

Lytic enzymes as selectivity means for label-free, microfluidic and impedimetric detection of whole-cell bacteria using ALD-Al₂O₃ passivated microelectrodes

N. Counio^{a*}, T. Vanzieleghe^b, J. Rasson^a, N. Van Overstraeten-Schlögel^a, O. Poncelet^a, J. Mahillon^b, L. A. Francis^a, D. Flandre^a

^aICTEAM Institute, Université catholique de Louvain (UCL), Place du levant 3-L5.03.02, 1348 Louvain-la-Neuve, Belgium

^bELI Institute Applied Microbiology (ELIM), Université catholique de Louvain (UCL), Croix du Sud 2-L7.05.12, 1348 Louvain-la-Neuve, Belgium

Abstract

Point-of-care (PoC) diagnostics for bacterial detection offer tremendous prospects for public health care improvement. However, such tools require the complex combination of the following performances: rapidity, selectivity, sensitivity, miniaturization and affordability. To meet these specifications, this paper presents a new selectivity method involving lytic enzymes together with a CMOS-compatible impedance sensor for genus-specific bacterial detection. The method enables the sample matrix to be directly flown on the sensor surface without any pre-treatment, and considerably reduces the background noise. Experimental proof-of-concept, explored by simulations and confirmed through a setup combining simultaneous optical and electrical real-time monitoring, illustrates the selective and capacitive detection of *Staphylococcus epidermidis* in synthetic urine also containing *Enterococcus faecium*. While providing capabilities for miniaturization and system integration thanks to CMOS compatibility, the sensors show a detection limit of ca. 10⁸ (CFU/mL)*min in a 1.5 μL microfluidic chamber with an additional setup time of 50 min. The potentials, advantages and limitations of the method are also discussed.

Keywords: Real-time biosensing; Impedance spectroscopy; Interdigitated microelectrodes; Whole-cell bacteria; Lytic enzymes; Urinary infections

1. Introduction

Recently brought to huge medical interest, especially in the struggle against antibiotic-resistant bacteria, lytic enzymes are molecules that specifically digest the cell wall of most Gram-positive bacteria. They can be produced by either bacteriophages or bacterial cells. In the first case, bacteriophage lytic enzymes, also called *endolysins*, break bonds in the thick cross-linked peptidoglycan to enable phages to inject their genetic material in their host to infect it (Fischetti, 2005). In the second case, bacteria produce their own lytic enzymes, also called *autolysins*, which are tightly controlled and necessary for cell wall rearrangements during cell division. Finally, in some cases, evolution has driven bacteria to develop lytic enzymes to eliminate species competing for a specific environmental niche. This is notably the case for *Staphylococcus simulans*, which produces lysostaphin, a peptidoglycan hydrolase active against almost all *Staphylococcus* species.

Besides their medical relevance, lytic enzymes and bacteriophages have also proven to be powerful tools to achieve high-selective impedimetric detection of bacteria (Zourob and Ripp, 2010), in a growth-dependent or -independent way. In the first case, bacterial growth monitored through ion release is strongly affected in a culture media containing phages, since target bacteria are continuously digested (Chang et al., 2002). In the growth-independent method, phages are immobilized on the electrode surface and subsequently capture and digest bacteria in a selective way, releasing ions in the outer medium that are monitored by the impedimetric sensor (Shabani et al., 2008). Another electrochemical technique detects peptidoglycan fragments, products of the bacterial lysis, that have specifically bound on an antibody layer grafted on the sensor surface (Yoon et al., 2013).

For Point-of-Care (PoC) diagnostics, growth-independent impedance detection provides a lower detection time. Despite essentials for Lab-on-Chip (Loc) systems, compactness, simplicity and

autonomy remain challenging for the following reasons. First, most studies report gold microelectrodes hardly compatible with the Complementary Metal-Oxide-Semiconductor (CMOS) process. To benefit from low-cost, system integration and miniaturization capabilities of CMOS, microelectrodes can be patterned in the last CMOS metal layer and covered with an $\sim 0.1\ \mu\text{m}$ -etched (Ghafari-Zadeh et al., 2008; Wang and Lu, 2010) or $\sim \mu\text{m}$ -intact (Prakash and Abshire, 2007; Romani, 2004) passivation layer. However, this thick oxide layer causes a massive drop in sensitivity.

Secondly, grafting a biorecognition layer, e.g., lytic enzymes, bacteriophages or antibodies, on the sensor surface typically faces problems such as reproducibility, uniformity and stability over time (Van Overstraeten-Schlögel et al., 2014). Although already complex, the functionalization protocol further needs to be adapted to every surface material and grafting molecule. In addition, the biorecognition layer is subject to nonspecific bindings in complex samples, requiring dedicated negative controls.

Thirdly, the largest sensitivity of bacterial impedance sensors is typically achieved in low-salt buffers of electrical conductivity close to $100\ \mu\text{S/m}$, such as 0.1% peptone water (Radke and Alocilja, 2005), 100 mM mannitol solution (Suehiro et al., 2006; Varshney, 2007), or even in deionized water (Yang, 2008). Resuspension of bacteria in these buffers is then typically performed but requires at least three centrifugation steps to remove most ions contained in the initial matrix, which is time-consuming ($> 1\text{h}$) and requires lab equipment incompatible with PoC and LoC applications. Furthermore, great care should be taken in the result interpretation, since observed shifts can either be due to bacterial cells or the presence of remaining ions from the initial buffer.

In this paper, three notable innovations are brought together to solve the aforementioned problems for direct and selective detection of bacteria towards PoC and LoC applications. First, a new selective method based on lytic enzymes was developed. Free of selective surface functionalization and centrifugation steps, the whole procedure is then simple, straightforward and reproducible. In short, once the sensor surface is totally covered with various bacteria, sensor levels before and after the application of specific lytic enzymes are compared; if different, some target bacteria have been lysed and the sample can be qualified positive. We demonstrate this concept with a selective detection of *S. epidermidis* in synthetic urine, also containing *E. faecium* as a negative control, to mimic urinary infections.

Second, CMOS-compatible microelectrodes, made in aluminum and covered with a very thin layer of atomic-layer-deposited (ALD) Al_2O_3 , have been used. The 30 nm-thick Al_2O_3 layer protects the underlying aluminum from corrosion and has a moderate impact on bacterial sensitivity, compared to a micrometer-sized oxide layer. As a 80 nm electrochemically-anodized Al_2O_3 layer has previously been demonstrated for bacterial detection in dried conditions (Tang et al., 2011), this paper extends the use of this type of passivated electrodes to detection in liquid.

Finally, an innovative setup combining simultaneous real-time optical and electrical monitoring of sensor was built. Compared to most studies where electrical shifts are interpreted without optical control, this original setup provides a way to accurately link electrical phenomena to surface events such as binding of bacterial cells and considerably decreases the risk of misinterpretations.

2. Materials and methods

2.1. Micro-fabrication and encapsulation of the interdigitated microelectrodes (IDE)

Fig. 1 sketches the process flow. First, 3-inch Pyrex wafers were immersed in a freshly-prepared Piranha solution ($\text{H}_2\text{O}_2:\text{H}_2\text{SO}_4$, 2:5) during 10 min for cleaning, followed by two continuously renewed immersions in DI water baths during 20 min. Afterwards, aluminum was evaporated in a rotate mode to deposit a $1\ \mu\text{m}$ -thick layer. A first optical lithography with positive photoresist then provides finger masking during the subsequent Al plasma etching. After removing resist, the whole wafer was covered with 33 nm of plasma enhanced ALD Al_2O_3 at room temperature (RT) with trimethylaluminum and oxygen as precursor. The argon flow through plasma source was 200 sccm and the oxygen flow for the plasma step was 30 sccm during 20 sec. Afterwards, a second optical lithography with positive photoresist was used to define pad area and etch the subsequent Al_2O_3 layer

after 15 sec immersion in IPA:HF 70% (3:1). Finally, a last optical lithography with negative photoresist KMPR 1025 was used to define 30 μ m-thick walls to support the microfluidic cap. The interdigitated microelectrodes (IDE) are 2 μ m-wide, 4 μ m-spaced, 1 μ m-thick and define a 300 μ m-diameter circle (Fig. 2a-b).

For the microfluidic cap, a 270 μ m-thick KMPR 1050 layer was patterned by optical lithography on a clean 3-inch silicon wafer. This mold was then covered with a thin hexamethyldisilazane (HMDS) layer to easily take off the polydimethylsiloxane (PDMS) cap subsequently. Inside a Petri dish, the PDMS was then flown on the mold wafer and incubated at 60°C overnight. To ensure the watertight sealing of the system, a transparent pressure tool was used (Fig. 2c). As the PDMS cap is pressed on the chip, the whole microfluidic channel including inlets and outlets is 1 mm-wide, 5 mm-long and 300 μ m-thick, resulting in a total channel volume of 1.5 μ L, while the immediate volume above the 300 μ m-diameter sensor is 21 nL.

2.2. Chemical and reagents

Solutions and buffers used in this study were: Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Lysogeny broth (LB), synthetic urine, polydopamine solution, 1 μ M lysostaphin solution, 0.01 M phosphate buffer saline (PBS) and PBS diluted 1:10, PBS 1:100, PBS 1:1000 by volume in DI water, respectively. Detailed protocols to prepare these solutions are available in Supplementary Information.

2.3. Bacterial samples

S. epidermidis ATCC 35984 and *E. faecium* ATCC 19434 were used as reference strains for this study. Detailed protocols for culture, resuspension and CFU counting are available in Supplementary Information.

2.4. Experimental setup and impedance measurements

The device was positioned on an inverted microscope (DMI6000, Leica, Belgium) enabling real-time imaging of the sensor surface during electrical measurements (Fig. 2c). Samples were flown through the microfluidic channel by a peristaltic pump. An impedance analyzer (LCR 4284A, Agilent, USA) was connected to electrical probes and remotely controlled through LabVIEW® to perform an automatic sweep from 100 Hz to 1 MHz, at voltage amplitude of 50 mV. Before impedance measurement, an open calibration was performed by positioning electrical probes above aluminum pads, without any electrical contacts.

Once electrical probes contacted to pads, the sensor was first rinsed and measured in PBS 1:1000 at 125 μ L/min, during 10 min at RT. Then, the polydopamine solution was incubated during 30 min at dark condition, without flow and electrical measurements. Afterwards, the sensor was washed with PBS 1:1000 during 5 min at 250 μ L/min followed by 5 min at 125 μ L/min under electrical measurements to reach a constant value. At this point, the sensor is ready to be experimented with bacterial cells. But, as they are contained in PBS 1:1000 or in synthetic urine, two different protocols must be considered (Sections 2.4.1 and 2.4.2).

2.4.1. Real-time detection of *S. epidermidis* in PBS 1:1000

Before flowing bacterial suspensions, PBS 1:100, PBS 1:10, PBS and PBS 1:1000 solutions were successively flown at 125 μ L/min during 10 min and for each, reference measurements were performed. Suspensions from 10⁶ to 10⁹ CFU/mL of stationary-state *S. epidermidis* resuspended in PBS 1:1000 were then injected at 1 μ L/min, each followed by the same washing procedure with sterile PBS 1:1000 as previously described. Finally, PBS 1:100, PBS 1:10 and PBS were flown again under electrical measurements to extract shifts in different conditions of salinity, but with exactly the same number of adherent *S. epidermidis* on the sensor surface, as verified by our optical setup.

2.4.2. Selective detection of *S. epidermidis* in synthetic urine, in the presence of *E. faecium*

For this experiment, the suspension of *E. faecium* (negative control) in synthetic urine was first introduced at 1 $\mu\text{L}/\text{min}$, followed by a 10 min wash with PBS 1:1000 as previously described. These two steps were repeated with exponential-state *S. epidermidis* (target) contained in synthetic urine, for experiments requiring both species on the sensor. Finally, lysostaphin was incubated 30 min at 1 $\mu\text{L}/\text{min}$, followed by a final wash with PBS 1:1000. To assess reproducibility, three totally independent experiments were performed for both negative controls, i.e. sensors with *E. faecium* only, and target samples, i.e. sensors with *E. faecium* and *S. epidermidis*.

2.5. Automatic bacterial counting algorithm

Based on microscope images, a MATLAB® program was implemented to automatically count the number of attached bacteria on the sensor surface. A mask was first obtained after black and white (B&W) conversion and electrode dilatation, compensating the blur effect on IDE edges. Using the mask, the electrode region was set to a zero value and the whole image was converted to B&W with an optimal threshold to distinguish bacteria. Finally, the number of pixel clusters was automatically counted.

3. Results and discussion

Impedance results are displayed in terms of the normalized admittance Y/ω [F], with Y [S] the sensor admittance, i.e., the inverse of impedance, and $\omega=2\pi f$ [rad/s] the angular frequency. The capacitance denotation is not used because the impedance phase is slightly different from -90° . Before investigating selectivity to *S. epidermidis* in synthetic urine, real-time detection of *S. epidermidis* resuspended in the low-salt buffer PBS 1:1000 is first investigated to optimize sensor performances. In all experiments, a polydopamine layer, known as biological glue (Lee, 2007), covers the sensor surface (Al_2O_3) to increase and homogenize bacterial binding.

3.1. Real-time detection of whole-cell *S. epidermidis* resuspended in PBS 1:1000

3.1.1. Shifts and slopes in real-time

During the initial wash of the polydopamine-covered sensor with sterile PBS 1:1000, the normalized admittance Y/ω showed stability and exhibited no drift (Fig. 3a). Once samples of *S. epidermidis* resuspended in PBS 1:1000 were flown on the sensor, Y/ω systematically increased by three successive and different mechanisms (Fig. 3a). The first is an immediate shift Δ_c [F], observed just after bacterial injection and attributed to the slight difference in ionic content, i.e., electrical conductivity, between sterile PBS 1:1000 and bacterial resuspension in PBS 1:1000. Indeed, centrifugation steps lead bacterial cells to release ions due to osmotic pressure and damaged cell walls (Peterson et al., 2011). Despite its dependence on bacterial concentration, two reasons make Δ_c unsuitable for bacterial sensing. First, Δ_c is strongly affected by experimental procedures such as manipulation, contamination and temperature, all affecting the baseline sample conductivity (see the artifact on 10^6 CFU/mL in Fig. 3a). The use of a reference conductimeter within the microfluidic channel could solve this problem, but increases the system complexity. Second, Δ_c is useless for real applications dealing with saline solutions, whose high electrical conductivity is hardly impacted by bacterial ion release. For these reasons, Δ_c -based sensing should be avoided.

During the next 20 min bacterial incubation, Y/ω was shown to continuously grow with increasing bacterial surface coverage, defining the real-time slope $s_{\pi} = \partial(Y/\omega)/\partial t$ [F/min] (Fig. 3a, dotted blue linear curve). Thanks to simultaneous optical and electrical measurements, the direct link between s_{π} and the bacterial real-time binding was assessed (Fig. S1a). Furthermore, a linear dependence between

s_{rt} and the bacterial concentration is highlighted in Fig. 3b. The related “real-time shift” Δ_{rt} [F] was extracted as the difference between Y/ω values after the incubation phase and after the initial conductivity peak. Its linear dependence on the bacterial density [$\#/mm^2$] was quantified to 319 aF per adherent bacterium, resulting from the presence of both adherent and non-adherent, but close to the sensor surface, bacterial cells (Fig. 3c). However, both s_{rt} and Δ_{rt} are strongly reduced in more saline bacterial samples for reasons described in Section 3.1.4.

After the bacterial incubation, a wash with sterile PBS 1:1000 flushed non-adherent bacteria and excessive ions away, so that Y/ω stabilized at a lower equilibrium value (Fig. 3a). This level minus the pre-incubation Y/ω value in sterile PBS 1:1000 defines the “shift after wash” Δ_w [F]. A linear dependence between Δ_w and the bacterial surface coverage was experimentally evaluated to 101 aF per adherent bacterium (Fig. 3c) and confirmed by simulations in Fig. S1b (Couniot et al., 2013). In contrast to Δ_{rt} , only the remaining adherent bacteria on the sensor affect Δ_w , justifying its smaller value. On the other hand, Δ_w is highly stable and reliable since it purely originates from dielectric properties of bacterial cell only. Indeed, as measured only at equilibrium under flow with sterile PBS 1:1000, Δ_w is not subject to ionic release or contamination. In addition, wash procedures can be added as using saline bacterial samples to recover Δ_w . For these reasons, next discussions are thus only based on Δ_w .

3.1.2. Limit of detection

The intrinsic limit of detection (LOD) of surface-based sensors is defined as the minimum number of adherent bacteria required on the sensor surface to generate a signal Δ_w five times larger than the noise σ_n . It is possible to express the LOD in term of the bacterial concentration C_b [CFU/mL], which can be linked to the non-saturated bacterial density S_b [$\#/mm^2$] by the following formula, for a 20 min incubation time: $\log_{10}(S_b) = -3.25 + 0.92 \cdot \log_{10}(C_b)$ (Fig. 3b). As the noise source from the electrical readout was characterized to $\sigma_n = 1$ fF in optimal conditions, approximately 50 bacteria on the sensor surface are therefore needed to have $\Delta_w > 5 \cdot \sigma_n$, corresponding to a bacterial density of 707 bacteria per mm^2 , i.e., 0.06% of surface coverage. From the previous formula, the LOD is approximated to 10^8 (CFU/mL)*min, which means either 10^8 CFU/mL within 1 min of bacterial incubation, or 10^7 CFU/mL within 10 min, or equivalently 10^6 CFU/mL within 100 min.

3.1.3. Effect of buffer salinity

To quantify sensor performance in various conditions of salinity and at a fixed bacterial density of $3 \cdot 10^4$ $\#/mm^2$, pre- and post-incubation washes were performed with various dilutions of PBS, whose electrical conductivity spans from 1.4 mS/m to 1.4 S/m (see Section 2.4.1). For each PBS solutions, the sensitivity $S(\omega) = \Delta_w / (Y_0 / \omega)$ [%] was computed from Δ_w and from the initial normalized admittance Y_0/ω . Its mean $\overline{S(\omega)}$ and standard deviation σ_s were obtained through time averaging of at least 10 successive measurements in steady state. At the frequency maximizing the signal-to-noise ratio $SNR = 20 \cdot \log_{10}(\overline{S(\omega)} / \sigma_s)$ [dB], the sensitivity was shown to decrease from 9% to 2% as the ionic strength increases (Fig. 3d, black bars). Indeed, the electric field gets more confined in surface and the insulator capacitance dominates (see Section 3.1.4 for more explanation). In contrast, maximal sensitivities without SNR consideration comprise extremely large error bars (Fig. 3d, red bars), unsuitable for accurate interpretation. In conclusion, the largest sensitivity is achieved with low-salt washing solutions.

3.1.4. Analytical model and simulations

In absence of bacteria, the sensor impedance can be modeled with the insulator capacitance C_{ins} [F], the double layer (DL) capacitance C_{DL} [F], the solution resistance R_{sol} [Ω] and the solution capacitance C_{sol} [F] (Fig. 4a). Since PBS 1:1000 has electrical conductivity $\sigma_{sol} \approx 1.4$ mS/m and relative

permittivity $\epsilon_{r,sol} = 80$, the Debye length λ_D is approximately 32 nm, resulting in $C_{DL} \approx 10 \cdot C_{ins}$ as the insulator thickness and relative permittivity are $t_{ins} = 30$ nm and $\epsilon_{r,ins} = 9$, respectively. Therefore, C_{DL} can be neglected in series and two cutoff frequencies govern, as confirmed experimentally (Fig. 4b):

$$f_{c,1} = \frac{1}{2\pi \cdot R_{sol} (C_{ins} / 2)} \approx \frac{1}{\pi} \cdot \frac{t_{ins}}{d} \cdot \frac{\sigma_{sol}}{\epsilon_0 \epsilon_{r,ins}} \approx 42 \text{ kHz}$$

$$f_{c,2} = \frac{1}{2\pi \cdot R_{sol} C_{sol}} \approx \frac{1}{2\pi} \cdot \frac{\sigma_{sol}}{\epsilon_0 \epsilon_{r,sol}} \approx 225 \text{ kHz}$$

where d is the electrode gap [m] and ϵ_0 the vacuum permittivity [F/m]. For applied frequencies $f < f_{c,1}$, C_{ins} dominates while C_{sol} prevails at $f > f_{c,2}$. Between these two cutoff frequencies, the impedance is more resistive due to R_{sol} dominance.

With adherent bacterial cells on the sensor surface, the global impedance was experimentally shown to decrease, highlighting increasing capacitive behavior especially at large frequencies where the sensitivity $S(\omega)$ was maximized (Fig. 4c). At such frequencies, the cytoplasm conductance dominates the bacterial impedance Z_b [Ω] and shortens electrical field lines, resulting in larger capacitance due to the smaller path. In contrast, the sensitivity is extremely small at low frequencies since C_{ins} is not affected by bacterial cells. As shown in Fig. 4c, these analytical and experimental considerations were confirmed by finite-element simulations of the 2D model comprising one bacterial cell (Fig. 4a), modeled with a Gram-positive two-shell representation whose dielectric values are given in (Sanchis et al., 2007).

3.2. Specific detection of *S. epidermidis* in synthetic urine

To provide bacterial selectivity in complex samples with lytic enzymes, our method involves five steps (Fig. 5a). First, the matrix sample containing bacterial cells is flown during 20 min on the clean sensor covered with a polydopamine layer. After that, the sensor is washed with PBS 1:1000 to fully remove non-adherent species and enable sensitive and accurate measurements at 1 MHz (see Section 3.1 for justification). Then, lytic enzymes are flown during 30 min atop the sensor and selectively lyse target bacteria, if present on the sensor surface. Then, a second wash with PBS 1:1000 is performed to sweep enzymes away before measuring the normalized admittance Y/ω again. A shift in Y/ω occurs between the two measurements only if target bacteria, if present on the sensor surface, have been lysed. An absence of shift means that the surface state with adherent bacteria is unchanged, i.e., target bacteria were missing from the sensor surface.

Fig. 5b-c depicts the typical evolution of Y/ω at 1 MHz, for both the negative control, i.e., synthetic urine with only *E. faecium*, and the test sample, i.e., synthetic urine with both *S. epidermidis* and *E. faecium*. Only parts in PBS 1:1000 used for wash are shown since low-sensitivity occurs in saline solutions (see Section 3.1.3), whose out-of-range parts do not interfere with subsequent shifts Δ_1 , Δ_2 and Δ_3 measured at equilibrium in PBS 1:1000. As shown by Δ_1 and Δ_2 in Fig. 5b-c, both adherent *E. faecium* and *S. epidermidis* on the sensor surface strongly affect the normalized admittance thanks to their dielectric properties at 1 MHz (see Section 3.1.4). Absolute shifts Δ_1 and Δ_2 are significant compared to the temporal noise computed on at least 10 successive measurements, but vary between the three independent sensors because of the different numbers of adherent bacteria (Fig. 6a). As shifts are normalized by the number of bacteria counted on the sensor surface, *E. faecium* and *S. epidermidis* present reproducible normalized shifts spanning from 52 to 59 aF per bacterium and from 77 to 97 aF per bacterium, respectively (Fig. 6b). The larger sensitivity to *Staphylococci* may be explained by their dielectric properties, probably increased by a higher ionic cytoplasmic content thanks to the heavily cross-linked cell wall.

After the incubation of lysostaphin, i.e., a lytic enzyme that specifically digests *Staphylococcus* spp., sensors with only *E. faecium* on the surface showed a slight decrease $\Delta_3 \in [2 \text{ fF}, 21 \text{ fF}]$ of the normalized admittance, despite intact *E. faecium* cells as observed optically (Fig. 5b and 6a). This

slight drop can be attributed to enzymes binding onto the polydopamine layer, which typically sticks any biomolecules containing aromatic rings. On the other hand, sensors covered with *S. epidermidis* showed a significantly larger decrease $\Delta_3 \in [163 \text{ fF}, 299 \text{ fF}]$ of Y/ω (Fig. 6a), whose larger variability results from the different amounts of lysed *S. epidermidis* on the sensor surface, for each experiment. As Δ_3 is divided by the number of lysed bacteria, a reproducible normalized shift spanning from 68 to 88 aF per lysed *S. epidermidis* is obtained. Despite the almost complete destruction of *S. epidermidis* cells, the sensor does not recover its initial impedance value with *E. faecium* only. Our interpretation is that cellular debris (DNA, proteins and peptidoglycan) remains on the sensor surface after cell lysis and interacts with the electric field to cause measurable shifts in Y/ω .

3.3. Advantages and limitations

The selective method of Section 3.2 presents several advantages among others. First, the matrix can directly be flown on the sensor without any pre-treatment steps such as centrifugation or dilution. The possible presence of large cells (diameter $> 10 \mu\text{m}$) in the matrix is likely not a problem since they should be washed away by strong shear forces at flow rate of $250 \mu\text{L}/\text{min}$, as confirmed optically with large bacterial cell clusters. A filtration method, e.g., membrane at the channel inlet, could alternatively be added to filter them out.

Second, in contrast to affinity-based surface sensors, our selective method does not suffer from background noise due to non-specific binding of biomolecules on the sensor surface, which strongly facilitates its use in real matrix samples. Compared to antibodies, the use of lytic enzymes can be easily extended to all Gram-positive bacteria (using *endolysins*) and strongly reduces cost at a large-scale production, since peptidoglycan hydrolases can be obtained by fermentation. The risk of false negatives due to mutation is also relatively low, because peptidoglycan hydrolases target highly conserved bonds in the bacterial cell wall.

Third, washing with low-conductivity media enables the stable and sensitive monitoring of intrinsic dielectric properties of adherent bacteria, avoiding time-consuming centrifugation required for ion release based techniques. Furthermore, the electrical conductivity of the bacterial sample does not need to be controlled, as it is preferably the case for ion release technique.

Finally, since the electrode materials ($\text{Al}/\text{Al}_2\text{O}_3$) are CMOS-compatible, the method can be extended to microelectrodes patterned in the last metal layer of a CMOS circuit, benefiting from system miniaturization and low manufacturing cost, both essentials for LoC applications.

On the other hand, our method exhibits several limitations. First, great care with PBS 1:1000 wash is needed since ionic contamination can easily occur and provide false positives. The fluidic setup must be optimized to avoid any cross contamination from saline to non-saline buffers.

Second, osmotic-sensitive bacteria may burst during an osmotic shock. In this case, the sensor would provide false positives during the rinsing procedure if burst bacteria are not those targeted. Due to their thick cell wall ($\sim 30 - 50 \text{ nm}$), Gram-positive bacteria are less subject to this phenomenon than Gram-negative bacteria characterized by a 2-8 nm-thick cell wall. To resolve the two aforementioned issues, a higher ionic medium could be used for measurements, but the electrical frequency should be increased accordingly to keep the same sensitivity.

Third, reproducible cell adhesion on the sensor surface is crucial to avoid false negatives, in the case bacteria do not adhere to the surface. To address this problem, a polydopamine layer was used in this work to improve and homogenize bacterial binding in saline and non-saline buffers. Though other coatings could also provide adequate or better results, they should be investigated in a separate study. Furthermore, the proposed method could be combined with well-known concentration techniques such as dielectrophoresis (Suehiro et al., 2006) or magnetic beads (Varshney, 2007), to enhance sensitivity and decrease detection time.

Finally, a comparison with other significant works dealing with impedimetric detection of bacteria in solution is provided in Table S1 and supports our conclusions.

4. Conclusion

In this paper, an innovative selective method for impedimetric detection of bacterial cells was presented. It is based on the use of lytic enzymes to selectively destroy target bacteria, anchored on the sensor surface after the flow of a treatment-free sample matrix. This principle was demonstrated by selectively detecting *S. epidermidis* in synthetic urine samples also containing *E. faecium*, as a model for real urinary infections. This method is a step towards Lab-on-Chip (LoC) and Point-of-Care (PoC) systems, since it enables the direct flow of the sample matrix and the integration with CMOS readout circuit on the same chip. Furthermore, the selectivity principle can easily be extended to all Gram-positive bacteria using bacteriophage lysins, which have similar enzymatic activity as lysostaphin used in this paper. Finally, real-time detection of *S. epidermidis* resuspended in low-ionic buffer was also investigated, enabling key comprehension for sensor optimization. All electrical interpretations were confirmed by real-time simultaneous optical monitoring of the sensor surface thanks to an innovative setup.

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Figures

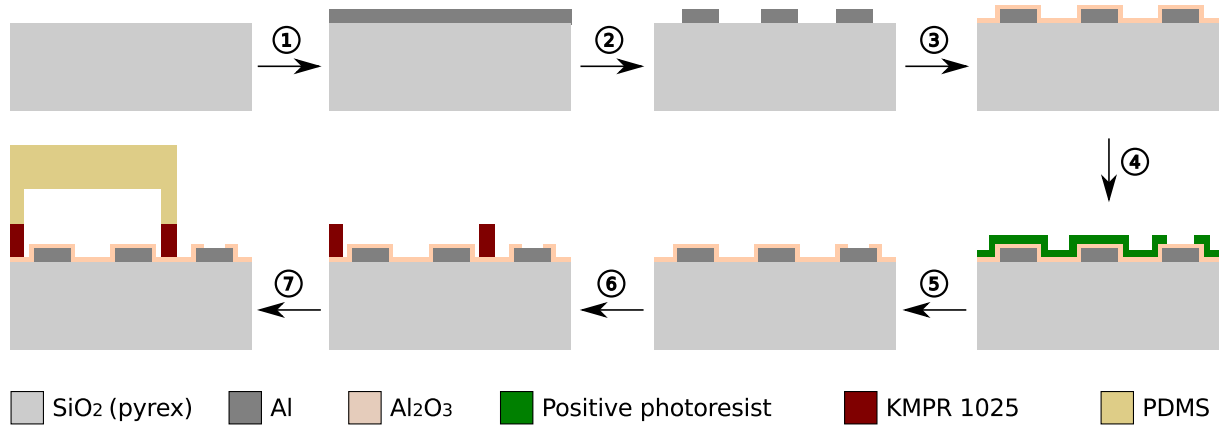


Fig. 1 – Sensor micro-fabrication steps: (1) aluminum deposition, (2) positive optical lithography followed by aluminum plasma etching, (3) deposition of an ALD Al₂O₃ layer, (4) positive optical lithography to open pads, (5) etching Al₂O₃ with IPA:HF 70% (3:1), (6) positive optical lithography to define thick KMPR walls and (7) PDMS cap pressure.

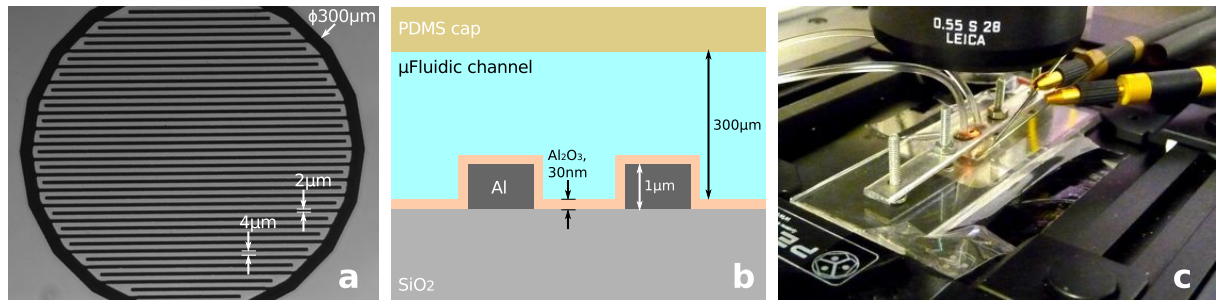


Fig. 2 – (a) Sensor microphotograph, (b) schematic cross-section of the encapsulated sensor and (c) photograph of the encapsulated sensor positioned on an inverted microscope and contacted through electrical probes.

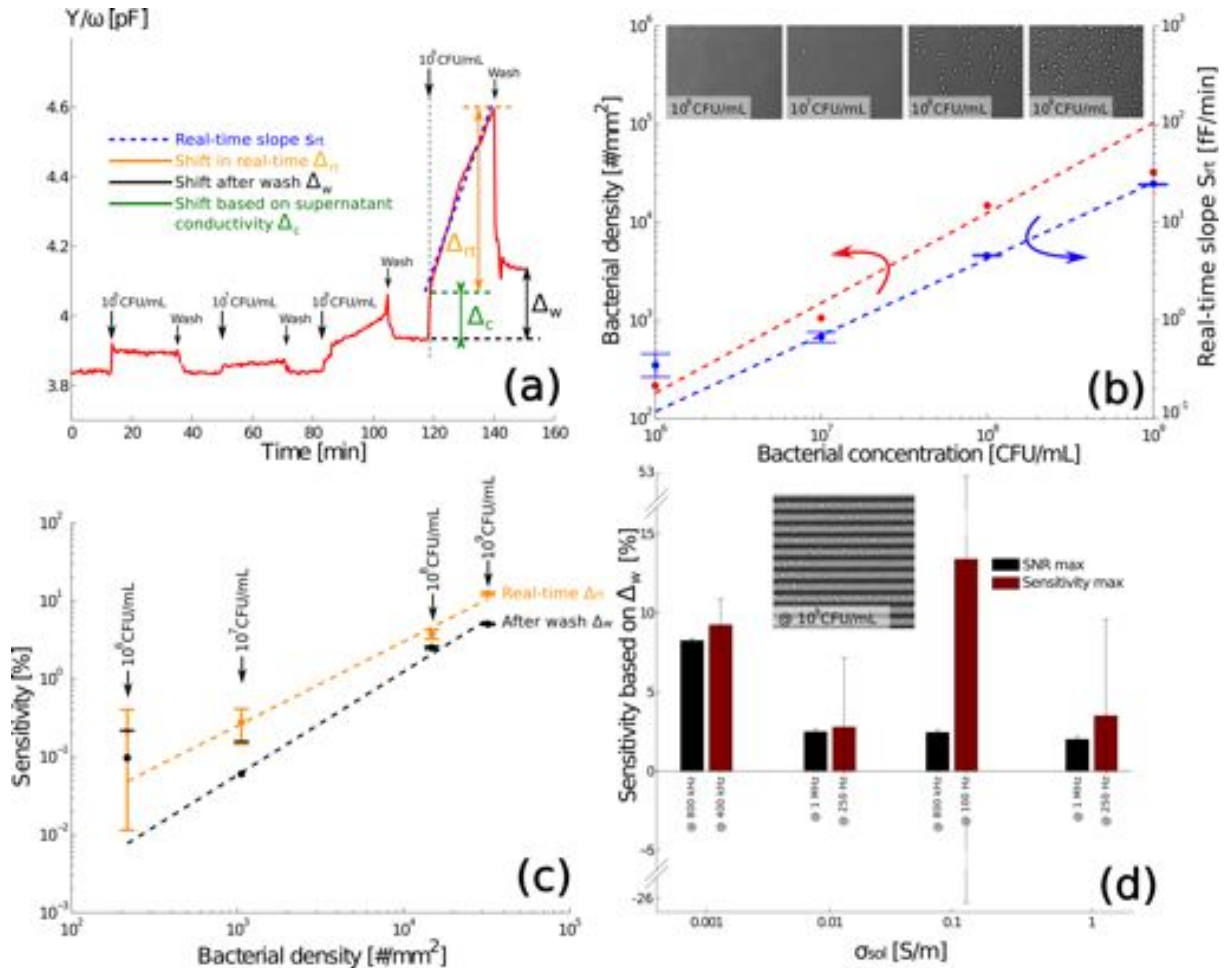


Fig. 3 – (a) Real time monitoring of the normalized admittance Y/ω at 1 MHz as increasing *S. epidermidis* concentrations from 10^6 to 10^9 CFU/mL in PBS 1:1000 are successively injected. (b) Dependence of the bacterial density measured optically after 20 min incubation and the 1 MHz admittance slope s_{rt} with the bacterial concentration. (c) Dependence of the sensitivity, expressed as the relative change of Y/ω in percent, with the bacterial coverage at 1 MHz for different conditions illustrated in (a). (d) Dependence of the maximal sensitivity and the sensitivity maximizing the SNR with the conductivity σ_{sol} of the buffer used for measurements, for the shift Δ_w and at a fixed bacterial coverage of $\sim 3 \cdot 10^4$ #/mm². Error bars express temporal noise computed from at least 10 successive measurements of corresponding shifts at steady state.

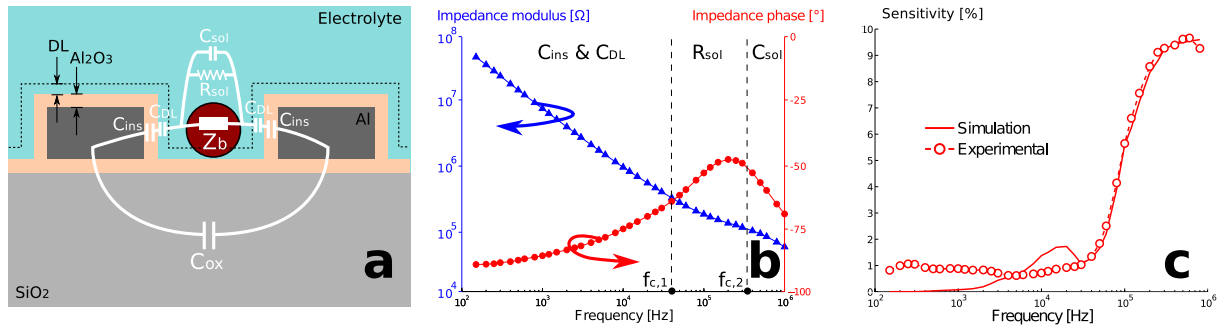


Fig. 4 – Sensor modeling: (a) Equivalent electrical circuit including bacterial cells, (b) experimental impedance modulus and phase in PBS 1:1000 without bacteria versus the applied frequency and (c) comparison between experimental and simulated sensitivity $S(\omega)$ to *S. epidermidis* versus the applied frequency.

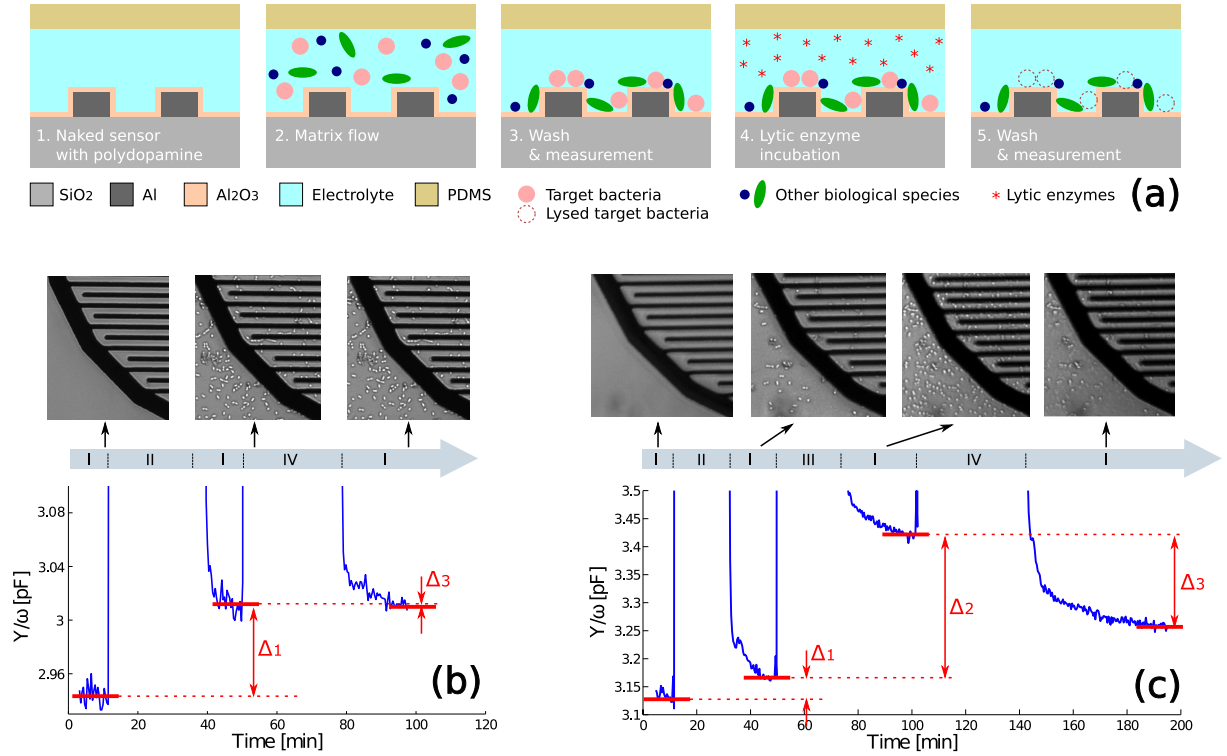


Fig. 5 – Selectivity method: (a) principle scheme, (b) real-time evolution of the normalized admittance Y/ω at 1 MHz for the negative control, *E. faecium* in synthetic urine, and (c) for the target sample, *S. epidermidis* and *E. faecium* in synthetic urine. The events mentioned in the timeline are: (I) washing with PBS 1:1000, (II) incubating 5.10^8 CFU/mL *E. faecium* in synthetic urine, (III) incubating 5.10^8 CFU/mL *S. epidermidis* in synthetic urine and (IV) incubating lysozyme. Shifts after wash Δ_1 , Δ_2 and Δ_3 are evaluated after the addition of *E. faecium*, *S. epidermidis* and lytic enzymes, respectively.

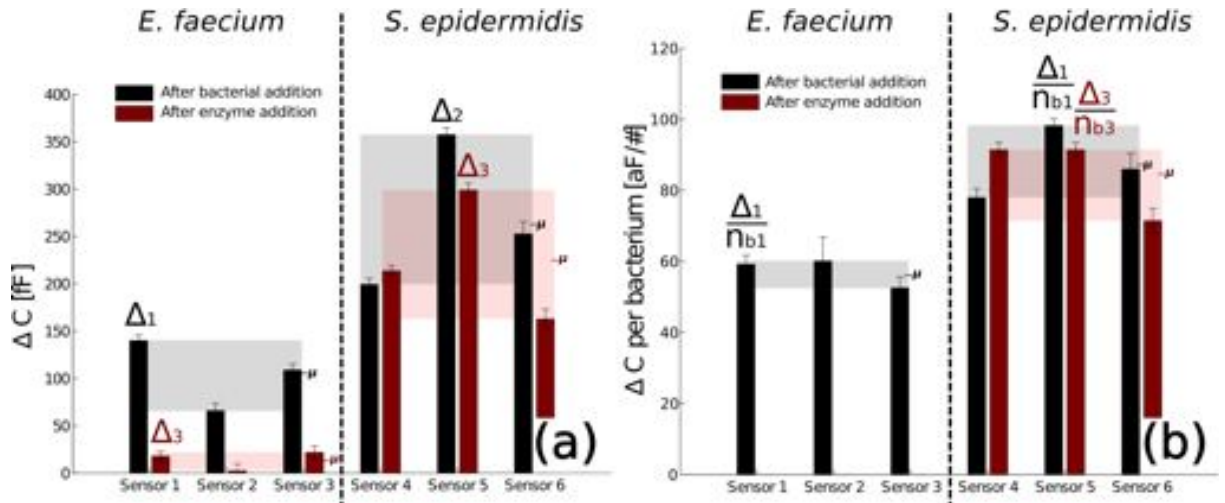


Fig. 6 – Comparison of impedance measurements on *E. faecium* (negative control) and *S. epidermidis* (target) at 1 MHz and for three different sensors in each case: (a) absolute capacitive shifts ΔC after *E. faecium*/*S. epidermidis* binding (in black) and after enzyme incubation (in red), (b) relative capacitive shifts normalized to the number of bound bacteria after incubation or to the number of destroyed bacterial cells after lytic enzyme incubation. Error bars at each block refer to temporal noise obtained after averaging at least 10 successive temporal measurements on one sensor in a steady-state window. Translucent boxes depict the measurement span in a given condition, whose μ corresponds to the mean value of the 3 sensors.