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An Impedimetric Sensor for Real-Time Detection of Antibiotic Resistance Genes Employing Rolling Circle Amplification

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In this paper, we present a method for the detection of antibiotic resistance genes belonging to the diverse group of β-lactamases based on real-time monitoring of a rolling-circle amplification (RCA), and a simple electrochemical sensor for capacitatively coupled contactless conductivity (C^4) measurements. Impedance spectra are presented to describe the underlying principles of the sensor and to find optimum operating conditions. Conductivity changes induced by the formation of amplification products were monitored. By comparing the time responses we were able to distinguish amplification reactions starting with DNA target concentrations as low as 0.42 nM from negative controls containing no target DNA.

Keywords: real-time amplification monitoring; impedance sensing; C^4 sensor, conductivity

1. INTRODUCTION

The next generation of nucleic acid (NA) based diagnostic test devices will deliver fast and reliable results at the point of care (POC) and enable rapid and effective treatment. Due to the limits of detection of existing NA biosensor technologies and the low abundance of target NA in clinical samples, a molecular NA amplification is required in most cases. Compared to conventional polymerase chain reaction (PCR), the instrumental setup for isothermal amplification techniques is much simpler, which makes them attractive for the integration in POC devices. Today, several such techniques exist, offering high amplification factors and excellent sensitivities [1]. The integration of existing amplification technologies with sample preparation, NA extraction and detection is probably the greatest challenge in nucleic acid testing POC product development.

In contrast to conventional end-point PCR, where the reaction products are detected after the amplification step is finished, real-time PCR, continuously monitors the generation and accumulation of the PCR products. Today, real-time amplification monitoring is usually performed with optical

detection methods involving complex architectures and relatively expensive instrumentation. Because of their simplicity, and especially in view of the demands for an increased level of system integration in future POC devices, electrochemical sensors are an attractive alternative for real-time NA amplification monitoring. Within the last years, significant improvements have been achieved with methods detecting the presence of NA in the sample volume [2-5] as opposed to conventional electrochemical methods based on surface functionalized electrodes [6]. Most of these approaches make use of intercalating redox reporters, whose activity at the electrode surface changes with progressing NA polymerization. Detection limits in the order of 10^3 DNA copies and below have been reported using electrochemical amplification monitoring, a recent overview is given in [7].

An alternative way to monitor the amplification process is by measuring the electrical conductivity of the amplification mix. Jiang *et al.* [8] demonstrated the real-time measurement of DNA amplification in a loop-mediated isothermal amplification (LAMP) reaction, using a sensor with gold-coated electrodes to monitor the conductivity of the LAMP-mix during the reaction. By addition of cationic dyes to the mix which were bound to the NA molecules, they were able to optimize their measurement sensitivity. Fang *et al.* used contactless impedance measurements with passivated electrodes to monitor strand-displacement and RCA [9,10]. Their sensor system was fabricated employing microengineering techniques comprising thin film interdigitated electrodes and a silicon microfluidic chip with integrated thin film heater and temperature sensor.

In this paper, we describe a simple impedimetric sensor and demonstrate its suitability for online monitoring of RCA in a standard 200µL Eppendorf tube.

2. THEORETICAL BACKGROUND OF C^4 SENSORS

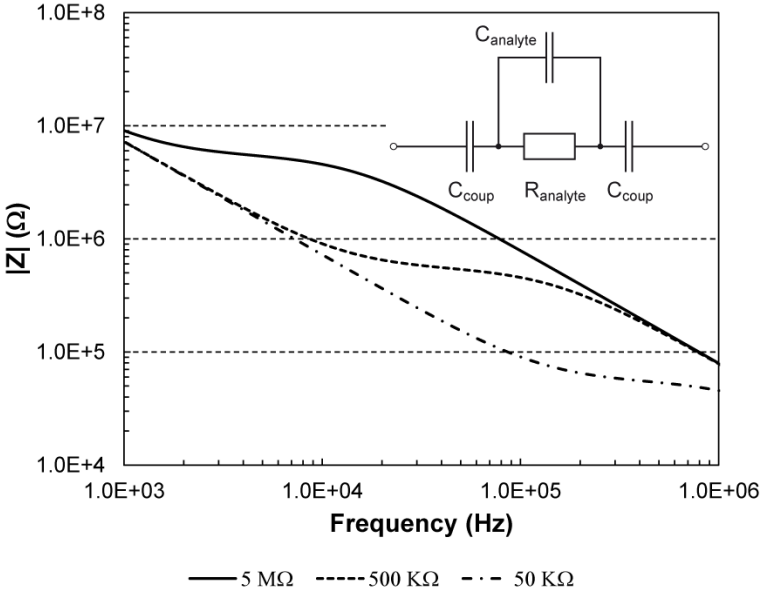

Figure 1. Calculated impedance spectra of a C^4 sensor. Values for C_{coup} and $C_{analyte}$ are close to those found experimentally in our sensor (44 pF and 2.2 pF, respectively). Spectra were calculated using Matlab.

Capacitatively coupled contactless conductivity (C^4) sensing is a relatively new method to determine the conductivity of liquids by measuring the electric impedance between two passivated electrodes which are immersed in the analyte [11]. At the moment, C^4 sensors are mainly applied and studied in analytical chemistry for measurements of ionic concentrations in capillary electrophoresis [12].

As compared to conventional conductivity measurement systems employing electrodes in direct electrical contact with the analyte, passivated electrodes have the advantage to avoid measurement errors caused by corrosion or polarization effects at the electrode surfaces. This allows the creation of reliable and reusable conductivity sensors which do not require extensive chemical or electrochemical electrode cleaning before use.

The major shortcoming of C^4 sensors is that a measured impedance value is not simply determined by the conductivity of the analyte but also by the capacitive components and the measurement frequency. A typical equivalent electric circuit is depicted in the inset of Figure 1.

The ohmic resistance of the liquid ($R_{analyte}$) is connected to the electrodes via the coupling capacitance of the passivation (C_{coup}). $C_{analyte}$ represents the capacitance of the liquid. At low frequencies, the total impedance Z is determined by C_{coup} and decreases accordingly inversely proportional with the frequency, until a point is reached, where the impedance of C_{coup} becomes smaller than $R_{analyte}$. In this frequency range, the impedance spectra show a characteristic shoulder where the impedance takes values close to $R_{analyte}$. For even higher frequencies, $R_{analyte}$ is shunted by $C_{analyte}$ and the impedance is – just as in the low-frequency range – independent of the conductivity of the analyte.

These fundamental properties of C^4 sensors limit their dynamic range and require a proper choice of the measurement frequency. The interrelationship between the suitable frequency range and the conductivity of the analyte therefore makes a detailed spectral characterization of C^4 sensors indispensable for their successful implementation in a measurement system.

3. SENSOR FABRICATION AND METHODS FOR CHARACTERIZATION

The sensor was custom-made for the detection of NA amplification in a 200µL Eppendorf tube (Fig. 2). Holes were drilled into the lid to create feedthroughs for the electrodes and to fix a breadboard to the lid for better handling. Polyurethane enameled copper wire was used to fabricate the electrodes. The thickness of the insulation was examined with a scanning electron microscope and was found to be approximately 6 µm. Two wire loops were formed and inserted into the tube. One end of each loop was fixed on the top of the breadboard without making contact. The other end was electrically connected to an Agilent 4284A precision LCR-Meter, which we used for sensor readout. The maximum frequency of this instrument is 1 MHz. A DIP-switch was placed between the measurement cable leading to the LCR-meter and the electrodes, allowing open/short measurements to compensate for the impedance of the cable. All impedance data presented in this paper are corrected. In order to protect the electrodes and to improve the robustness of the sensor, two glass capillaries were placed over the electrodes and fixed to the lid.

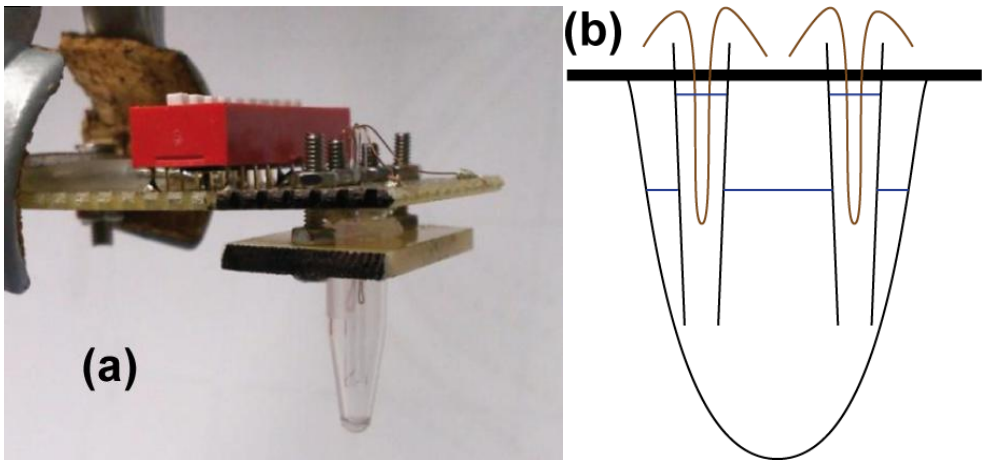

Figure 2. (a) Photo and (b) schematic representation of the sensor.

Nucleic acid amplification products were analyzed with gel electrophoresis using a SYBR Safe DNA stained 2% agarose gel. The pockets of an electrophorese chamber were filled with 8 µL of DNA samples. Electrophoresis was carried out at 160V for 50 minutes, the results were visualized with a UV-imaging system (BioSpectrum 310).

4. RESULTS AND DISCUSSION

4.1. Preliminary sensor tests

Several solutions of KCl in deionized water were prepared serving as test analytes with different conductivities for a general sensor characterization. The sensor was also tested with φ29 buffer (Fisher Scientific Austria GmbH), which is a constituent of the RCA mixture we later used for our real-time monitoring experiments. Since this buffer dominates the conductivity of the RCA mixture due to its high ionic concentration, we used it here to verify the feasibility of the sensing concept for the purpose of real-time RCA monitoring.

The Eppendorf tube was filled with 180µL of the test analyte and the sensor was mounted. Before each measurement, the passivation was tested with an ohmmeter. The values for the DC-resistance between the electrodes were typically well above 100 MΩ. To avoid any electrochemical reactions on the electrode surface, impedance spectra were measured with an excitation voltage of 10mV. After each measurement, the sensor was thoroughly rinsed with deionized water and blown dry.

The impedance spectra for the various test analytes are depicted in Figure 3 showing the expected characteristics discussed above. For a 10 mM KCl solution, the impedance has a distinctive plateau at 210KΩ for frequencies around 85 kHz (Fig. 3a), where the phase attains its maximum (Fig. 3b). This shows a high contribution of the ohmic resistance of the liquid to the total impedance, as opposed to the left and right hand side, where the capacitive components are dominant and the phase is closer to -90°. The spectra of 10 mM KCl solution and φ29 buffer are very similar. Due to their higher conductivities, the impedance plateaus are lower and the phase maxima are shifted to the right hand side of the spectrum. Suitable frequencies for conductivity measurements can be estimated to lie above

the onset of the shoulder at approximately 200 kHz. The conductivity of 1 M KCl is almost too high to be measured within the given frequency range. Although the increase of the phase indicates the onset of capacitive coupling to the solution, the impedance spectrum is almost a straight line. Higher measurement frequencies would be required to access this conductivity range with our sensor.

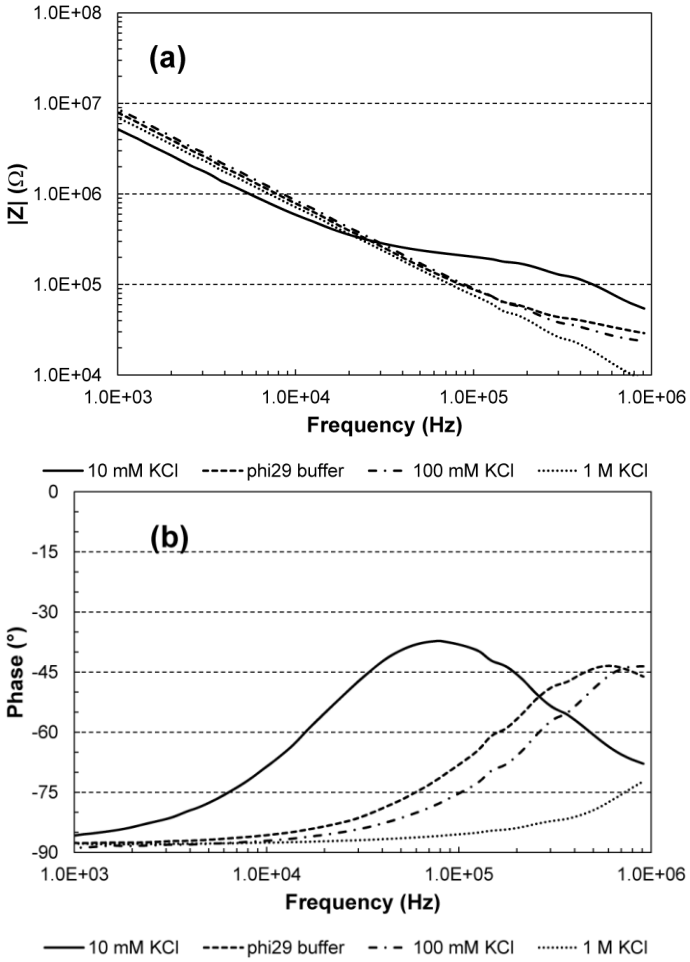

Figure 3. Measured impedance spectra for analytes with different electrical conductivities. (a) Absolute value of the impedance, (b) phase angle.

4.2. Real-time monitoring of RCA

To demonstrate real-time monitoring of NA amplification, we used an assay that has been recently developed in our group for the multiplex detection of β-lactam antibiotic resistance genes. The experimental details of this 100-plex reaction detecting the 31 clinically most important β-lactamase genes are described in a previous publication [13]. In short, this assay is based on padlock probes which are ligated in the presence of a DNA target and subsequently amplified using RCA. Based on the detected resistance genes, the β-lactam susceptibility of the analyzed clinical isolate can be predicted and the antibiotic treatment optimized [14]. To use the impedimetric sensor for pathogen characterization in multiplex reactions, all padlock probes targeting the β-lactamase genes that confer resistance to the same antibiotic class are pooled in a single reaction mixture (e.g. the carbapenemase

genes bla_{KPC}, bla_{VIM}, bla_{OXA-48}, bla_{NDM}, bla_{IMP}, etc. responsible for resistance against the antibiotic class of carbapenems). If any of the β-lactamase genes is present in the sample, then the sensor will give a positive signal and indicate resistance against the corresponding antibiotic class.

In the experiments, 176.5 µL of the RCA reaction was filled in the Eppendorf tube. The positive controls contained 8.3 nM synthetic target DNA in the amplification reaction. Negative controls without target DNA were used to verify the results. The tube was placed in a thermo-mixer set to 37°C, the lid with the sensor was mounted, and the impedance measurement was started. Spectra comprised 20 different frequencies in the range from 100 Hz to 1 MHz, the measurement time of a single scan was approximately 12 sec. After some drift during thermalization, the impedance spectra remained stable. NA amplification was triggered by pipetting 3.5 µL of φ29 polymerase through a small hole in the lid. After approximately 45 minutes, the measurement was terminated, and the sensor cleaned as described earlier. After the amplification was stopped by a 2-minute incubation at 65°C, which inactivated the of φ29 polymerase, a total of 20 µL of the reaction products was taken from the tube to control the presence of amplification products. To further enhance the DNA concentration, a digestion step was performed, followed by a second RCA as described in [13]. The obtained product was then analyzed via agarose gel electrophoresis.

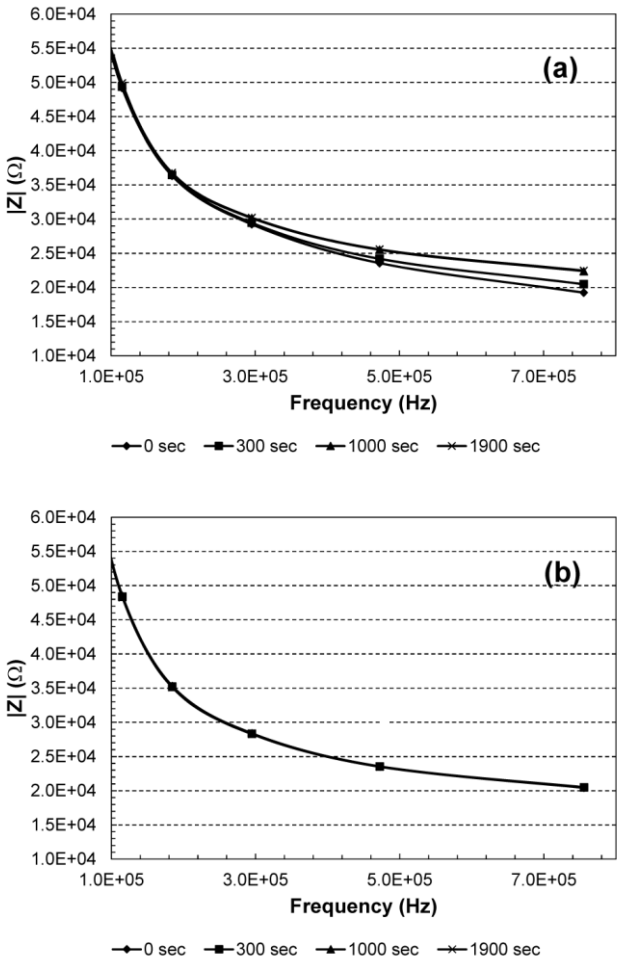

Figure 4. Impedance spectra measured at different reaction times for positive samples (a) and negative controls (b).

Impedance spectra taken at different points in time after the addition of the polymerase are shown in Figure 4. For a positive sample (Fig. 4a), the impedance increases in the first minutes of the reaction. The impedance change becomes more significant with increasing measurement frequencies. Below 200 kHz, the sensor is not sensitive. In contrast to the positive sample, the impedance spectra remain constant in time for a negative control (Fig 4b).

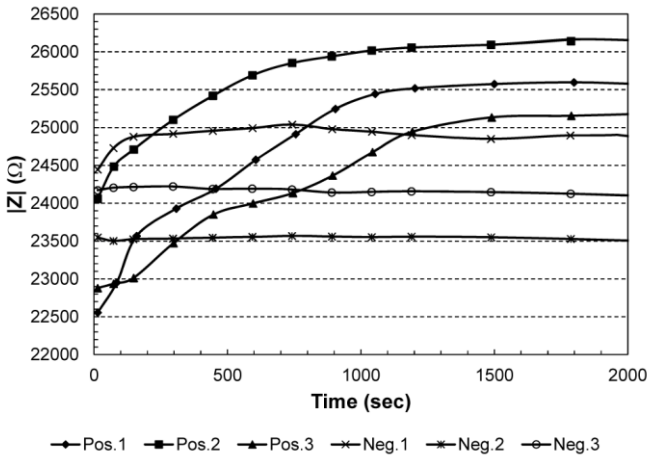

Figure 5. Time dependence of the measured impedance during RCA for three positive samples and three negative controls at 472 kHz.

A more detailed picture of the temporal evolution of the impedance at 472 kHz is given in Figure 5 for three positive samples and three negative controls. All positive samples show a significant decrease of the conductivity of the RCA mixture during the amplification reaction. After approximately 1200 seconds, the impedance becomes constant, indicating the end of NA polymerization. The findings from the conductivity measurements are in good accordance with the results from gel-electrophoresis shown in Figure 6.

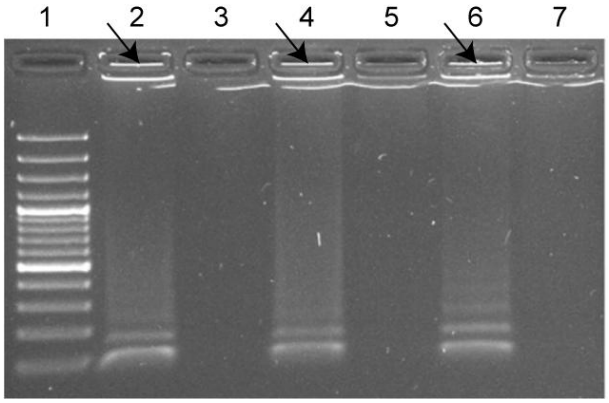

Figure 6. Image of agarose gel-electrophoresis; 1:100 bp DNA-Ladder, upper bright bar: 1000 bp, lower bright bar: 500 bp. 2, 4, 6: Positive samples; 3, 5, 7: Negative controls. Amplification products only occurred in positive samples. Long single-strand DNA can not enter the gel and remains in the pocket (indicated by arrows). Shorter single-strand DNA fragments of various lengths were created in the digestion step and appear as multiple bands.

The observed decrease in conductivity can be explained by a net decrease of the mobility of the NA components as the deoxyribonucleoside monophosphates are polymerized to form bigger DNA molecules.

It needs to be stated, however, that this explanation is speculative and needs further clarifying. The experimental results and models in literature are contradictive. In contrast to our results, Fang *et al.* [9,10] observed an increase in conductivity for different amplification methods. This increase was ascribed to the release of a pyrophosphate ion and hydrogen ions each time a deoxyribonucleoside monophosphate is added to an amplicon from a corresponding deoxyribonucleoside triphosphate. Jiang *et al.* [8], however, pointed out the variability of their experimental curves with temperature, pH and ionic concentration. In general, they observed a decrease of the conductivity, but some of the measurements presented in their work also showed the opposite tendency.

The dependence of the impedance change on the concentration of target DNA before starting the amplification (Fig. 7.) reveals a sharp linear increase for small concentrations ranging from 0 to 1.0 nM. For higher concentrations, we observe a nonlinear behavior. The smallest concentration we used in our experiments (0.42 nM) corresponds to a 2.1% impedance rise which is clearly distinctive from the negative controls (0.15%). While this value compares well to the 1 nM target DNA concentration Fang *et al.* [10] were able to detect in their microfluidic setup with an impedimetric sensor, significant improvements of the sensitivity will be required to make the system fit for practical use. Higher sensitivities using real time monitoring based on conductivity measurements have already been demonstrated by Jiang *et al.* [8], who were able to detect the presence of 10 CFU/ml of *Escherichia coli* in a LAMP reaction. We believe a careful optimization of the assay parameters will be required to fully exploit the potential of the detection principle. In general, while maintaining a high efficiency of NA amplification, ionic background concentrations should be reduced to maximize the effect of amplification product formation on the electrical conductivity. Further studies will also be required to clarify the reason for the nonlinearity of the concentration dependence. At present, we assume that a misbalance between the high concentrations of target DNA and φ29 polymerase may be responsible for this behavior.

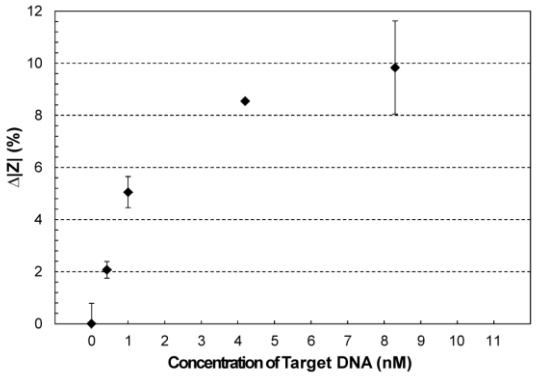

Figure 7. Impedance change after 45 minutes amplification time for different DNA target concentrations. The measurement frequency was 472 kHz. The error bars indicate the standard deviation of at least 3 different measurements, except for the point at 4.2 nM, which is a single measurement.

5. CONCLUSION AND OUTLOOK

A simple and reusable impedimetric sensor for conductivity measurements in liquids was fabricated and the frequency dependence of the sensor output was characterized. We successfully demonstrated the label-free detection of antibiotic resistance genes by real-time monitoring of a rolling-circle amplification. The sensor responded reproducibly to the decreasing conductivity of the medium, and can be used to optimize the sensitivity of assays based on real-time conductivity monitoring in isothermal amplification reactions.

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