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Single-Cell Pathogen Detection with a Reverse-Phase Immunoassay on Impedimetric Transducers

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Abstract

The risk of infectious diseases has compelled some industries to establish a zero-tolerance standard for the presence of microorganisms in a given sample. Here, we address this issue with a novel reverse-phase immunoassay on impedimetric transducers for the specific detection of extremely low numbers of pathogens (less than 10 cells). After simply spotting the sample onto the electrodes, physisorbed analytes were targeted with urease-labeled antibodies, and the urease on the pathogens hydrolyzed urea to ionic species with a concomitant decrease of the resistivity of the solution. By this methodology, the limit of detection (LOD) based on the 3σ criterion was 1 *Escherichia coli* cell with an assay time under 1 h. However, the precise number of cells present in highly diluted samples is uncertain, making it difficult to assess the final LOD of the sensor. We overcome this problem by using an atomic force microscope to deposit and image in situ the exact number cells on the transducer. After performing the immunoassay, a single *E. coli* cell was successfully detected without ambiguity in the number of cells even in the presence of a 10^4 excess of a competing microorganism, thus demonstrating the outstanding LOD and selectivity of the proposed reverse-phase immunoassay.

The social alarm of infectious disease propagation is pushing the analytical community hard to develop suitable methodologies for the detection of the extremely low number of pathogens in complex samples.^{1–5} The rapid growth rate of these hazardous microorganisms makes it mandatory to find the detection system that can specifically determine the presence of a target pathogen before it multiplies and becomes a severe health risk, in some cases with the requirement of assessing its total absence to satisfy zero-tolerance policies.⁶ To fulfill this objective, such a system should be not only fast but also robust and easy to use so that the detection could be accomplished without any tedious sample pretreatment, performed in-field, and easily accessible to unskilled users. Nowadays, the detection of a single pathogen cell relies on the use of time-consuming cell culturing and PCR protocols, which are not suitable in decentralized studies for the rapid determination of pathogens. Alternatively, new approaches based on the use of nanosensors have demonstrated outstanding sensitivity by detecting a single cell in a shorter period of time;^{7–12} however, the use of a nanometer-sized transducer makes it mandatory to use complex microfluidics to direct the analyte to the sensing part of the device, and their use in complex samples is still to be tested.

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SUPPORTING INFORMATION AVAILABLE

Electrode layout, single cell deposition with DPN, simulations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Here, we introduce a new approach for the determination of the extremely small number of pathogens in complex samples based on the combination of an easy-to-use reverse-phase immunoassay with the outstanding sensitivity of an enzyme-amplified impedimetric transduction mechanism. In the reverse-phase configuration, the sample is directly spotted onto a suitable substrate and dried so that all the materials present in the drop remain physisorbed to the surface; subsequently, the presence of the target analyte is detected by incubating the substrate with specific antibodies (Figure 1). This approach, formerly proposed for the detection of ultralow amounts of protein in cell lysates,¹³ is perfectly suited for its application in sensors, since it simplifies the sensor fabrication and analytical procedure by circumventing the immobilization of the biorecognition element to the transducer and reducing the incubation steps to just one. In the present work, *Escherichia coli* cells are manually spotted onto polysilicon interdigitated transducers and, after drying, their presence is detected by using urease-labeled antibodies. Upon addition of the urea enzyme substrate, the urease bound to the pathogens by the immunoreaction hydrolyzes the molecule to ionic species, thus inducing a concomitant decrease of the resistivity of the solution between the electrodes.¹⁴ The electric field lines generated by the interdigitated transducers are mostly confined in the region extending a few micrometers over the electrodes, thus making these transducers particularly sensitive to the variations of the electrical properties of the solution occurring in this region.^{14,15} As a result, the local generation of ions by urease is efficiently detected by the sensor, which is able to detect a single *E. coli* cell even in the presence of a vast excess of competing microorganisms with an assay time under an hour, thus proving its usefulness for the point-of-care application. The demonstration of this ultimate LOD and selectivity is a serious issue, since the use of inaccurate methods for cell quantification along with the error associated to the serial dilution of the sample makes it difficult to assess the exact number of cells in highly diluted solutions. Here, we overcome this problem by using an atomic force microscope (AFM) to deposit and image in situ the exact number of cells onto the transducer, so that the immunoassay can be performed without ambiguity in the number of cells present on the electrodes. By using this so-called dip-pen nanolithography approach (DPN),^{16,17} the sensor performance is comparable to the manual spotting procedure, thus validating the use of the reverse-phase immunoassay on impedimetric transducers for the detection of the extremely low number of pathogens.

EXPERIMENTAL SECTION

Preparation of Cells Solutions

Bacterial cells were centrifuged at 10,000 rpm for 5 min, and the resulting pellet was suspended in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH 7.4 tablets, Sigma). Sterile disposable materials were used for the manipulation of cell suspensions. Buffers were prepared with ultrapure water and filtrated with 0.2 mm diameter polypropylene porous membranes. Glass materials were acid-clean. The concentration of the resulting cell solutions was determined with a McFarland standard.

Conjugation of Urease to Antibody

Urease was conjugated with anti-*E. coli* by the following procedure. The buffer in the goat polyclonal anti-*E. coli* (1 mg/mL, Abcam) was exchanged with a desalting column (P10, Sephadex) for a phosphate buffer (PB pH 7, 10 mM). The protein-rich fractions were identified by measuring the absorbance at 280 nm. Then, sulfo-SMCC (4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt, Sigma) was added to set the final concentration to 1 mg/mL. After the overnight incubation at 4 °C, non-reacted sulfo-SMCC was removed by using the P10 desalting column. The protein-rich fractions were mixed with Jack Beans urease (10 mg/mL, Sigma) and incubated at 4 °C

overnight. The resulting urease-labeled antibodies were aliquoted and stored at -20°C before their use.

Reverse-Phase Immunoassay

Polysilicon interdigitated electrodes with a finger width of $3\text{ }\mu\text{m}$ and a pitch of $13\text{ }\mu\text{m}$ were used as impedimetric transducers. The fabrication and characterization of the electrodes has been published elsewhere.¹⁸ The electrode layout is available in the Supporting Information, Figure S1. Cells were serially diluted in the PBS solution to the desired concentration, and a $0.5\text{ }\mu\text{L}$ drop was manually spotted onto the interdigitated area of the electrodes. After drying for 5 min, the sensors were incubated in a BSA-PBS solution (bovine serum albumin, 50 mg/mL) for 15 min to avoid non-specific adsorption of biomolecules in subsequent recognition steps. Then, the sensors were incubated with urease-labeled anti-*E. coli* in the PBS solution (1:10 in volume) for 30 min. This dilution was selected to maximize the sensitivity of the detection step (maximal analyte signal with minimal background signal). Subsequently, these sensors were washed with a PBS-Tween solution (0.1%), followed by rinsing with a glycine buffer solution (50 mM). Next, they were immersed at a fixed position in a beaker containing the glycine buffer, which was stirred with a magnetic stirrer at a fixed velocity. Finally urea was added to the desired final concentration every 30 s, and the variation of the resistivity due to the enzymatic reaction was monitored by measuring the real part of the impedance at 12.6 kHz .¹⁸

Modification of AFM Tips with *E. coli* Cells

Gold-coated AFM tips were modified with thiolated polyethylene glycol molecules (1 mg/mL in absolute ethanol, MW 5000, Nektar). After overnight incubation, they were washed with fresh ethanol and dried with the help of a filter paper. This treatment is meant to assist releasing the pathogens smoothly during subsequent deposition steps. The tips (Tap300GB, Budget Sensors) had a nominal radius smaller than 10 nm , and resonant frequency and force constant were around 300 kHz and 40 N/m , respectively. After a solution of *E. coli* cells ($2\text{ }\mu\text{L}$, 10^7 cells/ml) in PBS was spotted onto the tip and let dry to force the deposition of the cells, the tips were thoroughly washed with deionized water and the excess solvents on the tip were removed with a filter paper.

Dip-Pen Nanolithography Approach

After modification with *E. coli* cells, AFM tips were loaded on the holder of a stand-alone MFP-3D AFM (Asylum Research, Santa Barbara, CA). First, the surface of the sensors was imaged in the AC mode of AFM. The term “AC mode” is an alternative designation of the Tapping Mode. Then, by using the MicroAngelo software the pathogen on the AFM tips were transferred to the edges of electrodes in the contact mode with the force of 300 nN and the maximum tip velocity of $0.5\text{ }\mu\text{m/s}$. Subsequently, the same area was imaged again in the AC mode to confirm the number of pathogens immobilized on the electrodes. AFM images were treated with WSxM software (Nanotec Electronica) to enhance the contrast.¹⁹

RESULTS AND DISCUSSION

To theoretically demonstrate the suitability of the interdigitated transducers to detect a single pathogen, the effect of one *E. coli* cell covered by urease onto the electrodes was simulated (details about the simulations are available in the Supporting Information). The spatial distribution of ionic species generated by the enzyme bound to the pathogen via immunoreaction and the variation of resistance measured by the electrodes were taken into account. Under agitation, a laminar flow on the chip surface is produced (Supporting Information, Figure S2), and a stable concentration pattern generated by transport (diffusion and convection) of the products of the enzymatic reaction is quickly settled. As shown in Figure

2a, the ion concentration next to the electrode surfaces is the maximum around the pathogen and extends laterally because of the presence of the laminar flow. This characteristic ionic fingerprint results in a variation of the resistivity of the solution in this region that yields an increase of the density of currents when the electrodes are excited at constant voltage amplitude (Figure 2, panels b and c). Working under substrate saturation conditions and considering a perfect coverage of the cell surface by urease, a theoretical decrease of the resistivity of 0.2 k Ω , as compared with the initial situation, is obtained by simulation. This value is well above the variation observed for blank experiments in absence of the enzyme,¹⁷ and therefore, it suggests that the proposed impedimetric immunoassay could be adequate for the determination of a single pathogen bound to the surface of the electrodes.

Next, the capability of the reverse-phase immunoassay on impedimetric transducers for the detection of low pathogen numbers was experimentally tested. The sample containing the *E. coli* cells was serially diluted, and drops containing different cell numbers from 0 to 7 were spotted onto different transducers, where they quickly dried (Figure 1a). Since the area covered by the drop was smaller than the interdigitated area of the transducer (2 mm², see electrode layout in the Supporting Information), after drying, all the materials present in the drop are forced to interact with the sensing part of the chip. In Figure 1b, the randomly distributed physisorbed cells are targeted with urease-labeled anti-*E. coli*; upon addition of the enzyme substrate, the production of ionic species by urease results in a local decrease of the resistivity that can be monitored by measuring the real part of the impedance (Z') at a proper frequency, which corresponds to the resistance of the solution between the electrodes (Figure 1c).¹⁸ Figure 3a shows a representative example of the sensor response to solutions containing either 1 or 0 *E. coli* cells. From the slope of the linear part of these plots, the increase in the enzyme activity with respect to the zero point was plotted as a function of the number of cells present in the drop (Figure 3b). Since the number of surface-bound enzymes is proportional to the number of bacteria via the recognition of conjugated antibodies, the increase of the enzyme activity is consistent with the increased *E. coli* numbers on each electrode. The whole procedure was repeated three times on different sensors and for different *E. coli* solutions to study the variability of the assay. By this methodology, the limit of detection defined as three times the standard deviation of the blank signal was 1 *E. coli* cell.

In Figure 3b, several factors are expected to have an impact in the variability of the assay. On one hand, ambient factors such as temperature drift and the unspecific adsorption of the enzyme to the electrode surface can randomly alter the signal measured by the transducer. On the other hand, the exact number of cells present in the drops used to calibrate the sensor is unknown, and therefore there is always an error associated to the x -axis of Figure 3b that cannot be assessed and has a great impact on the response of such a sensitive sensor. Even if the transducers were imaged with a microscope after the cell deposition step, it would be difficult to be absolutely certain about the number of cells of randomly distributed pathogens, since the pathogens could be mistaken for impurities present on the surface before the spotting procedure. As a consequence, the statement of the single-cell detection is compromised unless the exact number of cells present on the sensor is known. To overcome this issue and unambiguously determine the detection of a single pathogen, instead of the manual spotting procedure we used a dip-pen nanolithography approach to deposit and image in situ the exact number of *E. coli* cells at the transducers' surface. Figures 4 a–c illustrate the procedure for the controlled deposition of bacterial cells by DPN. By this approach, the bacterial cell was deposited as ink (Figure 4d) on the electrode with an AFM cantilever for a pen. The electrodes digits have a thickness of 500 nm.¹⁸ In the contact mode of the AFM, the friction between the AFM tip and the edge of the electrode digits could be used to release one *E. coli* cell (Figure 4c and Supporting Information, Figure S2), and this single cell deposition step was confirmed by detecting the typical rod-shape of *E. coli*¹⁹ in the AC mode of the AFM (Figure 4f). To make sure that the imaged object is the result of the deposition step, the same area was imaged

with the same tip and immediately after the DPN process. By repeating this procedure, *E. coli* cells were placed one by one to precisely control the number of cells on the surfaces of respective transducers. By this methodology, transducers with 1, 2, 3, or 5 *E. coli* cells were prepared, and the immunoassay was performed on these sensors as above (Figure 5a). Each sensor was prepared and measured on a different day, and therefore a blank experiment without *E. coli* was performed for each sensor measurement. From these measurements, the signal of a single *E. coli* cell was well above three times the standard deviation of the blank signal, and this result validated the proposed transduction mechanism on impedimetric transducers for the detection of a single pathogen cell. Moreover, results obtained by DPN in Figure 5a were within the variability of the results obtained by using the manual spotting procedure in Figure 4b, thus indicating that the major contribution to the error in Figure 4b was the uncertainty in the number of cells during the calibration procedure.

Although the use of DPN could unambiguously demonstrate the detection of a single cell, in real samples coabsorption of complex matrixes could have an influence on the detection limit of the sensor. To simulate a complex matrix, we examined control experiments for detecting *E. coli* at the single cell level in the presence of a vast excess of an interfering microorganism, *Salmonella typhimurium*. Both *E. coli* and *S. typhimurium* are gram-negative bacteria with similar antigenic characteristics, thus providing an excellent test for determining the specificity of the immunoassay. A single *E. coli* cell was deposited on different sensors by DPN as shown in Figures 3a–c, and then drops containing 10^2 , 10^3 , or 10^4 cells of *S. typhimurium* were added as contaminants onto these electrodes where they quickly dried. By this procedure, there is no uncertainty in the number of *E. coli* cells bound to the sensor, and therefore variations in the sensor response can be unambiguously related to the presence of the competing microorganism. Subsequently, the enzyme-conjugated antibody for *E. coli* was incubated to the set of electrodes, and the impedance responses were measured after triggering the enzymatic reaction. In Figure 5b, the transducer response remained close to the single cell level even under the interference of highly concentrated *S. Typhimurium*, and this result indicated that the specificity of the impedimetric reverse-phase immunoassay could be high enough to selectively detect a single pathogen in complex matrixes.

In the present sensing configuration there are two crucial factors for achieving such outstanding LOD and selectivity. First, urease only generates the impedance signal for the target pathogen via the specificity of the conjugated antibody. Second, the dimensions of the interdigitated transducer and the applied frequency were tuned to be more sensitive to the variation in the electric properties of the solution occurring in the region near the surface, but not limited at the solution–electrode interface.^{14,21} Measurements of electrical parameters at the electrode interface tend to introduce instability and interference from contaminants and microorganisms that adsorb non-specifically to the surface. However, our detection system is not so susceptible to these factors since we measure the variation of the resistivity of the solution between the electrodes, which only depends on the concentration and mobility of charged species in the solution. Since contaminants on the electrode surface do not produce any ions to significantly change the resistivity, it is consistent with the proposed detection mechanism that these contaminants do not have a great impact on the signal detected by the transducer as shown in Figure 5b.

CONCLUSIONS

In summary, simulations and experimental data demonstrated that a single *E. coli* cell could be detected by interdigitated impedimetric transducers, even in the presence of a vast excess of a competing microorganism. The capability of interdigitated electrodes to locally detect changes in the resistivity of the solution, along with the specificity of the antibody for the target and the efficient and selective generation of the signal by the enzyme, were responsible for the

outstanding LOD and selectivity of the assay. The adoption of a reverse-phase configuration could simplify the analytical procedure by directly spotting the sample onto the transducer to bind the analyte, and by using only one antibody for targeting the cells, as compared to former sandwich-based proposals. In contrast with other approaches for a single pathogen detection based on the use of nanosensors, this simplified procedure could be performed because the interdigitated transducers exhibited excellent sensitivity with a relatively large sensing area, which enabled the sample deposition via the manual spotting procedure. Furthermore, the adaptation of the DPN technique for depositing and in situ imaging the *E. coli* cells onto the electrodes could confirm the final performance of the sensor without ambiguity in the number of analytes, which is a common problem when assaying highly diluted solutions. The dynamic range of the assay could be increased by serially diluting concentrated samples and measuring each dilution with an array of electrodes. The outstanding LOD and selectivity of this easy-to-use, rapid immunoassay, along with the easy mass fabrication of the transducer, make this sensor an appealing alternative for those in-field sensing applications where a zero-tolerance standard of pathogen detection has to be met.⁶

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Figure 1.

Scheme of the reverse-phase immunoassay on impedimetric transducers. (a) A drop containing the pathogens is spotted onto the interdigitated area of the electrodes, where it quickly dries. (b) Physisorbed cells are targeted with urease-labeled antibodies. (c) Urease catalyzes the conversion of urea to ionic species, and the solution resistivity between the electrodes decreases.

Figure 2.

(a) Simulated distribution of the ionic species around the pathogen, at 1 μm from the sensor surface (Z axis). Vertical stripes represent the interdigitated electrodes. Arrows indicate the direction of solution flow. (b) Current density in a plane orthogonal to the flow direction and for a voltage of 1 V across the electrodes in the absence of the pathogen. (c) Current density at the cell position in the presence of the pathogen upon triggering the enzymatic reaction.

Figure 3.

(a) Change in the real part of the impedance (Z') with the concentration of urea after addition of 1 *E. coli* cell (black, $y = -0.062x + 0.04$, $r^2 = 0.97$) or no cells (red, $y = -0.037x + 0.06$, $r^2 = 0.89$). (b) Increase of the enzyme activity detected after *E. coli* cells were bound to the sensor surface from 1 to 7 cells with the serial dilution of the sample, and those cells were targeted by enzyme-conjugated antibodies (reverse-phase immunoassay). Error bars are the maximal and minimal values obtained from three different electrodes modified with three different *E. coli* solutions. Standard deviation of the blank: $6 \Omega mM^{-1}$ ($n = 3$).

Figure 4.

Deposition and imaging procedure of individual *E. coli* cells one at a time via the DPN technique. (a) *E. coli* are manually spotted onto an AFM tip. (b) The AFM tip is moved to the edge of the electrode. (c) A single *E. coli* is released at the edge in the contact mode. (d) A SEM image of an AFM tip containing *E. coli* for the step (a). Scale bar: 2.5 μm . (e) An AFM image (topography) of the microelectrode surface in the AC mode. Scale bar: 600 nm. (f) An AFM image (topography) of a single *E. coli* cell attached at the edge of the electrode in the AC mode (marked with red arrows). Scale bar: 600 nm.

Figure 5.

(a) Increase of the enzyme activity detected after *E. coli* cells were bound to different sensors from 1 to 5 cells with DPN and targeted with enzyme-conjugated antibodies. Standard deviation of the blank: $7 \Omega \text{ mM}^{-1}$ ($n = 4$). (b) Increase of the enzyme activity for a single *E. coli* cell in the presence of 10^2 , 10^3 , and 10^4 *S. typhimurium* cells. Red dotted lines indicate the signal for 1 or 2 *E. coli* cells in the absence of *S. typhimurium* cells.