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Monitoring antibacterial permeabilization in real time using time-resolved flow cytometry

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ABSTRACT

Despite the intensive study of antibiotic-induced bacterial permeabilization, its kinetics and molecular mechanism remain largely elusive. A new methodology that extends the concept of the live–dead assay in flow cytometry to real time-resolved detection was used to overcome these limitations. The antimicrobial activity of pepR was monitored in time-resolved flow cytometry for three bacterial strains: *Escherichia coli* (ATCC 25922), *E. coli* K-12 (CGSC Strain 4401) and *E. coli* JW3596-1 (CGSC Strain 11805). The latter strain has truncated lipopolysaccharides (LPS) in the outer membrane. This new methodology provided information on the efficacy of the antibiotics and sheds light on their mode of action at membrane-level. Kinetic data regarding antibiotic binding and lytic action were retrieved. Membrane interaction and permeabilization events differ significantly among strains. The truncation of LPS moieties does not hamper AMP binding but compromises membrane disruption and bacterial killing. We demonstrated the usefulness of time-resolved flow cytometry to study antimicrobial-induced permeabilization by collecting kinetic data that contribute to characterize the action of antibiotics directly on bacteria.

1. Introduction

Most gold-standard methods used to study the efficacy of antibiotics are time consuming and provide limited information on the kinetics of bacterial killing or inactivation, which hampers retrieving information that may be useful to deduce the molecular mechanisms involved. The permeabilization/killing kinetics provides valuable information on the molecular mode of action, target and specificity of the antibiotic. In this study, we describe a methodology that automatically detects the kinetics of antibiotic-induced bacterial permeabilization. This methodology uses a commercially available mixture of two dyes (Syto-9 and Propidium iodide [PI]) that allows to quantify the fraction of dead bacterial cells in a population [1,2] using time-resolved flow cytometry. The action of pepR, an antimicrobial peptide (AMP) developed in our labs, against *Escherichia coli* was studied through the application of the method. AMPs are small, structurally diverse and usually cationic amphipathic peptides that interact directly with lipids [3–6] permeabilizing Gram-positive and Gram-negative bacteria including drug-resistant strains [7,8], although few exceptions have been reported [4,6,7]. By targeting membranes, AMPs induce a rapid death in bacterial cells with low chances for resistance development [4,6,7]. The fast kinetics of permeabilization is a very demanding technical challenge that has to be specifically addressed when aiming at time-resolved data.

Altogether, this work describes and characterizes a novel and robust technique, time-resolved flow cytometry, to evaluate the antimicrobial effect of AMPs, which may be extended to other membrane-targeting antibiotics or to evaluate cellular cytotoxicity. The kinetic detail obtained with this technique may help to contribute to clarify the mode-of-action of antibiotics against different bacterial species and mutant bacteria. Gaining deeper insights on the mode of action of antibacterial agents may lead to a more rationally guided drug design, which is extremely important to avoid the emergence of multi-drug-resistant bacterial strains as a consequence of antibiotic misuse [3,9,10].

2. Materials and methods

2.1. Materials

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and sodium chloride (NaCl) were purchased from Merck Millipore (Darmstadt, Germany). Luria-Bertani agar was from Applichem LiveScience (Darmstadt, Germany), LB broth from Fisher Scientific (Waltham, MA, USA) and isopropyl alcohol from VWR BDH Prolabo (Leicestershire, England). LIVE/DEAD *Bac*Light Bacterial Viability Kit,

Abbreviations: AMP, antimicrobial peptide; FRET, Förster resonance energy transfer; PI, propidium iodide; LPS, lipopolysaccharide

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for quantitative assays (L7012) was purchased from Life Technologies (Carlsbad, CA, USA). Dengue virus strain 2-derived peptide pepR (LKRWGTIKKSKAINVLRGFRKEIGRMLNILNRRRR-amide) was synthesized by solid-state synthesis as described elsewhere [11]. pepR stock solution (500 μM) was prepared in sterile Milli Q water. All other laboratory used reagents were of the highest quality available commercially. All the experiments were performed at pH 7.4 at 22 °C ± 1 °C.

2.2. Methods

2.2.1. Preparation and culture of bacterial strains

The *E. coli* strains used for the studies were ATCC 25922, K-12 wild-type (*wt*) (CGSC Strain 4401) and JW3596-1 (CGSC Strain 11805) [12,13], from now on abridged as 25922, K-12*wt* and JW3596-1, respectively. Bacteria were maintained as stock cultures at −80 °C and revived by growing on Luria–Bertani agar plates overnight at 37 °C. For each bacterial strain, two suspensions with 10^8 cfu/mL were prepared in LB broth by direct suspension of colonies to minimize stress conditions until experimental procedures. Bacterial suspensions were then centrifuged for 10 min at 4000 ×g twice and resuspended in either HEPES buffer, pH 7.4, containing 150 mM NaCl, for live bacteria, or in HEPES buffer after the first centrifugation and 70% isopropyl alcohol after the second centrifugation to induce bacterial death. The suspensions for live and dead bacteria were afterward incubated at room temperature for 1 h, mixing every 15 min. One final centrifugation was performed and bacteria were resuspended in HEPES buffer. All bacterial suspensions were diluted in HEPES buffer to 10^6 cfu/mL.

2.2.2. Bacterial live/dead assay using flow cytometry

Flow cytometry experiments were performed using a BD LSR Fortessa from BD Biosciences (San Jose, CA, USA), which is equipped with 3 lasers (violet—405 nm, blue—488 and red—640 nm), forward and side scatter detectors and 9 fluorescence emission detectors (530/30 and 695/40 channels were used for Syto-9 and PI detection, respectively). The Live/Dead kit assay is composed of two fluorescent dyes, Syto-9 and PI. Syto-9 translocates cell membranes being able to complex with bacterial DNA; in contrast, PI is only able to interact with bacterial DNA if the lipid bilayer is damaged, indicating that PI-positive bacteria are fragilized or dead. The kit assay used to evaluate the antimicrobial activity of pepR on *E. coli* was obtained from Life Technologies (L7012) and bacterial stains were performed according to the manufacturer's instructions. Briefly, 1.5 μL of both Syto-9 and PI was added at each test tube with 1 mL of bacterial suspension (10^6 cfu/mL). For laser calibration purposes and gating selection, unstained and single-stained Syto-9 or PI Live and Dead bacteria were also recorded, and a standard curve for bacterial death detection with Live/Dead staining was plotted (Fig. 1) [2,14].

2.2.2.1. Steady-state flow cytometry. For the pepR antimicrobial activity assays against 25922, K-12*wt* and JW3596-1, concentrations of pepR up to 20 μM were added to 1 mL of a bacterial suspension at 10^6 cfu/mL labeled with both Syto-9 and PI. The mixture was incubated for 1 h, and 15,000 events were recorded from each solution (three independent assays were performed for each condition). The percentage of dead bacteria in each solution was calculated according to Eqs. (1) and (2) in Section 3.

2.2.2.2. Time-resolved flow cytometry. In the kinetic acquisition mode, the Syto-9 and PI channels of the 10^6 cfu/mL bacterial suspensions were recorded for 60 min immediately after addition of 20 μM pepR (Fig. 2, Figs. S1 and S2). The average fluorescence intensities $\langle I_{f,g}\rangle$ and $\langle I_{f,r}\rangle$ of the events recorded at each time point, t, was calculated. Due to AMP activity, as bacterial permeabilization progresses, the average fluorescence signal of Syto-9 ($\langle I_{f,g}\rangle$) decreases and $\langle I_{f,r}\rangle$ increases [15] (Fig. 2B). The ratio between both signals (R, Eq. (2)) can be directly correlated to the % of cell death if one normalizes for a 0%–100% ($a - b$) fluorescence ratio interval (Eq. (1)). Performing this procedure over time, one calculates the evolution of the percentage of bacterial death over time induced by the antibiotic (Fig. 2C), $R(t)$. The $R(t)$ of a bacterial suspension with no addition of pepR was also calculated to account for natural bacterial death during the 60 minutesmin of experiment and for additional unspecific fluorescence signal background correction. During the 60 min, the number of viable cells did not change (Supp. Fig. S3 – —green circles and Supp. Video S1).

2.2.3. Zeta-potential of bacteria

Zeta potential studies were performed in a Zetasizer Nano ZS (Malvern instruments, Worcestershire, UK) equipped with a 633-nm HeNe laser using disposable Zeta cells with gold electrodes. The protocol used was adapted from Alves et al. [11] to ensure optimal measurement conditions for the different *E. coli* strains. Briefly, the bacteria cells were suspended in filtered HEPES buffer at 10^7 cfu/mL, dispensed into the disposable zeta cells and allowed to equilibrate for 15 min at 25 °C before measuring. The experiments were carried out in two different days using independently grown cultures ($n > 3$). Measurements were repeated in the same conditions with the addition of 20 μM pepR.

2.2.4. Data analysis

The experimental values of % dead bacteria are calculated from Eq. (1) and plotted over time. Eqs. (3) and (4) are then combined to fit the data and retrieve k_0, k_2 and f. These parameters contain information on cooperativity (f), kinetics of binding of the antibiotics to bacterial targets (k_0) and kinetics of killing (k_2). Data fitting analysis was performed with GraphPad Prism software v5.0. Mean ± SD from 3 independent experiments for each condition is represented. The validation of the fitting models was achieved by an extra sum-of-squares F-test in order to choose the simplest model to describe the antimicrobial action against the bacteria tested ($p < 0.01$) between fittings with Eqs. (3) and (S12) (see supporting information for more detail). The goodness of the fit was analyzed with R^2, sum-of-squares and residual plot analysis with normality test (D'Agostino-Pearson test with $p < 0.0001$) (Supplementary Section 3, Fig. S3).

3. Theory

Syto-9 is a green-fluorescent (510–540 nm) nucleic acid stain that is able to penetrate both healthy and damaged bacterial cells. After incubation with Syto-9, virtually all bacteria emit green fluorescence upon excitation with blue light enabling counting total cells. In contrast, PI is unable to penetrate healthy bacteria. Its red emission (620–650 nm) is only associated to permeabilized bacteria (Fig. 1A). When both dyes are present in the same cell, the emission of Syto-9 is decreased due to the displacement of one stain by the other and apparent quenching by Förster resonance energy transfer (FRET) [15]. The emission spectra of bacterial suspensions may be integrated in the 510–540 nm and 620–650 nm intervals and the ratio of the corresponding values is compared to a calibration curve obtained with isopropyl alcohol-killed bacteria to calculate the percentage of permeabilized bacteria [15] (Fig. 1B). Although this assay provides useful information on antibiotic efficacy, it does not enable retrieval of kinetic information. Application of these principles to flow cytometry is currently based on steady-state conditions [15] and dependent on user-defined population gating [16]. Nevertheless, flow cytometry allows the analysis of thousands of single events in minutes and detects the evolution of a spectroscopic signal over time [17–20]. Despite this potential, the application of flow cytometry to kinetic studies has been overlooked and the applications described in the literature are limited to the detection of Ca^{2+} intracellular flux [20]. We have used the time-resolved capabilities of flow cytometry to evaluate bacterial susceptibility to antibiotics over time using the fluorescent dyes Syto-9 and PI. Briefly, when a cell passes through the excitation beam, the following data is registered: time since the experiment started, fluorescence intensity in detector 1 ("green," 530/30 BP), fluorescence intensity in detector 2 ("red," 695/40 BP) and forward/side scatter (Fig. 2A). The data are grouped according to regular time intervals (typically 1 s),