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Interfacial origin of label-free potentiodynamic fingerprints during bacterial growth in blood culture

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KEYWORDS

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ABSTRACT

Bloodstream infections require rapid microbiological diagnosis, yet conventional blood culture primarily detects microbial growth without providing direct information on pathogen identity.

We previously reported a label-free potentiometric platform integrated into blood culture bottles that generates microorganism-specific electrochemical fingerprints, although the physicochemical origin of these signals remained unclear. Here, we investigated whether these fingerprints arise from bulk metabolic changes or from bacteria-electrode interfacial processes. A compartmentalized blood culture system physically separating *Escherichia coli* from the sensing electrodes showed that diffusible metabolites and bulk acidification alone did not reproduce the potentiometric signature observed in the presence of bacterial cells. Time-resolved electrochemical impedance spectroscopy performed at the evolving open-circuit potential further revealed marked interfacial changes coincident with potentiometric detection. At 0.1 Hz, bacterial growth produced decreases in impedance modulus of approximately 125 k Ω and 8 k Ω on polyaniline and iridium oxide electrodes, respectively, together with material-dependent phase changes. These observations indicate that the electrochemical fingerprints primarily reflect dynamic bacteria-electrode interactions rather than bulk pH variations alone. The results support a model involving bacterial adhesion, local metabolic activity and early biofilm-associated interfacial reorganization, and provide a mechanistic basis for label-free electrochemical monitoring of bloodstream infections.

1. Introduction

Bloodstream infections (BSI) remain a major cause of morbidity and mortality worldwide, frequently progressing to sepsis and septic shock when appropriate antimicrobial therapy is delayed^{1,2}. Rapid detection and identification of causative pathogens are therefore critical for patient survival. Conventional blood culture systems rely primarily on indirect measurements

of microbial metabolism, such as CO₂ production, which require several hours before reaching detectable thresholds^{3,4}. Although molecular diagnostic approaches have significantly reduced identification time, they often require complex sample preparation, costly reagents, and prior knowledge of the target organism^{5,6}.

Electrochemical sensing strategies have emerged as attractive alternatives for real-time bacterial monitoring due to their high sensitivity, miniaturization potential, and compatibility with complex biological matrices. Various electrochemical techniques, including amperometry^{7–12}, potentiometry^{8,13}, differential pulse voltammetry^{14,15} and electrochemical impedance spectroscopy (EIS)^{7,16–18}, have been explored to detect microbial growth or biofilm formation. Many approaches rely on surface functionalization with antibodies^{19–24}, aptamers²⁵, or nucleic acid²⁶ probes to achieve selectivity. While effective, these strategies inherently limit multiplexing capability and increase system complexity.

In a previous study published in *Sensors & Actuators: B. Chemical*²⁷, we presented a multi-material potentiometric platform integrated directly into a blood culture bottle (**Figure 1**). The initial objective of this platform was to detect the growth of microorganisms in human whole blood by measuring the pH of the culture medium using polyaniline and iridium oxide electrodes, which are pH-sensitive materials. However, we observed that bacterial growth generates specific electrochemical behaviors in these two materials: the potentiometric measurement yields reproducible, pathogen-specific electrochemical fingerprints (ELFs) that enabled discrimination at Gram-type and genus levels, using machine learning algorithms. Importantly, this approach did not rely on exogenous redox mediators, labeling strategies, or operator intervention.

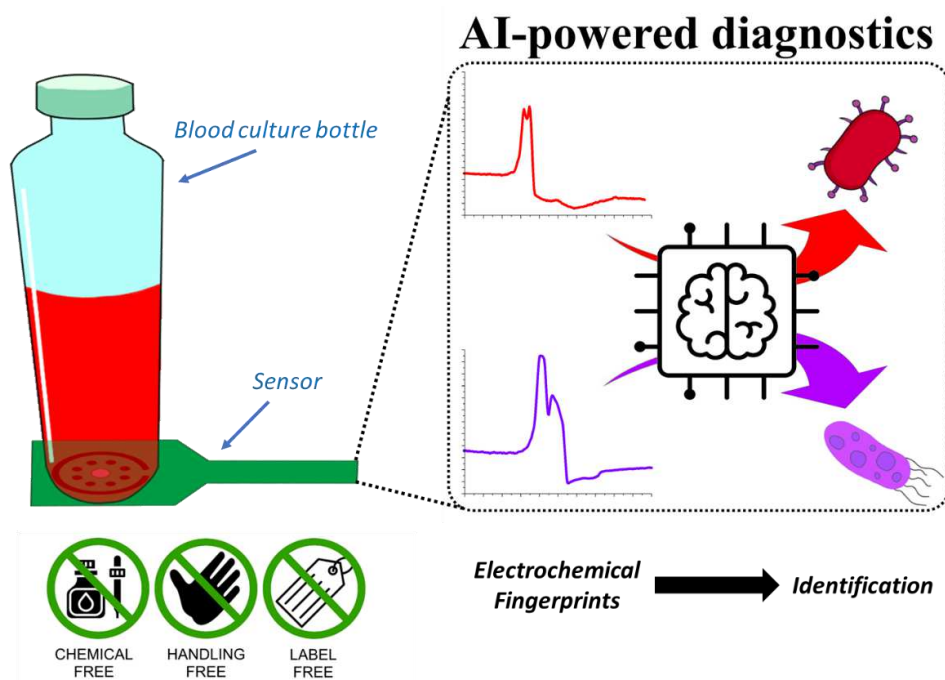


Figure 1. Scheme of the principle of bacterial diagnosis using potentiometry²⁷. The electrochemical fingerprints (ELFs) are specific to the detected bacteria; a machine learning model is trained to recognize these fingerprints in order to automate identification.

Although the diagnostic performance of the system was demonstrated for 14 species and 9 genera, the origin of the potentiometric fingerprints observed is not yet understood. The magnitude and temporal evolution of open-circuit potential variations could not be fully explained by pH changes alone, as electrodes unresponsive to pH generate their own potentiometric response. While bacterial metabolism produces CO₂ and organic compounds capable of modifying solution composition, previous reports suggest that surface-associated phenomena may dominate electrochemical responses during microbial growth^{8,28,29}.

Bacterial adhesion to abiotic surfaces is a well-characterized process involving initial reversible attachment, followed by irreversible adhesion and production of extracellular polymeric substances (EPS), ultimately leading to biofilm maturation³⁰. Biofilms are structurally

heterogeneous assemblies composed of bacterial cells embedded within a hydrated matrix of polysaccharides, proteins, lipids, RNA, and extracellular DNA³¹. Numerous studies have demonstrated that biofilm formation can alter interfacial electrochemical properties, including double-layer capacitance, charge transfer resistance, and mass transport characteristics^{32–34}. For instance, impedance spectroscopy has been widely used to monitor biofilm growth on metallic and carbon electrodes, often revealing depressed semicircles and distributed capacitive behavior indicative of heterogeneous surface coverage^{28,29,35}.

However, most electrochemical biofilm studies are performed under potentiostatic control in well-defined electrolyte solutions, frequently using exogenous redox probes such as ferri/ferrocyanide¹² to quantify charge-transfer resistance and steric hindrance. By contrast, real biological matrices remain highly complex, protein-rich, and dynamically evolving systems in which bacterial proliferation and electrode surface chemistry change simultaneously and timely. Measurements performed at the natural open-circuit potential (OCP) preserve the native thermodynamic state of the system but also complicate impedance interpretation, because the interfacial state follows a temporal evolution associated to the biofilm growth^{29,32,35}.

Recent studies on biofilm impedance have shown that interfacial responses depend strongly on the evolved structure of the biofilm, the frequency domain considered, and the evolving properties of the electrode–biofilm interface. In parallel, a recent work on tuning bacteria–electrode interfaces highlights that the electrochemical signal depends on how microorganisms colonize and interact with the sensing surface³⁶. Even for bacteria that do not perform direct extracellular electron transfer to the electrode, local metabolic activity can alter ionic composition and interfacial polarization, thereby reshaping the measured impedance response.

Based on these considerations, we hypothesized that the electrochemical fingerprints previously observed do not originate solely from diffusible metabolites in the bulk medium, but rather from dynamic interactions between proliferating bacteria and the electrode surface. In particular, we propose that adhesion and early-stage biofilm formation modulate the effective interfacial impedance and contribute significantly to the potentiometric signature. To test this hypothesis, we decided to focus our analysis on a well-studied bacterium: *Escherichia coli*. We developed a compartmentalized blood culture bottle allowing to physically separate bacterial cells and soluble metabolic products. In parallel, time-resolved impedance spectroscopy measurements performed at the evolving open-circuit potential were used to characterize interfacial modifications during bacterial growth. By combining spatial separation between microorganisms and electrodes with frequency-domain electrochemical analysis, this study aims to elucidate the mechanistic origin of label-free electrochemical fingerprints in complex biological media.

2. Materials & Methods Experimental section

2.1. Chemicals and reagents

All the chemicals were purchased from Sigma-Aldrich with the highest purity grade existing and used without any further purification. Polyaniline ink was prepared by dilution (1:1 in distilled water) of a doped polyaniline ink (Rescoll)³⁷. The mixture was vortexed during 1 min before each use. Iridium oxide/carbon ink was prepared by adding Iridium oxide (Premion 99,99% – Alfa Aesar) to carbon ink BQ242 (Dupont) and Ethylene Glycol Diacetate as previously described³⁸. The carbon ink BQ242 (Dupont) alone, already used in the Iridium

oxide/carbon ink blend, was also used without any modification. The reference electrode material used is an Ag/AgCl ink (Dupont 5874). To prevent from silver leakage (which is bactericidal) and increase stability of the reference electrodes, it was protected with a polyurethane membrane, homemade by dissolving polyurethane (Selectophore™, Sigma-Aldrich) in THF. The porous polytetrafluoroethylene membrane used for the two chambers blood culture bottle was purchased from Merck Millipore (JGWP04700).

2.2. Bacterial strains and growth conditions

Two strains of *Escherichia coli* were used: strain ATCC 8739 to study the correlation between pH and potential, and strain ATCC 25922 for the other experiments (supplier reference, *Microbiologics*). In each experiment, bacterial colonies were derived from a working culture on Columbia agar (5% v/v sheep blood, VWR) seeded 24 hours earlier and incubated at 37°C. Colonies were taken from this working culture and placed in 3 mL of pharmaceutical diluent (VWR) to obtain 0.5 McF (between 0.8 and 0.12 absorbance). This amount generally corresponds to a bacterial concentration of 2×10^8 cfu/mL for rod-shaped Gram-negative bacteria. As this concentration is too high for our experiments, a cascade dilution protocol was used to decrease the concentration of the sample vial to a target value. These dilutions were performed in pharmaceutical diluent (VWR). A volume of 0.4 mL of a bacterial suspension, ranging in concentration from 2×10^2 to 2×10^3 cfu/mL (depending on the experiment), was added to a mixture of 10 mL of blood and 30 mL of culture medium, in our homemade blood culture bottle (initial concentration inside the bottle ≈ 10 cfu/mL). The human blood used comes from Etablissement Français du sang (EFS). For legal reasons, each blood sample comes from only one patient. No experiments were performed with the same blood. Defibrinated horse blood was provided by Thermo Scientific Oxoid™ (OXSR0050C). Culture media used in our

instrumented blood culture bottles come from commercial blood culture bottles (BacT/ALERT® FA Plus – Ref 410851 - bioMérieux). The blood culture bottles were incubated at 37°C with shaking at 90 rpm in an Infors HT Ecotron incubator. To count colonies, numerating cultures in triplicate were grown in Columbia agar (5% v/v sheep blood, VWR) for 24 h after inoculation. This method provides the quantity of bacteria inoculated at $t = 0$.

2.3. pH and biomass measurements during a blood culture

The measurement of pH, in real time, can be performed in a blood culture flask by immersing a commercial pH electrode but, in order to measure the microbial biomass in the medium, a sampling method is necessary. In order to limit the risks related to the handling of needles (to collect) and blood of human origin, defibrinated horse blood was used for this experiment.

A blood culture bottle instrumented with our electrochemical device is fitted with a cap into which a septum has been previously inserted. The bottle is filled with 80 mL, *i.e.*, twice as much liquid as in a standard blood culture, in order to allow the immersion of the electrodes despite the regular sampling of solution. The proportions remain the same as in all standard blood culture experiments: $\frac{1}{4}$ blood (20 mL) to $\frac{3}{4}$ BacT/ALERT® medium (60 mL). The blood/culture medium mixture is inoculated with approximately 600 cfu of *Escherichia coli* (ATCC 8739), bringing the bacterial concentration in the vial to approximately 7.5 cfu/mL at the start of the measurement. The culture is carried out at 37°C under stirring at 90 rpm.

At regular intervals, 2 mL of medium are sampled. A volume of 1 mL allows a pH measurement of the medium, which is measured with a conventional glass electrode and a pH meter (inoLab™ pH 7310). Another volume of 1 mL is diluted in cascades (Pharmacopoeia Diluent – 9 mL tube - VWR) up to concentrations compatible with PFU counting on plates.

2.4. Electrochemical platform

We used a print circuit board (FR4) manufactured by the company Cibel (Bellême, France). The top layers of all electrodes and connectors were made of gold (Au bounding). The electrode array manufacturing process has been described previously^{27,38} and is shown in **Figure 2 - a, b & c**.

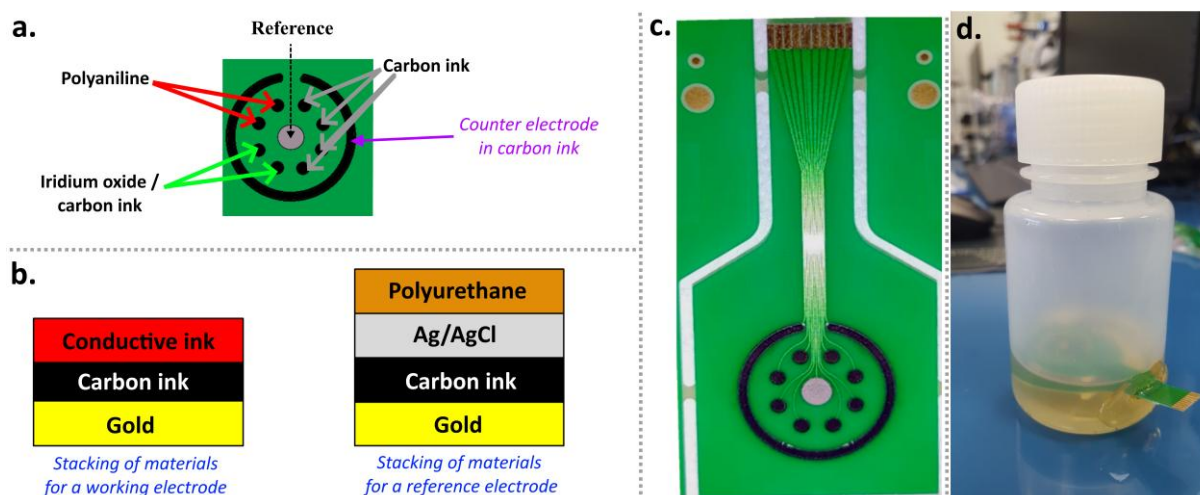


Figure 2. (a.) Schematic of the arrangement of the different electrode materials on a generic platform. (b.) Stacks of materials on a working electrode and a reference electrode. A carbon ink undercoat is present on all electrodes. (c.) Photograph of a generic platform covered with conductive inks. (d.) Photograph of an instrumented blood culture bottle (before it is filled with blood).

The platforms were embedded in polypropylene flasks **Figure 2 - d**. The bottles were filled with BacT/ALERT® medium then autoclaved to ensure the absence of contamination. A few minutes before starting the experiment, 10 mL of human blood or horse defibrinated blood was added to the vial with a known biomass (depending on the purpose of the experiment).

2.5. Electrochemical measurements

Potentiometric electrochemical measurements were performed with an AUTOLAB potentiostat (Autolab Bv, The Netherlands) equipped with a multiplexer, controlled by Nova 2.11 software.

Electrochemical impedance spectroscopy was performed with a PalmSens4 potentiostat (PalmSens) equipped with a multiplexer, controlled by PSTrace 5.10 software. The impedance spectra were recorded at open-circuit potential by applying a 10 mV (rms) voltage-modulated signal over a frequency range of 500 kHz to 0.1 Hz. The impedance spectra shown in the figures in the article are systematically limited to the range 0.1–1,000 Hz, which corresponds to the frequency range in which biofilm formation can be observed²⁹.

2.6 Data analysis

Data analysis was performed using Origin software (Version 6.0) and a homemade Python program for 3D EIS plots.

3. Results and Discussion

We have previously described a potentiometric method for the diagnosis of bloodstream infections²⁷. By simply analyzing the potential differences recorded across several electrode materials, the pathogen growing in the blood culture can be identified without any reagent. Two of the studied materials are well known to be pH sensitive^{39,40}, namely polyaniline and iridium oxide.

The results presented in our previous article highlighted a change in the interfacial potentials of each material which cannot be correlated, as anticipated, with the acidification resulting from CO₂ production during microbial growth. In order to illustrate this lack of correlation between the change in the potential of our redox electrodes – IrOx and PANI, respectively – and the pH

of the solution, we carried out a potentiometric measurement whilst *Escherichia coli* (ATCC 8739) was growing in defibrinated horse's blood, taking regular samples of the medium to analyse them using a commercially available pH meter. (**Figure 3 - Left**).

As shown in **Figure 3 - Right**, the evolution of the potentials recorded using our pH-sensitive materials do not follow the pH variation in the medium. Indeed, according to the calibration curves shown in **Figures S1 and S2**, the potentials of polyaniline and iridium oxide should increase by 64 and 54 mV, respectively, during the growth of *Escherichia coli*, corresponding to a decrease of one pH unit as exemplified in the aforementioned experiment.

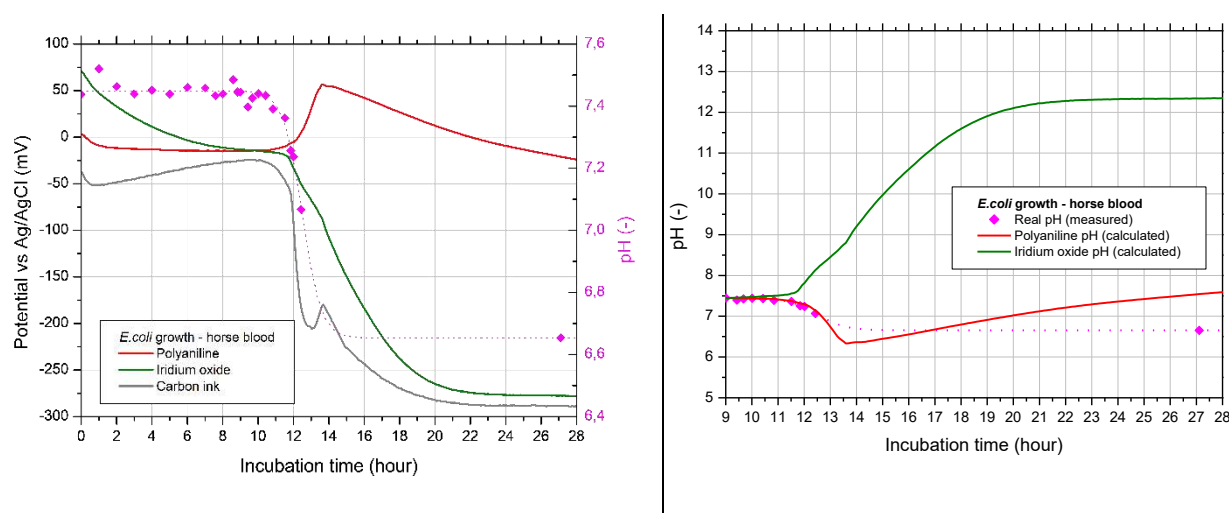


Figure 3. (Left) Potential-pH correspondence during growth of *E. coli* (ATCC 8739) in a BacT/ALERT® FA Plus + defibrinated horse blood mixture with one polyaniline electrode (red line), one iridium oxide electrode (green line) and one carbon ink electrode (black line). (Right) Difference between the measured pH (glass pH electrode) and the pH calculated from the potentials of polyaniline (red line) and iridium oxide (green line) electrodes according to their previous calibration in standard solutions.

However, the measured change in acidification (typically 1 pH unit) is temporally correlated with the occurrence of potential variations associated with the specific electrochemical signatures of the detected microorganisms.²⁷, as well as with the exponential growth phase that we were able to measure by sampling and counting in Petri dishes (**Figure S3**). We therefore hypothesized that the polyaniline and iridium oxide electrodes respond not only to pH changes, but also to redox-active species (associated to the bacteria growth) present in the solution (such as auto-inducers^{41,42}) or / and to phenomena occurring at the bacteria–electrode interface. To explore this phenomenon further and to specify the main mechanism, we performed two complementary experiments. First, we used a compartmentalized culture system to separate the bacterial cells from the compartment where the electrochemical response was measured. Second, we performed impedance spectroscopy during bacterial growth to assess whether interfacial changes accompany the potentiometric fingerprints. These two experiments should make it possible to determine whether the phenomena in question are linked, respectively, to the diffusion of metabolites or to interfacial behaviour.

3.1. Electrochemical detection of diffusive metabolites

3.1.1. Prototype of a two chambers blood culture bottle

The initial hypothesis proposed to explain the behavior of the polyaniline and iridium oxide electrodes was based on the production of redox-active metabolites by the bacteria. To examine this possibility, we developed a compartmentalized vial (**Figure 4**) in which one chamber is inoculated with *Escherichia coli* while the other remains sterile. The two chambers are connected through a porous polytetrafluoroethylene membrane (80 % porosity) generally used for sterilising filtration. The pore size (0.2 μm diameter) was chosen so that soluble molecules can diffuse through the membrane, whereas bacterial cells are retained.

If diffusible metabolites alone were responsible for the electrochemical fingerprints, the same behavior should appear in both chambers, with a delay corresponding to diffusion across the membrane.

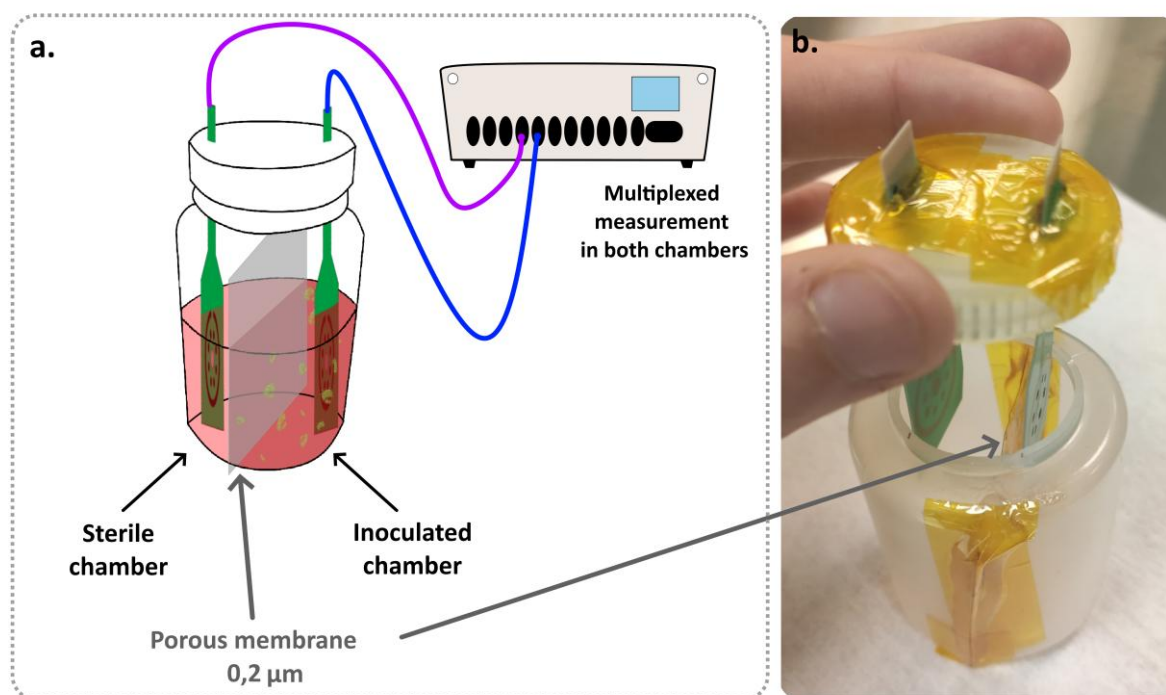


Figure 4. Explanatory scheme (a.) and picture (b.) of the prototype compartmented vial allowing a double electrochemical measurement of the potentiometric signature of bacteria added into an inoculated compartment and the detection of diffusive redox species and pH variations in a sterile compartment. Human blood culture in BacT/ALERT® FA Plus, 37°C, 60 rpm.

3.1.2. Biofilm detection or pH detection

In the inoculated compartment, we observed the same behavior as that shown in **Figure 3**. For polyaniline, bacterial growth produced an increase in potential followed by a decrease (**Figure 5 - solid line**). For iridium oxide, the potential decreased concomitantly to the exponential growth phase.

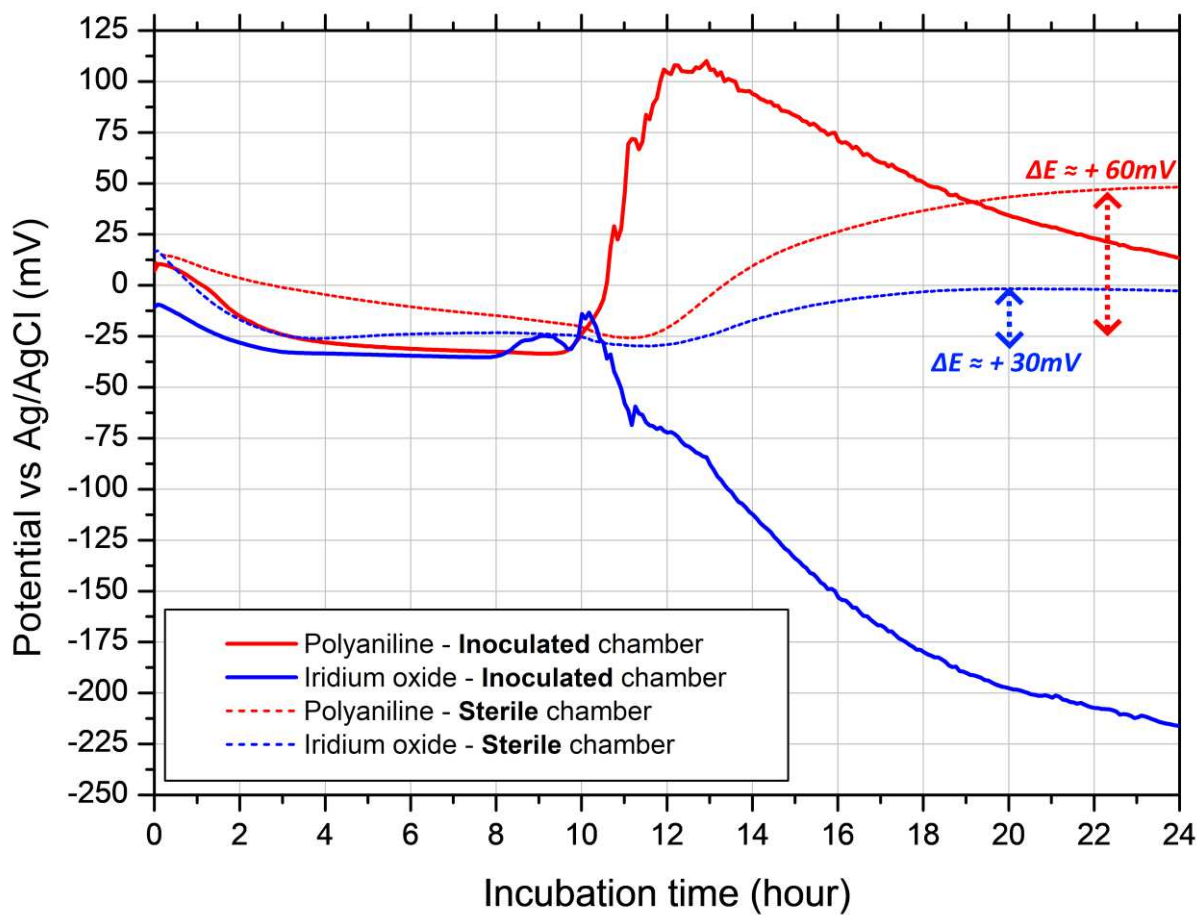


Figure 5. Potentiometric curves of polyaniline (red lines) and iridium oxide (blue lines) electrodes placed in two compartments: one with *E. coli* growth ATCC 25922 (solid lines) and one sterile (dot lines). Human blood culture in BacT/ALERT® FA Plus, 37°C, 60 rpm. The noise visible in the curves is due to the lack of uniform stirring, which is impossible to achieve without contaminating the sterile compartment due to spillover from the inoculated compartment.

In the sterile chamber, by contrast, the potentials of polyaniline and iridium oxide increased (**Figure 5 - dashed line**). This increase occurred with a time lag (typically 2 hours) compared with the changes in the electrochemical potentials detected in the inoculated compartment, which corresponds to the time required for the chemical species to diffuse through the

separating membrane into the sterile compartment. The evolutions of the interfacial potentials of the IrOx and PANI electrodes dipped in the sterile chamber are compatible with a change in pH, since both inks are pH-sensitive.

These observations indicate that bacterial metabolites present in solution do not, by themselves, reproduce the electrochemical fingerprints observed when bacteria are in direct contact with the electrode surface. The presence of microorganisms near the electrode is therefore necessary to generate the full potentiometric signature.

In a second step, the sterile compartment was mixed with the content of the inoculated chamber, *i.e.*, blood and inoculated culture medium. The previously sterile electrodes then displayed the electrochemical behavior expected during bacterial growth, confirming that the signal depends on the local biological state of the medium and on the presence of microorganisms at the interface (**Figure S4**).

3.2. Monitoring of bacterial culture thanks to impedance spectroscopy

3.2.1. Ohmic drop of polyaniline in presence of bacteria

To analyze the impact of bacterial cells on the electrochemical behavior of the electrodes, we monitored *Escherichia coli* growth by impedance spectroscopy in parallel with potentiometry. Two polyaniline working electrodes were used. One electrode served as a potentiometric control, while the second was used for potentiometric and then impedance measurements, with one measurement cycle (potentiometry + impedance spectroscopy) every 15 minutes (**Figure S5**). For practical reasons, the culture was performed at room temperature, which lengthened the detection time (**Figure S6-S9**).

In this study, impedance spectroscopy was performed at the open circuit potential of the electrodes (that is evolving with the bacterial growth as previously shown), rather than at a fixed imposed potential. This unconventional approach preserves the native electrochemical state of the system and avoids perturbing microbial growth with exogenous redox probes

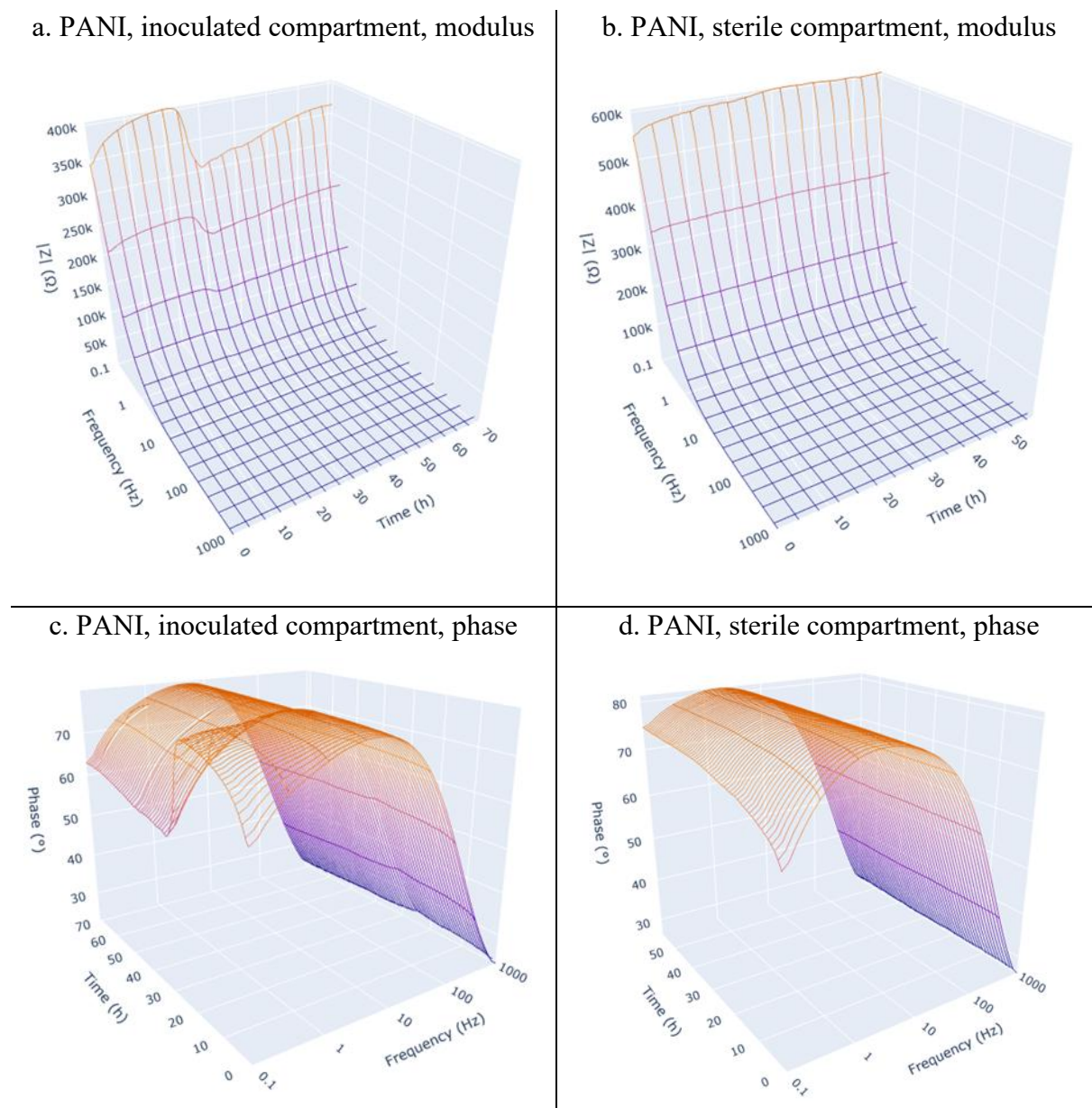


Figure 6. For a polyaniline ink electrode, between 1 kHz and 0.1 Hz, (a.) evolution of smoothed (using a three-point moving average, $k = 3$) impedance modulus during *E. coli* growth; (b.) evolution of smoothed impedance modulus ($k = 3$) during the incubation of sterile medium; (c.)

evolution of smoothed ($k = 3$) impedance phase during *E. coli* growth; (d.) evolution of smoothed ($k = 3$) impedance phase in the sterile compartment. Human blood cultures in BacT/ALERT® FA Plus, 20°C, 100 rpm.

During the exponential growth phase, the impedance modulus and phase of polyaniline dropped abruptly (**Figure 6 - a & c**). In the sterile blood culture (**Figure 6 - b & d**), by contrast, both parameters increased slowly over the incubation time. These observations are also apparent in the Nyquist plots shown in **Figure S10**.

These results suggest that bacterial growth induces two electrochemical behaviors of the electrodes. An initial increase in impedance is likely caused by biofouling, corresponding to progressive coverage of the surface by biological material. After several hours, the impedance decreases, as if bacterial adhesion and early biofilm development promoted more efficient charge transfer. This decrease may reflect the metabolic activity of the bacteria close to the electrode surface. A similar change in impedance is also visible on an electrode based on iridium oxide ink (**Figure S11 & S12**).

3.2.2. Low frequencies study for biofilm detection

As shown in **Figure 6**, low frequencies, typically below 1 Hz, are more adapted for observing changes in impedance during bacterial growth, for modulus as well as for phase. We therefore chose to focus the analysis on 0.1 Hz. At this frequency, both the normalized modulus and phase of the impedance of the polyaniline decrease during the exponential phase of growth at the same time as the potentiometric signal changes (**Figure 7**). This temporal coincidence supports the idea that the electrochemical fingerprint is linked to interfacial processes occurring during bacterial proliferation²⁷.

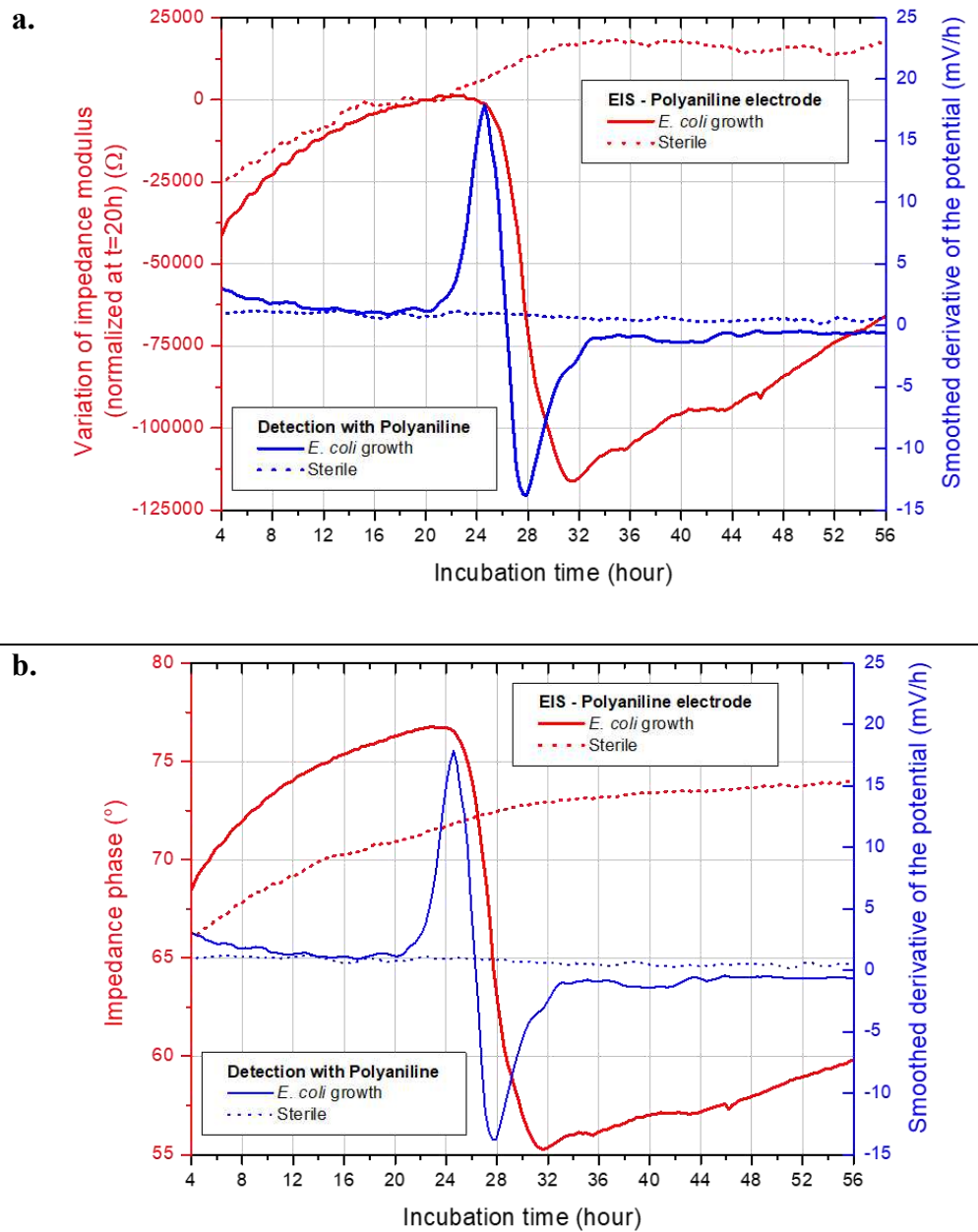


Figure 7. Time-dependent changes in the normalized modulus at 20 h (a) and the phase (b) of the impedance (red lines) measured at 0.1 Hz, for *E. coli* growth (solid lines) and for a control experiment (dot lines), using a polyaniline electrode. The changes in impedance are temporally correlated with the potentiometric variations (blue lines). Human blood cultures in BacT/ALERT® FA Plus, 20°C, 100 rpm.

This abrupt change in the (normalized) impedance modulus is also observable on the iridium oxide electrode (**Figure 8**). For this material, the phase also exhibits a variation that is time-correlated with the potentiometric detection, but with a very small amplitude.

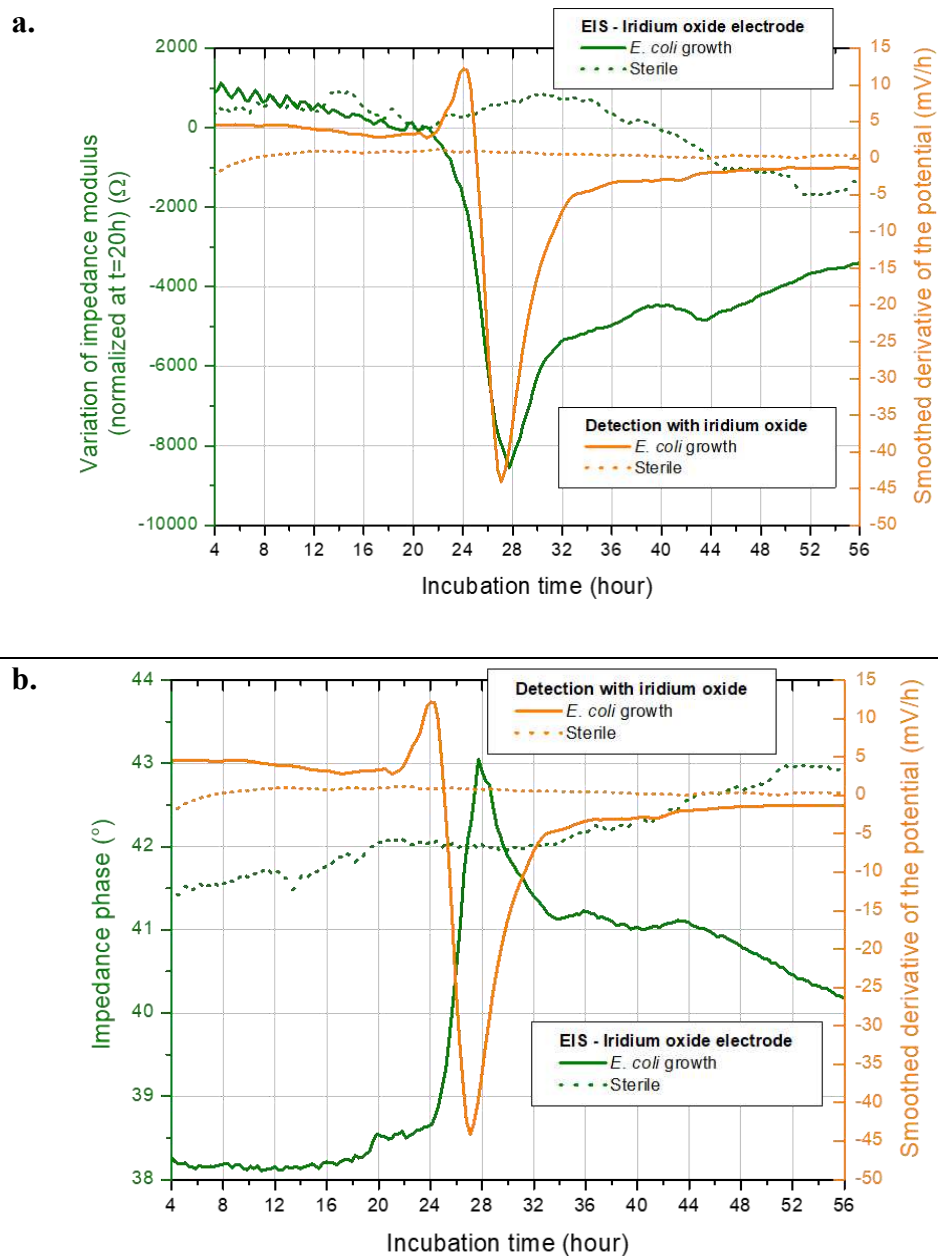


Figure 8. Time-dependent changes in the normalized modulus at 20 h (a) and the phase (b) of the impedance (green lines) measured at 0.1 Hz, for *E. coli* growth (solid lines) and for a control

experiment (dot lines), using an iridium oxide electrode. The changes in impedance are temporally correlated with the potentiometric variations (orange lines). Human blood cultures in BacT/ALERT® FA Plus, 20°C, 100 rpm.

The fact that the impedance drops sharply at the very moment when bacterial growth becomes detectable – whereas purely passive fouling would, on the contrary, leads to an increase of the interfacial barrier, especially at low frequencies^{43,44} (**Figure 7 - a, dot lines**) – suggests that the system is not responding to a phenomenon of passive biological fouling (*i.e.*, a blocked electrode). Blood components – in the absence of biomass – may indeed help to strengthen the interfacial barrier. However, through potential measurements carried out in the absence of bacteria, we were able to show that this adsorption has no influence on the measured potential. Thus, at the open circuit potential, these species have only a passive impact, corresponding to a slow and gradual increase in interfacial impedance over time (**Figures 7 and 8, dot lines**). Conversely, in the presence of biomass, the impedance follows a non-linear trend, with significant changes occurring concurrently with potentiometric detection (**Figure 7 and 8, solid line**). The pure adsorption could have been quantified using a ferrocene-type redox probe, but this would have disrupted microbial growth. When the bacterial population enters the exponential phase, the interface appears to evolve toward a biologically active state in which electrode colonization, local production of redox species, and the gradual formation of a biofilm alter charge transfer, a phenomenon observable at low frequencies for these materials (polyaniline and iridium oxide), whose electrochemical changes are reversible (capacity of to reversibly change their protonation, ion-exchange and redox states in response to local interfacial conditions). We have already demonstrated the importance of the presence of microorganisms in contact with electrode materials (cf. **Figure 5**) for obtaining the

potentiometric signals that allow us to detect and (ultimately) identify bacteria²⁷. From this perspective, the sharp decrease in the impedance modulus observed at 0.1 Hz for polyaniline and iridium oxide ($-125\text{ k}\Omega$ and $-8\text{ k}\Omega$, respectively) reflects a profound reconfiguration of the electrode–medium interface, where the resistive contribution decreases despite the growth of biological material on the interface. This behavior is consistent with previous reports^{45,46} on others microorganisms, especially *Pseudomonas aeruginosa*, where biofilm development has been associated with a reduction in the resistive component of the signal.

This interpretation is supported by the behavior of the impedance phase (**Figure 7 - b** & **Figure 8 - b**). On polyaniline, the drop in the phase (from 77° to 55°) at the time of detection indicates that the system is shifting away from strongly capacitive behavior classically observed for conductive polymers^{42,47} toward a mixed response (*i.e.*, the impedance spectra move from capacitive to Constant Phase Element comportment), with a more pronounced resistive component. On iridium oxide, the phase exhibits a more complex evolution, with a sharp increase (from $38,5^\circ$ to 43°) followed by a decrease, suggesting a transient reorganization of the interface before the establishment of a new electrochemical state. The phase does not merely describe an additional “value” of the signal: it provides information on the nature of the interfacial regime, and indicates here that bacterial growth does not simply lead to an insulating layer, but to a system whose RC response changes during colonization. This evolution is consistent with a simultaneous change in both the charge transfer resistance and the capacitance of biofilm, rather than with a simple increase in either of these components.

The contrast between polyaniline and iridium oxide is also particularly revealing. Polyaniline exhibits a much greater amplitude variation ($-125\text{ k}\Omega$), which can be explained by its nature as a conductive polymer, which is more sensitive to changes in redox state and to the local ionic

environment. Iridium oxide, on the other hand, exhibits a lower amplitude ($-8\text{ k}\Omega$) but distinct phase dynamics, suggesting that each material “reads” the same biological situation differently. This supports an interpretation in which the signal depends on both bacterial colonization and the surface’s own chemistry: the bacteria do not impose a universal effect; rather, they modulate an interface whose response depends on the material. The fact that impedance variations follow potentiometric detection in time reinforces the idea that the two measurements translate the same event from two complementary angles: potentiometry captures the overall signature, while impedance reveals the underlying interfacial reorganization.

4. Conclusion

This study aims at elucidating the physicochemical origin of label-free electrochemical fingerprints observed during bacterial growth in instrumented blood culture bottles. Indeed, understanding the interfacial processes governing these signals is essential not only for improving sensor performance, but also for establishing a robust physicochemical foundation for electrochemical bacteria detection and identification in bloodstream infections. The observed fingerprints are not solely due to bulk acidification or diffusible metabolic products. Although bacterial growth acidified the culture medium, the potentiometric responses of polyaniline and iridium oxide did not match the predicted pH variations. More importantly, physical separation of bacteria from the sensing electrodes using a porous membrane resulted in markedly different potentiometric responses between the inoculated and bacteria-free compartments, despite allowing soluble metabolites to diffuse between them. Together, these observations indicate that bulk physicochemical changes contribute to the electrochemical environment but are insufficient to reproduce the characteristic fingerprint observed during

bacterial growth. Instead, the proximity of microorganisms to the electrode is crucial for the measured response.

This distinction between bulk and interfacial contributions is particularly relevant in complex biological media. The physicochemical environment experienced by an electrode colonized by microorganisms is unlikely to be equivalent to that measured in the bulk solution. Bacterial adhesion creates a confined microenvironment in which proton concentration, ionic composition, dissolved gases, redox-active metabolites and extracellular polymeric substances may differ substantially from their bulk concentrations. Consequently, an electrode located within or immediately adjacent to this biologically active interface may respond to local conditions that are not captured by conventional measurements of bulk pH.

The delayed potential increase in the bacteria-free compartment supports the interpretation that soluble metabolic products diffuse across the membrane, contrasting with the complex fingerprint measured in the inoculated compartment. The compartmentalized experiment therefore suggests the coexistence of at least two contributions: a relatively homogeneous bulk component associated with bacterial metabolism and medium acidification, and a local interfacial component requiring the presence of microorganisms close to the sensing surface. The latter appears to be essential for generating the characteristic electrochemical fingerprint.

Time-resolved impedance measurements provide independent evidence that bacterial growth is accompanied by substantial reorganization of the electrode interface. In sterile blood culture medium, impedance evolved progressively over time, consistent with passive adsorption and fouling of the electrode by proteins and other blood components. In the presence of *E. coli*, however, this initial evolution was followed by an abrupt decrease in impedance modulus

coincident with the potentiometric detection window. At 0.1 Hz, the decrease reached approximately 125 k Ω for polyaniline and 8 k Ω for iridium oxide. The temporal correspondence between potentiometric and impedance changes strongly suggests that both measurements are sensitive to the same biological transition occurring at the electrode-medium interface.

Importantly, the decrease in impedance should not be interpreted simply as the opposite of biofouling. Biofilm-associated impedance responses are highly dependent on electrode material, microbial species, medium composition, frequency range and biofilm developmental stage²⁶. While passive adsorption of biological material often increases the interfacial barrier, metabolically active bacterial colonization can simultaneously modify local ionic conductivity, surface charge, dielectric properties and interfacial transport. Previous studies have similarly reported decreases in resistive contributions during bacterial colonization^{45,46}. Our observations are therefore consistent with a transition from predominantly passive fouling toward a dynamically reorganized bacteria-electrode interface during bacterial proliferation.

A further important observation is that the magnitude and phase dynamics of the impedance response differed substantially between polyaniline and iridium oxide. This material dependence argues against a purely solution-driven mechanism, which would be expected to affect all electrodes more uniformly. Instead, it suggests that bacterial growth modifies an interface whose response is conditioned by the physicochemical properties of the electrode material. Polyaniline is a proton-responsive conducting polymer whose oxidation and protonation states directly influence its electronic conductivity and interfacial capacitance^{40,48}, whereas hydrated iridium oxide exhibits proton-coupled redox and ion-exchange behavior⁴⁹. Local variations in proton activity, ionic composition, redox environment and surface coverage may therefore be transduced differently by the two materials.

This observation provides a possible mechanistic explanation for the discriminatory capability of the multi-material array reported previously²⁷. The electrochemical fingerprint may not arise from each material independently measuring a single bacterial parameter. Rather, bacterial growth generates a complex interfacial microenvironment that is transduced differently by electrodes with distinct surface and redox chemistries. The resulting multidimensional response could therefore encode microorganism-dependent differences in adhesion, metabolism and local physicochemical conditions.

The present electrochemical data alone cannot determine whether the observed interfacial transition corresponds to initial bacterial adhesion, microcolony formation, extracellular matrix production, or a more mature biofilm state. Nevertheless, the requirement for bacterial proximity to the electrode, together with the time-dependent impedance changes, is consistent with bacterial adhesion and early biofilm-associated interfacial development.

From a sensor-design perspective, identifying the interface and its colonization as major contributors to the signal changes the strategy for future optimization of the platform. If time-to-detection depends partly on the establishment of a biologically active electrode microenvironment, sensitivity may potentially be improved by controlling surface properties that influence bacterial proximity and adhesion. Electrode roughness⁵⁰, wettability, surface charge, porosity and microstructure could therefore become design parameters in addition to the intrinsic electrochemical sensitivity of the sensing material.

Several limitations should be noted. First, the experiments focused on *E. coli*, and it is unclear if the same processes apply to other species. Second, different blood matrices and conditions were used, which may affect bacterial growth and interfacial behavior. Finally, direct

quantitative relationships between bacterial surface coverage, impedance changes, and potentiometric fingerprints remain to be established.

In summary, our results show that bacterial metabolism alone cannot explain the observed electrochemical fingerprints. Instead, the signal arises from a dynamic bacteria-electrode interface, where local biological activity interacts with the physicochemical properties of the sensing material. This framework supports the use of multi-material electrochemical fingerprints for pathogen discrimination and highlights the bacteria-electrode interface as a key target for future optimization of smart blood culture systems. Furthermore, an approach involving electrochemical analysis based on multiple methodologies (cyclic voltammetry, EIS, surface conductivity and potentiometry), coupled with AI, could be implemented to enhance the robustness of the measurement and potentially broaden the range of detectable bacterial species.

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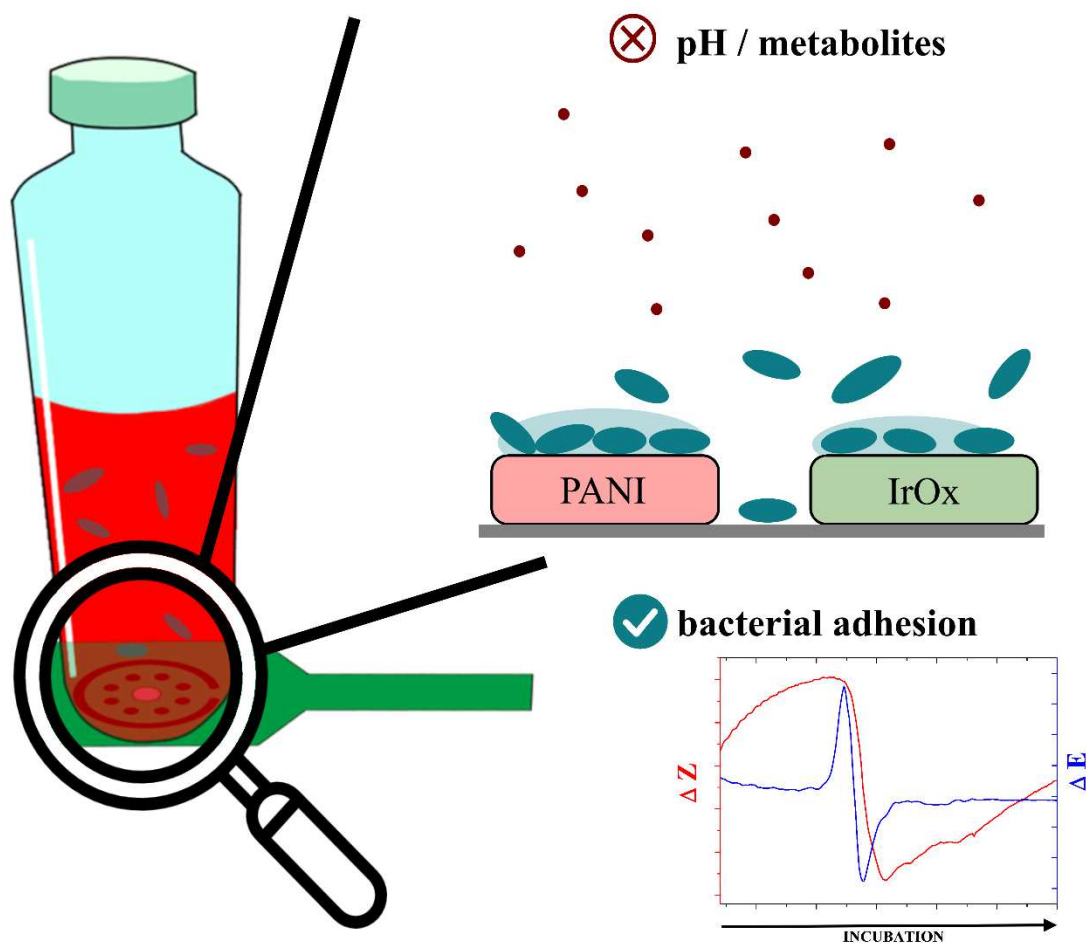
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