. A significant advance in the ferricyanide-based electrochemical cell respiration screen has already been realized here and future work with this sensing strategy is expected to improve applications for evaluations of microbial drug resistance.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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