HIGH-THROUGHPUT MASS MEASUREMENT OF SINGLE BACTERIAL CELLS BY SILICON NITRIDE MEMBRANE RESONATORS

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ABSTRACT

We present a technological approach to precisely measure the dry mass of many individual cells of a bacteria colony. In this technique, bacteria are transported from aqueous solution into gas phase and subsequently guided to the surface of a silicon nitride membrane resonator. Abrupt downshifts in the membrane eigenfrequencies are measured upon every bacterium adhesion and are related to the dry mass of the cell by theoretical methods. We measure the dry mass of *Escherichia coli* K-12 and *Staphylococcus epidermidis* with an unprecedented throughput of 20 cells/min and with a mass resolution of ~1%. Finally, we apply the Koch & Schaechter model to assess the intrinsic sources of growth stochasticity.

KEYWORDS

Nanomechanical mass spectrometry, high-throughput and high-precision membrane resonators, mass sensing, Koch & Schaechter model, bacteria growth stochasticity.

INTRODUCTION

In spite of decades of research, how bacteria are able to maintain their size remains an open question. Cell populations are intrinsically heterogeneous due to stochastic and deterministic mechanisms [1]. The heterogeneity of cell populations plays a fundamental role in the biological function. In order to understand the mechanisms of bacteria size regulation, techniques that can measure individual cells in populations are required. The dry mass, the mass of the non-aqueous content of the cell, is a particularly valuable parameter to quantify the size regulation of bacteria. It provides information of the amount of proteins, nucleic acids, carbohydrates and lipids present in a cell [2]. Despite its importance, the precise and rapid quantification of the dry mass of single bacterial cells remains technically challenging. Techniques such as quantitative phase imaging (QPI), microchannel resonators and mass spectrometry (MS) have been developed to measure the dry mass of single cells. QPI assumes a constant value for the ratio between the refractive index and the biomolecular mass density of cells [3]. While this ratio is similar for most globular proteins, it can vary by almost 20% for carbohydrates or lipids. However, the major limitation of QPI is the optical spatial resolution and phase noise, which limits the precision for measuring the dry mass of small cells below 1 μm, such as bacteria. Microchannel resonators measure the buoyant mass of suspended cells at sub-femtogram accuracy. The buoyant mass is the total mass of the cell minus that of the fluid displaced by the cell. Determination of the dry mass of the bacteria requires measuring the same bacterial cell in two fluids of different density, which is technically complex and limits the throughput of the assay [4]. Finally, MS approaches have measured the mass of individual biological particles, previously ionized. For instance, charge-detection MS (CDMS) simultaneously measure the charge and the mass-to-charge ratio of single ions with masses up to hundreds of MDa [5]. However, mass resolution decreases for large ions.

Nanomechanical mass spectrometry (NMS) has emerged as the only technique that can directly measure the mass of single biological entities from the nanometer to the micrometer scale, namely proteins, viruses and bacterial cells without requiring the measurement of the charge state of the ions [6]-[10]. In this technique, analytes are gently ionized from aqueous solution into gas phase by electrospray ionization (ESI) and guided to the surface of micro- and nanomechanical resonators, while retaining its original native structure as much as possible. When the analyte adheres to the resonator, abrupt downshifts in the resonance frequencies of the normal vibration modes of the resonator are measured, and can be related to the mass of the analyte. The fundamental limitation of the technique is the detection efficiency, defined as the ratio between the total nebulized particles and the particles that accrete on the resonator. This limitation comes from the trade-off between the resonator size and the minimum detectable mass. Smaller resonators are more sensitive but also dispose a smaller capture area. Here, we propose the use of ultrathin membrane resonators ($400\times350\times0.05\ \mu m$) for the measurement of the dry mass of bacterial cells. These structures present a high mass resolution together with a high capture area. Additionally, we have developed an inverse theoretical algorithm to determinate the dry mass of individual bacterial cells from the resonance frequency downshifts of several vibration modes. Finally, we have obtained the dry mass distributions of two bacteria species: *Escherichia coli* K-12 and *Staphylococcus epidermidis*.

RESULTS AND DISCUSSION

Nanomechanical mass spectrometer

The nanomechanical mass spectrometer comprises four differential pressure stages (Figure 1a, left). In the first

stage, a high voltage (2-3 kV) is applied to a solution with the bacterial cells to generate a Taylor cone at the electrospray ionization (ESI) source, which is at atmospheric pressure. The bacterial cells travel through the second stage at 10 mbar, where a capillary inlet operated at 150 °C promotes desolvation. Subsequently, the cells are transferred to an aerolens tube designed to laminarize the underexpanded gas flow. Numerical simulations by finite element method (FEM) show that the bacteria injected with supersonic speeds (600-700 m/s) into the bore of the aerolens are decelerated to a few tens of m/s inside the laminarized flow (Figure 1a, right). A pair of skimmer-shaped lens electrodes are used to focus the bacterial cells onto the surface of the membrane resonator, which is at the fourth pressure stage, at 0.1 mbar. The soft-landing of our instrument enables to retain the native conformation of the bacteria in the resonator surface, as confirmed by scanning electron microscope (SEM) images (Figure 1b).

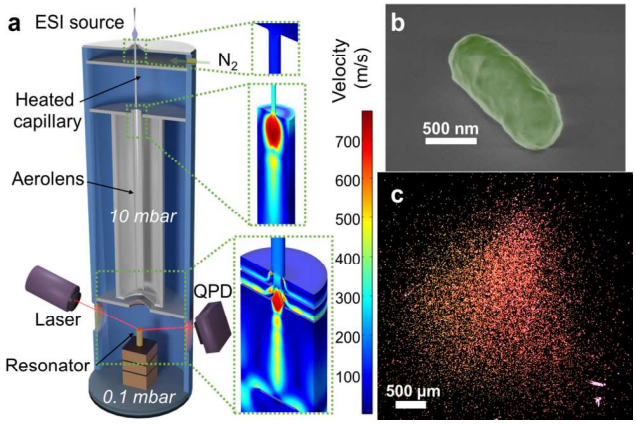

Figure 1: Nanomechanical mass spectrometer. a) Schematic of the instrument (left) and numerical simulation of the flow speed by FEM (right). The speed of the cells is very similar to the flow speed. b) Scanning electron microscopy image of an E. coli bacterium on a silicon surface. c) E. coli bacterial cells over a silicon surface placed at the resonator position.

Figure 1c shows a dark-field optical microscope image of *Escherichia coli* K-12 cells over a silicon surface placed at the resonator position. The full width at half maximum (FWHM) of the cells distribution is of about 2.2×1.7 mm for *E. coli* cells and 1.6×1.2 mm for *S. epidermidis* cells [10]. The transmission efficiency, defined as the ratio between the number of nebulized particles and the number of cells that reach the resonator stage, is of about 0.03%, which is similar to previous works [7][9]. Moreover, the capture efficiency, defined as the ratio between the effective sensing area of the resonator and the cross section area of the cells beam at the resonator position, is of about 5% [10], which represents an increase of two orders of magnitude with respect to previous NMS works in vacuum conditions [7][9].

Membrane nanomechanical resonators

The nanomechanical resonators used in this work are silicon nitride rectangular membranes with side lengths of L_x = 400 µm and L_y = 350 µm, and thickness of 50 nm (Figure 2a). The experimental vibration mode shapes can be described by linear elasticity theory (equation 1) [10].

$$\psi_{ij}(x,y) = 2\sin\left(\frac{i\pi}{L_x}x\right)\sin\left(\frac{j\pi}{L_y}y\right) \quad (1)$$

where *(i,j)* refers to the number of antinodes in *x* and *y* directions, respectively.

We have measured the resonance frequencies of six vibration modes simultaneously by phase-locked loop (PLL) systems and driving the resonator by a piezoelectric actuator placed underneath the membrane. In particular, these modes were (1,1), (2,1), (1,2), (3,1), (1,3) and (3,2). The spectra of these resonances are shown in Figure 2b, with frequencies of about 470.4, 714.6, 771.6, 996.6, 1104.2 and 1169.6 kHz and Q-factors of about 1500, 2700, 3000, 3000, 3500 and 4100, respectively.

The motion of the membrane resonator was measured by the laser-beam deflection technique. This technique is sensitive to the slope of the vibration modes shapes of the membrane [11]. Figure 2c show the squares of the theoretical partial slope of the beam displacement along the *x*-direction of the six vibration modes, respectively. In order to measure the six vibration modes simultaneously with optimal signal-to-noise ratios, the laser beam must be focused in one of the regions indicated by Figure 2d. Figure 2d represents the product of the square of the six partial slopes represented in Figure 2c. In Figure 2a, we show the four regions (red points) where our laser was arbitrarily focused to measure the six resonance frequencies. The frequency sensitivity of the membrane resonator may be estimated by the Allan deviation. The minimum detectable mass, calculated from the Allan deviation of the (1,1) vibration mode, is of about 0.7 fg (1 fg=10^{-15} g) for a particle located at the center of the membrane [10].

The rectangularity of the membrane, with an aspect ratio of about 1.14, makes the resonator different enough from a perfectly square membrane with aspect ratio of one to avoid frequency degeneration. It plays an important role in the frequency density of the vibration modes, since the pairs *(i,j)* and *(j,i)* split into two close, but uncoupled, modes. The frequency ratio between the modes (3,2) and (1,1), seventh and first modes in frequency respectively, is only of about 2.5, which facilitates the tracking of a large number of vibration modes simultaneously.

Measurement of the mass heterogeneity of cells

The adsorption of a particle in a highly tensioned membrane causes changes in the kinetic energy due to the added mass of the particle m_p, while the potential energy is negligibly affected by the particle's stiffness [10]. Thus, the relative frequency shift of a vibration mode due to a particle on the membrane at position (x_0, y_o) is given by equation 2 [10].

$$\frac{\Delta\nu_{ij}}{\nu_{ij}} = -\frac{m_p}{2M}\psi_{ij}(x_0,y_0)^2 \quad (2)$$

where M is the mass of the membrane resonator and $\Delta\nu_{ij}$ is the frequency shift induced by the cell.

A real time record of the fractional changes of the frequency of the selected vibration modes of the membrane during ESI of *S. epidermidis* cells is shown in Figure 3a. The landing events of individual bacterial cells cause abrupt 'jumps' in the tracked eigenfrequencies. The inset of Figure 3a shows a close-up view to 60 seconds of the measurement, where more than 30 jumps can be seen,

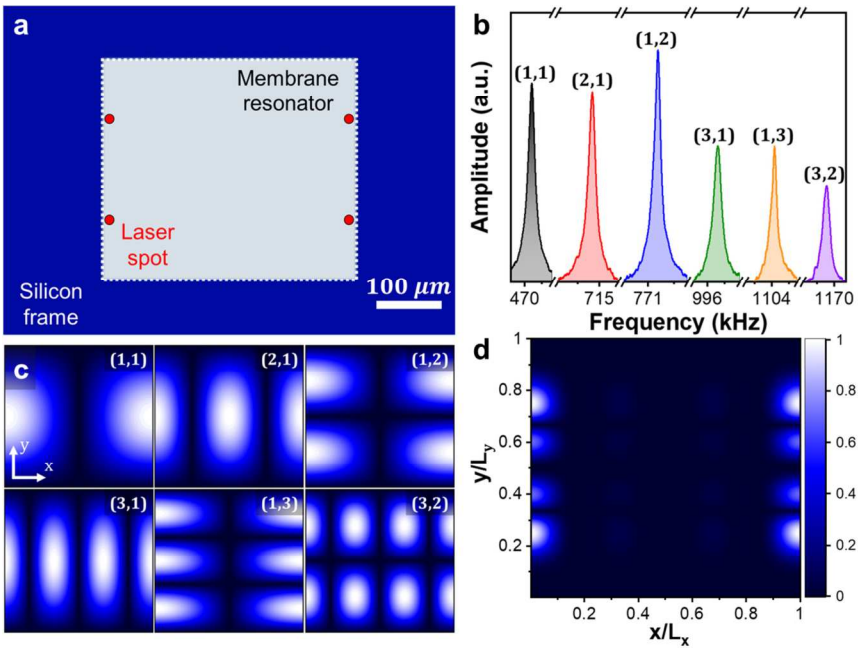

Figure 2: Silicon nitride membrane resonators. a) Optical microscope image of the resonator (400×350×0.05 μm) where the red points indicate the optimal measurement positions. b) Frequency spectra of the vibration modes (1,1), (2,1), (1,2), (3,1), (1,3) and (3,2). c) Squares of the theoretical partial slopes along x direction of the six vibration modes, respectively. d) Product of the square of the six partial slopes, which indicate the optimal positions for the laser beam.

which translates into around a jump each 2 seconds.

From the tentative events of *S. epidermidis* adsorptions of Figure 3a, represented by frequency shifts, about 70% are considered as valid events. We consider a valid event when: i) the obtained position of the particle by our inverse problem method is distanced more than 6% of the membrane length from the physical clampings and ii) the Pearson correlation coefficient between the experimental frequency 'jumps' and the square of the eigenmodes at the highest probability particle position is greater than 0.9. Further details about the inverse problem method used in this work can be found elsewhere [10].

Figure 3b shows the evolution of the normalized probability distribution function (PDF) of the dry mass as a function of the number of *S. epidermidis* cells detected. The final PDF of the dry mass has a mean and a standard deviation of 153±69 fg. The mass resolution of our measurements has a median of 3 fg, which can determine variations of 2% in the mass of single *S. epidermidis* cells.

We have also examined the dry mass heterogeneity of the bacterial cells. Here, we consider the model of cell division processes proposed by Koch and Schaechter [12]. In this model, the deterministic distribution for the mass of the steady-state populations of exponentially growing cells is given by $\theta(m)=m_d/m^2$, for $m_d/2 \leq m \leq m_d$ and zero otherwise, with m_d the mass at the instant of division. Equation 3 defines the effect that the stochasticity of the cell division has on the mass distribution.

$$\theta(m) = \frac{A}{m^2}\int_m^{2m} g(x)dx \qquad (3)$$

where A is a normalization constant and $g(x)$ is the probability density of the mass of the cells at division.

In Figure 4, we apply the Koch and Schaechter model to the mass PDF of *S. epidermidis* cells (in yellow) and of

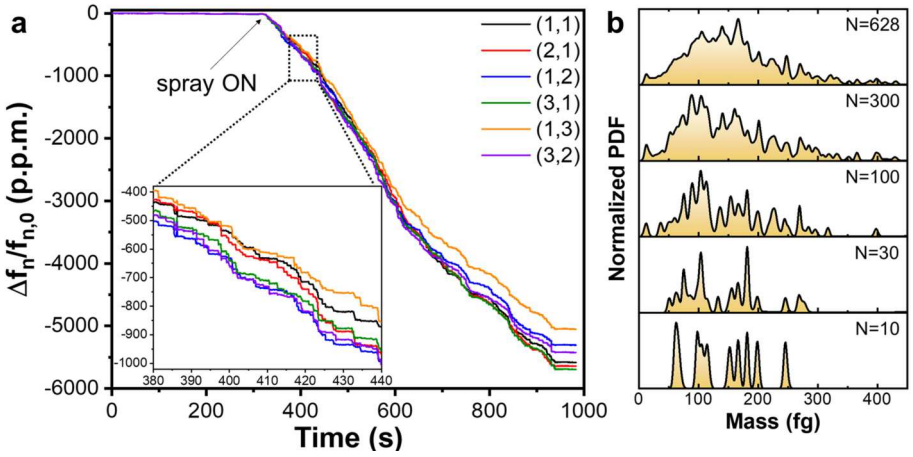

Figure 3: Nanomechanical spectrometry of S. epidermidis cells. a) Real time record of the fractional changes of the frequency of the selected vibration modes (inset: close-up view to 60 seconds of the measurement). b) Evolution of the normalized probability distribution function of the dry mass for the first 10, 30, 100, 300 and 628 cells.

E. coli K-12 cells (in green). The mass PDF of *S. epidermidis* is constructed with the 628 events of Figure 3b and the mass PDF of *E. coli* K-12 is constructed with 685 events. We have assumed normal distribution for the division mass of Equation 3 to obtain a coefficient of determination R-squared of about 0.96 for both bacteria species [10]. Koch and Schaechter model allows inferring the mean dry mass for both bacterial cells: 153 fg for *S. epidermidis* and 469 fg for *E. coli* K-12. The coefficient of variation can be also measured and is 0.46 for *S. epidermidis* and 0.37 for *E. coli*.

Koch and Schaechter model provides the mean mass at the instant of division of 248 fg for *S. epidermidis* cells and 763 fg for *E. coli* K-12 cells, and a CV of the division mass of 0.31 and 0.27 for the bacteria species, respectively.

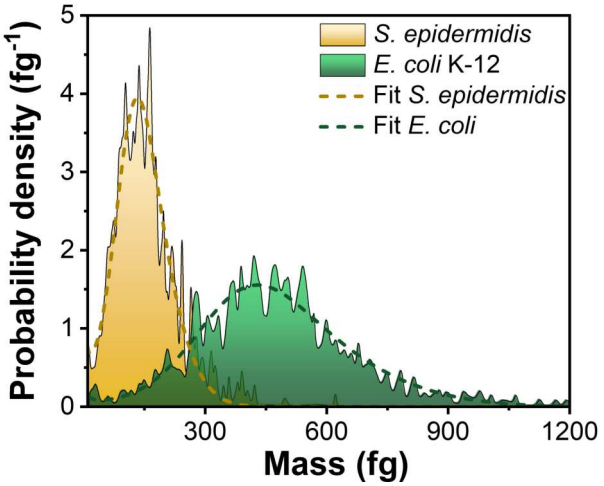

Figure 4: Stochasticity of the dry mass of the cells. Probability distribution functions of the mass of S. epidermidis cells (filled curve in yellow), N=628 events, and E. coli K-12 cells (filled curve in green), N=685 events, measured by our silicon nitride membrane resonators. The dashed lines represent the fitting to the Koch and Schaechter model, which leads to infer growth stochasticity parameters.

CONCLUSIONS

Cell growth and cell division are stochastic processes, and the mechanisms of cell size regulation are still not clear. Here we present a technique that can measure the dry mass of bacterial cells with high efficiency. Our membrane resonators can measure the dry mass of cell populations in a time span of few minutes with an unparalleled mass resolution of about 1%. We have applied the Koch and Schaechter model to obtain important parameters of the stochasticity of the cell growth. The extraordinary performance of our resonators leads to a better understanding on the mechanisms behind cell growth.

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