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A high sensitive microwave sensor to monitor bacterial and biofilm growth

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ABSTRACT

Bacterial biofilms have a significant economic and health impact in many different domains. In such films, the extracellular matrix prevents the diffusion of biocides, so antibiotic treatments require a concentration 500 to 1000 times higher than that used to eliminate the same bacteria when present as planktonic stage. Early detection of biofilms is therefore essential for effective eradication. In this paper, we present the development of a real-time and label-free radiofrequency biosensor dedicated to the monitoring of bacteria and biofilm growth. Its principle relies on an open-ended coaxial probe sensitive to the variation of the electrical conductivity of the probed medium in the microwave range. As shown, between 0.3 and 1 GHz, the high sensitivity of the method (2.3×10^4 CFU/mL) highlights the biofilm growing at the early stage of its formation. To demonstrate experimentally such effects, two model bacteria, *Vibrio natriegens* and *P. aeruginosa*, are considered. The proposed method should therefore be considered as a promising technique for biofilm monitoring in batch bioreactors or flow cells experiments.

1. Introduction

Bacterial biofilms are complex structures which are essentially composed of polysaccharides, proteins, nucleic acids and microorganisms such as bacteria or microalgae [1]. They enhances cells development, the expression of pathogenicity or toxins production and prevents external chemical compounds from reaching the cells [2–4]. In this form, microorganisms are much more resistant to antibiotic treatments, sometimes up to 1000 times more and increase their pathogenicity when biofilm reach a mature stage [5]. Consequently, biofilm formation strongly impacts several activities and applications. The development of innovative detection techniques of early stages of biofilm formation and associated microorganisms growing is of fundamental interest.

Bacteria growth is currently measured by standard culture-based laboratory assays or by molecular reactions. Such measurements require extensive sample preparations or expensive instrumentations and are not ideal. Biosensors allowing a real-time and label-free monitoring are proposed in the literature. They are based on optical devices, impedance spectroscopy, dielectric characterization or surface acoustic waves [6–9]. Among them, Electrical Impedance Spectroscopy (EIS) is a well-established method which operates from 10 mHz to 100 kHz to analyse interfacial properties between metallic electrodes and biological matter [10–12]. In this case, biofilm formation can be studied by electrical models [11]. The β-cellular membrane dispersion of cells that occurs between 0.1 and 10 MHz was also proposed to monitor the growth of bacteria [13]. A good correlation was obtained on biomass concentration and sporulation status using both the dielectric permittivity and optical density methods. However, due to the variety of polarization phenomena of biological matter which occur below 100 MHz, interpretation of dielectric permittivity spectra in this range remains a challenging task. Working in the microwave range where the polarization is dominated only by water dipolar polarization effects allows a more reliable scientific approach [14,15]. Artis et al. [16] proposed the use of a coplanar waveguide coupled to a microfluidic reservoir to probe the proliferation of cells at 20 GHz. This was done by considering the variation of power loss in the radiofrequency (RF) transmission line. In addition, another recent work used a coaxial transmission line to demonstrate a linear relationship between the real part of the complex

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permittivity and the bacterial concentration in solution at a frequency of 16.0 GHz [17]. However, the sensitivity of the method is about 10^8 CFU. mL^{-1} which is not ideal for the biofilm prevention, where bacteria needs to be detected at lower concentrations.

The application of the method in the frequency range 0.1 to 3 GHz is of particular interest for the development of low cost sensors since the technology is cheap in this range because of the existence of authorised frequency ISM bands. Interdigitated electrodes were demonstrated to be sensitive to the growth of bacteria [18]. Recently, bacterial growing was also monitored *via* the realization of high sensitive radiofrequency resonators [19]. This was achieved by measuring a variation of the amplitude and frequency of the resonance during *E. Coli* growth in the ISM band at 2.5 GHz. The experiment presented a variation of the resonant frequency of 3.4 MHz / log(OD_{600}), *i.e.* a frequency ratio variation of 1.36×10^{-3} / log(OD_{600}). The method was subsequently applied to the growing of bacteria in solid growth medium [20–23]. In another study, a capacitive microwave biosensor was shown to be able to detect 10^3 CFU/mL [24]. However, in these previous studies, no distinction between bacterial growth and biofilm formation was reported. Only Hoog et al. [25] proposed to monitor the biofilm formation by using a coaxial resonator filled by glass beads on which bacteria adhere. The method showed a change of both the frequency and the amplitude of the resonance indicating the variation of the dielectric permittivity and tangent loss associated with the biofilm formation.

The present work aims therefore at developing a microwave sensor which highlights the transition from bacteria growing to biofilm formation. This will be made in batch bioreactors which are extensively used for many applications including testing of treatment solutions. For this purpose, a microwave sensor based on an open-ended coaxial transmission line is first developed by electromagnetic simulations. The studied material is then positioned at the end of the probe. The influence of the variation of the electrical conductivity and of the real part of the dielectric permittivity associated with bacteria/biofilm growing on the sensor response is then discussed. To validate experimentally the sensor, two models of bacteria/biofilm growing are studied. The non-pathogenic marine bacterium *Vibrio natriegens*, has a very low duplication time (less than ten minutes) [10,26] and allows an investigation of bacteria growing without any biofilm formation. In contrast, the bacterium *Pseudomonas aeruginosa* which has been the subject of fairly comprehensive studies leads to the development of a biofilm [27–29]. The physiological properties of these two bacteria acquired since several decades make them suitable for study models [27]. Comparison of experimental results on these two bacteria together with electromagnetic simulations proves the ability of the sensor to separate biofilm formation from suspension growth.

2. Material and methods

2.1. Microorganism preculture and cell growth

Two gram-negative model bacteria were used: *P. aeruginosa* (Schroeter) (PA01) and *V. natriegens* (Payne) (ATCC 14048). *P. aeruginosa* and *V. natriegens* were inoculated from frozen stock into Teflon tube containing respectively: 50 mL of Tryptic Soy Broth media (TSB) or Nutrient Broth liquid medium (NB) supplemented with 1.5% NaCl. Strains were then incubated overnight at 37 °C for *P. aeruginosa* and at 28 °C for *V. natriegens*. In order to determine the correlation between absorbance and bacterial concentration (cells per mL), the optical density at 600 nm (OD_{600}) was measured every 2 h (in 8 replicates) during 24 h using a spectrophotometer (TECAN Infinite™) for both bacteria. After each reading, 100 µl of culture were sampled and plated onto Tryptic Soy Agar (TSA) or Nutrient Agar (NA) plates (respectively for *P. aeruginosa* and *V. natriegens*) in order to count the number of Colony Forming Units (CFU). For all the experiments, starting bacterial suspension was adjusted at 2×10^7 CFU/mL in TSB for *P. aeruginosa* and 2×10^5 CFU/mL in NB medium for *V. natriegens*.

2.2. Experimental setup

The manufactured probe is based on the principle of truncated coaxial probes, where an electric field is formed at the end of the line allowing a better sensitivity to the solution overhead. It consists of a cylindrical transmission line, composed of two conductive brass parts with a length of 15 mm. The inner and outer diameters measure 0.62 and 9.1 mm respectively and are separated by a dielectric (PTFE) with a diameter of 2.0 mm. A Teflon tank is located at the end of the line, which can be filled with bacterial culture solutions (the maximum volume is about 75 mL). This transmission line is connected to a vector network analyzer (VNA) to acquire the reflection (or S_{11} parameter) as function to the frequency or in a frequency range. The VNA is a handheld Anritsu MS2037C model, which frequency band is from 5 kHz to 15 GHz. The frequency of the incident signal was chosen from 0.1 to 3.0 GHz when the characteristic impedance of the line is 50 Ω. The electromagnetic field propagates along the coaxial line and reflection occurs when it encounters an impedance mismatch between the probe and the bacterial culture in the tank. The reflection depends on the solution conductivity and on its complex permittivity ε* seen at the end of the probe [30]. The permittivity can be defined as $\varepsilon^*(\omega) = \varepsilon'(\omega) - j.\left(\varepsilon''(\omega) + \frac{\sigma}{\omega.\varepsilon_0}\right)$ where ε' is the real part of the permittivity, ω the angular frequency, σ the electrical conductivity and ε_0 the permittivity of the vacuum. The sensor is connected to the VNA by a coaxial line that exits an oven (to regulate the different culture temperatures) through the front door seal.

2.3. Electromagnetic simulations and characterisations

A SOL (Short, Open, Load) calibration is performed at the end of the coaxial line. Growth monitoring was performed by inoculating bacterial preculture into 10 mL of culture medium (TSB or NB) and measures were taken every 30 min for 24 h. A lid held in place by parafilm was placed on top to maintain the sterility of the solution during the experiment and the whole sensor was then placed in an oven at 28 °C. For *P. aeruginosa,* the temperature was set at 37 °C; measurement were performed every 30 min for 48 h. The frequency range of the measurements is from 0.03 GHz to 3.0 GHz. To investigate reflection of a coaxial probe, electromagnetic simulations were realized using the software HFSS (High Frequency Structural Simulator) from ANSYS. It utilizes tetrahedral mesh elements to determine a solution to a given electromagnetic problem. Simulation sweeps were realized between 0.1 and 3.0 GHz.

2.4. Growth kinetics determination

Cells and biofilm formation kinetics were carried out for both bacteria. The pre-culture was inoculated in 60 mL of TSB to reach a concentration of 2×10^5 CFU/mL for *V. natriegens* and 2×10^7 for *P. aeruginosa*, in the same tank above the sensor. Every 30 min during 24 h and 48 h respectively, 500 µL of medium is collected and the OD_{600} is measured with a spectrophotometer (TECAN Infinite™). Biofilm formation kinetics was performed by labelling the biofilm with Crystal Violet in 96-well plates. At each measurement time, 50 µL of a Crystal Violet solution was added. After 10 min, the content of the wells was removed and a very delicate washing was performed by soaking the plates in a distilled water bath. Finally, 200 µL of ethanol was added to solubilize the stained adhered cells. The determination of the specific biofilm formation (SBF) value was calculated according to the method of Naves et al. [31].

3. Results and discussion

The development of radiofrequency sensors dedicated to bacteria growing monitoring is based on the variations of the real part of the complex dielectric permittivity (ε') and of the loss tangent or electrical conductivity (tan (δ) or σ). In this part, we focus on a sensor based on an

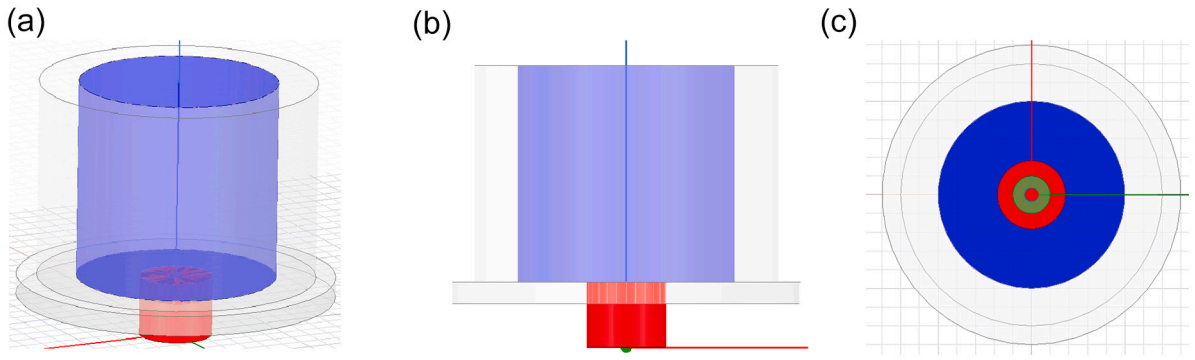

Fig. 1. Sketch of the probe. a) 3D view; b) side view and c) bottom view. The brass parts, the solution and the dielectric parts are respectively in red, blue and green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

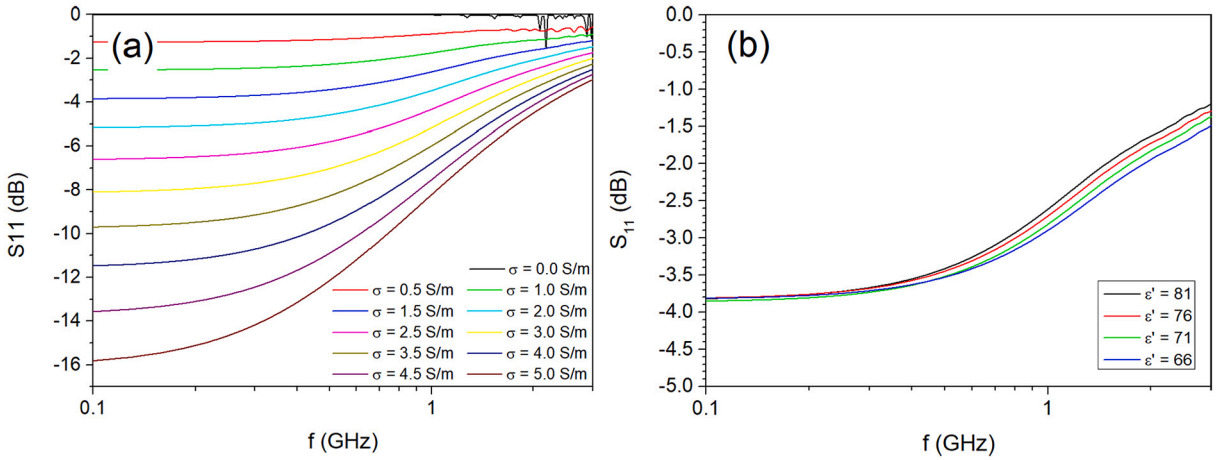

Fig. 2. HFSS simulations of S_{11} parameter. a) variation of the electrical conductivity σ for a fixed value of ε' = 81. b) variation of the relative dielectric permittivity ε' for a fixed value of σ = 1.5 S/m.

open-ended coaxial transmission line which displays an intense electric field at sensor/medium interface where a biofilm is expected to growth. Fig. 1 shows the design of the sensor. Fig. 1(a) represents the 3D view of the bioreactor, with the sensor in red and the solution in blue which can be filled with bacterial culture solution. The height and radius of the solution were both set to 50 mm (filled tank). Fig. 1(b) and Fig. 1(c) are 2D views. The sensor is based on the principle of an open-ended coaxial probe.

Fig. 2 displays the simulated variation of the S_{11} parameter with the electrical conductivity σ (Fig. 2(a)) and the real part of the dielectric permittivity ε' (Fig. 2(b)) of the surrounding solution. In Fig. 2(a), at low frequencies, the S_{11} values depend linearly on the conductivity. This is explained by an increase of dielectric losses (tan (δ)) initiated by an increase of σ. At frequencies above 1.0 GHz, the reflection parameter seems to tend towards a unique value, independently of the conductivity. This result is interpreted by a limited influence of the conductivity above 1 GHz. These results prove the interest of working with such device between 0.1 and 1.0 GHz to get a high sensitivity of S_{11} to the electrical conductivity. Fig. 2(b) evaluates the impact of ε' on the S_{11} parameter. Values between 66 and 81 are selected to cover a large variation of this parameter in agreement with previous works [16,32]. The electrical conductivity σ was set to 1.5 S/m, the value of the control TSB medium. As shown, the S_{11} values are almost constant in the 100/500 MHz range. The variation does not exceed 0.25 dB below 1.0 GHz. Above this frequency, some differences clearly appears on S_{11} parameter making difficult to differentiate the influence of both ε' and σ from the sensor response. These results demonstrate clearly the interest of working in the low frequency range with the proposed sensor to be only sensitive to σ without any influence of ε'.

The influence of bacteria and biofilm growth on the sensor response was studied experimentally. Measurements were made with an interval of 30 min after the inoculation of the bacteria. Fig. 3(a) shows the experimental S_{11} data as a function of frequency during the growth of *Vibrio natriegens* and for different incubation times. Measurements of the NB media are highlighted in red and show a value of −7.75 dB at 50 MHz which then increase from 0.3 GHz to 3.0 GHz. During the growing of bacteria, the same pattern as the control medium is displayed with a clear decrease of the S_{11} values. This behavior is observed in simulations and proves the strong correlation between the increase of electrical conductivity and bacterial concentration. Above 1.0 GHz, all curves merge progressively into a unique value indicating, in agreement with simulations, a loss of sensitivity to σ. Fig. 3(b) shows the ΔS_{11} (S_{11} normalized to the control media values) as function of the incubation time for several frequencies below 2 GHz. The same trend is observed at all frequencies, *i.e.* a clear decrease of the ΔS_{11} value during the first two hours of growth, then slows down to reach a constant value after 6 h of incubation time. As expected from simulations, the sensitivity of the method is enhanced at low frequencies leading to an initial value of −0.72 dB/h at 0.3 GHz against −0.22 dB/h at 2.0 GHz.

Fig. 3(c) shows the measured growth kinetics of the suspended bacteria and the biofilm formation of *Vibrio natriegens* strains made by optical density measurements (OD_{600}) and Crystal Violet staining. The planktonic development of the bacteria in these experimental conditions is fast and lasts about 7 h to reach a maximum concentration of almost 10^8 CFU.mL^{-1}. The amount of bacteria in the solution is then stable until the end of the experiment. As observed, very weak SBF values are

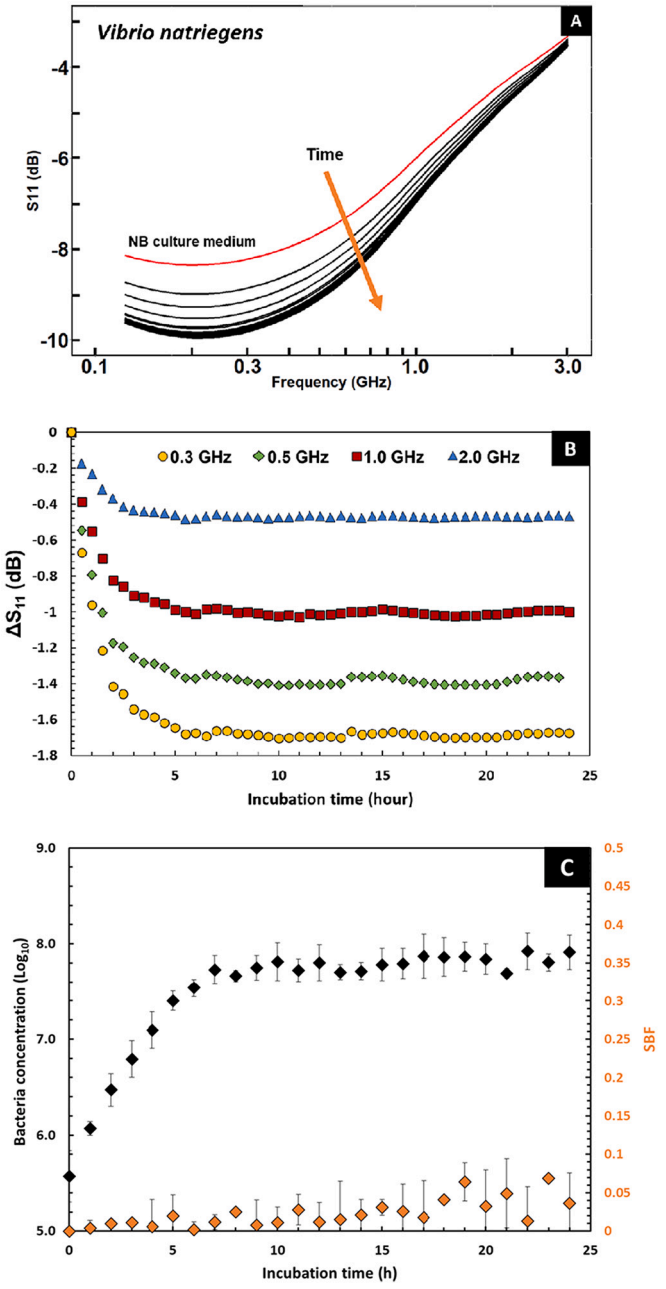

Fig. 3. Sensor response and growth kinetics of the bacterium *Vibrio natriegens*. a) S_{11} measurements of the bacterium during its growth during 24 h. b) Variability of the S_{11} normalized in function of the control NB culture media at the frequencies of 0.3, 0.5, 1.0 and 2.0 GHz. c) Growth and biofilm formation kinetics.

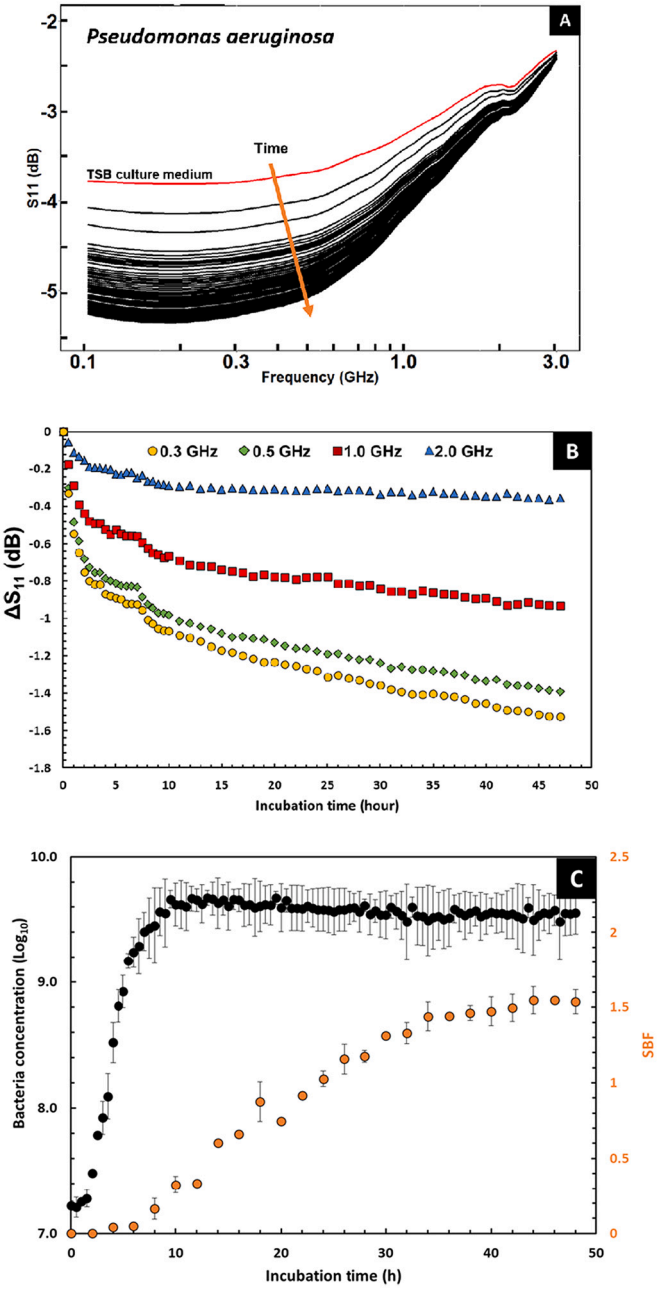

Fig. 4. Sensor response and growth kinetics of the bacterium *Pseudomonas aeruginosa*. a) S_{11} measurements of the bacterium during its growth during 48 h. b) Variability of the S_{11} normalized in function of the control TSB culture media at the frequencies of 0.3, 0.5, 1.0 and 2.0 GHz. c) Growth and biofilm formation kinetics.

measured indicating no biofilm formation. Such results proves that *V. natriegens* is a fast growing bacterium [33–35] but its biofilm formation remains negligible. Such results display a very good agreement between the sensor response and the conventional measurements performed on bacteria growth in a bioreactor, indicating the ability of the method to monitor in real-time bacteria growing. At 0.3 GHz frequency, the first two hours of growth show a decrease of 1.45 dB. Over this period of time, the bacterial concentration increases from 2.0×10^5 to 3.2×10^6 which corresponds to a slope of 0.95 dB/log(CFU) or 0.95 dB/log(OD_{600}). In the work of Narang et al. [19], a microfluidic microwave ring resonator is used to detect the presence of *E. coli* in a liquid medium under different pH conditions. A maximum sensitivity of 0.4771 dB/log (OD_{600}) was obtained indicating the high sensitivity achieved with the present method. Moreover, given the variations observed at the shelf level, the background noise seems to be close to 0.04 dB which corresponds to a sensitivity of 2.3×10^4 CFU/mL *i.e.* a sensitivity close to that obtained recently in Piekarz et al. work. [24].

The ability of the sensor to detect suspended bacteria in solution being established, similar experiment was performed with *P. aeruginosa*, a bacteria which is known to produce a biofilm. Fig. 4(a) displays the S_{11} parameter as function of the frequency for different inoculation times. As for *V. natriegens*, S_{11} values are decreasing during the growth of the bacteria. The different initial values of the S_{11} parameter and the shape of the curves below 500 MHz between the two experiments is ascribed to the two different media used.

Fig. 4(b) shows the ΔS_{11} parameter as function of the incubation

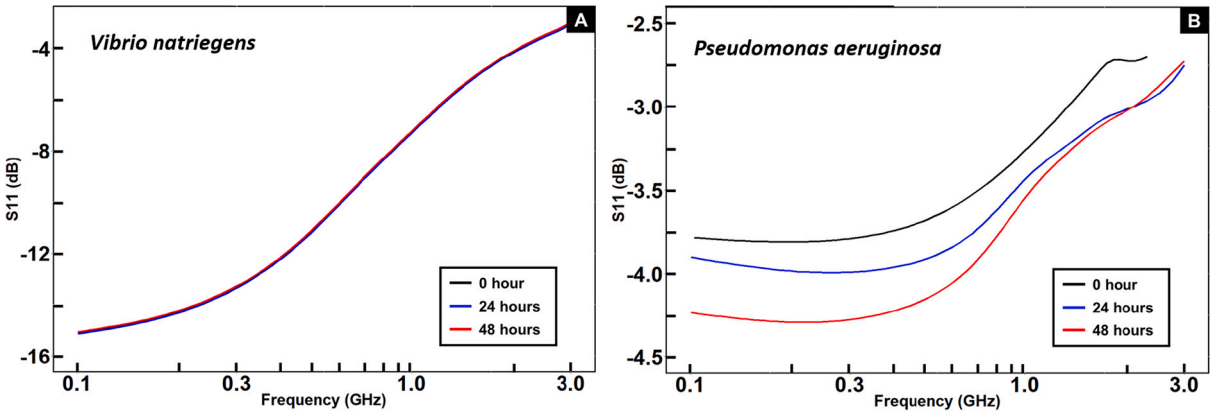

Fig. 5. Evaluation of the biofilm impact on the sensor response. The culture medium was replaced before each measurement to preserve only the attached bacteria.

time for several frequencies below 2 GHz. Initially, the variation is similar to the *Vibrio natriegens* one, with a decrease that slows down to a stationary phase after 6 h. However, at 7 h of incubation time, a further decrease clearly appears on the measurements. In contrast to bacteria growing, this process is a long time process and stops only after about 60 h of incubation for a stabilization (data not shown). Note that this further decrease can be observed only below 1 GHz due to the highest sensitivity of the sensor in this range. Fig. 4 (c) displays the growth curves of planktonic bacteria and biofilm. The growth of *P. aeruginosa* is a little longer than *V. natriegens* and lasts between 8 and 10 h, then a stationary phase appears with minor variations at a final concentration of 4.4×10^9 CFU/mL. The sensitivity for this bacteria is about 0.82 dB/log(OD_{600}) on the linear phase on the first 2 h. This difference with *V. natriegens* highlights the dispersion of results due to the nature of the cells studied, which had already been slightly discussed by Russel et al. [17]. Indeed, the physicochemical properties of the cells (size, membrane thickness, roughness) will strongly influence the measurements and can explain these differences in sensitivity which has not often been taken into account in studies.

In contrast to *V. natriegens*, this bacteria produce a large amount of biofilm. The SBF index starts to be significant from 6 h of incubation. The increase progressively slows down with time, showing a shelf at 48 h of growth. The different phases reported by the S_{11} parameter at low frequencies are therefore clearly related to changes in the growth of the bacteria and biofilm during the experiment. The rapid decrease during the first 6 h corresponds to the growth of suspended bacteria only. The next step is explained by the biofilm formation. Obviously, at 7–10 h incubation time, the two processes are active since it corresponds to the end of growing stage of bacteria and the start of biofilm formation.

These experimental results demonstrated that the sensor is thus able to track the growth of suspended bacteria and biofilm formation in a culture medium. To further prove the interest of the method, another experiment aiming at isolating the biofilm formation was carried out. Each bacteria was incubated during 48 h. In contrast to previous measurements, the culture medium was renewed just before the S_{11} measurements in order to simulate the effect of a flow cell. Fig. 5 shows the S_{11} values measured at 24 h and 48 h for both the bacteria. Fig. 5(a) corresponds to *V. natriegens* in the NB medium. The results indicate that there are no significant variation of S_{11}. Since the medium above the probe is similar for each runs, only attached bacteria, *i.e.* the biofilm has an influence on the S_{11} parameter. No biofilm formation is then confirmed. Concerning *P. aeruginosa*, results on the Fig. 5(b) indicate a significant drop of the S_{11} values over the frequency range below 1 GHz. Beyond 2 GHz, as displayed in Fig. 4a, the S_{11} parameters after 24 and 48 h are very close and there is even an inversion of the curves. However, this slight inversion is not significant due to the used process, which requires to renewal of the liquid. The decrease of the S_{11} over the 48 h is about 0.51 dB at 0.3 GHz which is very close to the variation of S_{11} measured in Fig. 4(b) from 7 h of growth to the end of the experiment. The impact of biofilm expansion on the sensor response is thus well established and this confirms that, on Fig. 4(b), the two growths are well identified. The slope was based on the evolution of the biofilm thickness, leading to a biofilm specific sensitivity equal to −0.012 dB/h at the frequency of 0.3 GHz.

4. Conclusion

The development of a sensor based on the electromagnetic waves reflection is presented for the monitoring of bacteria and biofilm growing. The principle of the sensor is based at about 300 MHz on the variation of the electrical conductivity (σ) or tangent loss (tan (δ)) while the influence of the dielectric permittivity on the data is negligible. Having the ability to detect biofilms in the early stages of formation is crucial. Key applications in aquatic microbiology are to find new antimicrobial treatments with low resistance rates to prevent the occurrence of disease, biofouling or bio-corrosion. This sensor brings a solution to this problem and could be used in many fields to support other techniques in biofilm prevention systems.

CRediT authorship contribution statement

Matthieu Longo: Investigation, Writing – original draft. **Stéphane Rioual:** Conceptualization, Writing – original draft, Supervision. **Philippe Talbot:** Investigation, Writing – original draft. **Fabienne Faÿ:** Conceptualization, Writing – original draft, Supervision. **Claire Hellio:** Conceptualization, Writing – original draft, Supervision. **Benoît Lescop:** Conceptualization, Writing – original draft, Supervision.

Declaration of Competing Interest

None

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