Impedance-based detection of extracellular DNA in wounds

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Abstract. Wound infection status is a relevant diagnostic parameter to enhance wound treatment towards better healing rate. Impedance evaluation is a powerful tool to measure the inflammatory response like the released DNA of neutrophils. In our research we investigated the dielectric behaviour of neutrophils settled on electrodes in vitro. The cells have been stimulated to react in the same way as in a wound infection. The result is a significant impedance deviation of about 50\% with comparable amount of cells like in an infected wound. Microscopic fluorescence verifications acknowledge these findings.

1. Role of extracellular DNA in wound healing

Evaluation of wound healing status is an ongoing issue in clinical praxis, especially considering the treatment of chronic wounds. Wound infections are one of the most feared complications because of the threat of invading pathogens in the blood, which possibly lead to systemic inflammatory response syndrome and finally to death [1]. An infection of a wound goes by with several immune reactions. Main actors are the leukocytes, e.g. granulocytes and monocytes, which invade the wound. One of the first reactions on pathogens is the release of deoxyribonucleic acid (DNA) packaged in chromatin by neutrophil granulocytes [2]. These neutrophil extracellular traps (NETs) are able to capture some bacteria and fungi to avoid further spreading. NETs are nearly always released, when a granulocyte senses a pathogen. They consist mainly of dispersed chromatin with double-stranded (ds) DNA, histones and other antimicrobial proteins [3]. Due to the massive release of NETs to the exudate and the immediate reaction of the neutrophils, NETs are an excellent marker. A technique to detect extracellular DNA would give the physicians a proper diagnostic value to decide whether an infection is going on in a wound. This again would lead to an improvement of wound treatment.

2. Impedance measurement in wounds

The impedance of wound fluid is affected by the different constituents and their dielectric behaviour. The constituents vary with the wound status and can be divided in three categories, which seem interesting from a dielectric point of view: ionic, molecular and cell components. As the origin of the exudate is blood, in the non-infected stage the exudate composition is close to blood plasma. During an infection, which means mainly an accumulation of harmful pathogens, leukocytes are enriched in the exudate. The first arriving neutrophils immediately form NETs. An impedimetric infection sensor must be sensitive to the dielectric properties of NETs and their components, mainly the DNA with bound proteins. The leukocytes itself and the varying ion strengths during the immune response affect
the measurement, too. The dielectric behaviour of dispersed DNA has been studied in various publications beginning from the 1980s [4] up to now, e.g. [5][6]. Most publications concentrate on DNA-detection principles based on hybridization, labelling, specific binding and combinations of these in lab environment. In vivo detection is more challenging due to the impure natural form of neutrophil derived DNA with bound proteins. Nevertheless, the basic dielectric behaviour of DNA is also of interest. To understand the dielectric behaviour it is important to know that DNA has a strong negative charge with a positive counter ion shell. The first impedance characterization experiments for DNA were done by Sakomoto et al. [4]. They found a low frequency dispersion (in the range of some Hz) of double-stranded DNA, which was later explained by Molinary et al. as a displacement of the counter ions around the polymer backbone of DNA [7]. A second dispersion between 0.5 to 70 kHz was described by Tomić et al. as a result of counter ion relaxations along a single DNA chain [8]. A third, smaller dispersion can be explained as a hopping of counter ions between DNA chains of one DNA molecule [8][9]. It occurs in the range between 0.1 MHz and 15 MHz.

As NETs do not consist of condensed DNA - ds DNA can be elongated over some hundreds of nanometres - only the interactions directly related to a single DNA stretch can be taken into account. One fibril of NETs contains several DNA chains in an uncommon conformation. Mainly the second dispersion in the medium kHz range is expected to encounter again. Before NET-release, the highly non-conductive cell membrane shields the intracellular space. Especially in the low frequency range dielectric behaviour is dominated by cell membrane effects. This again leads to the α-dispersion in the frequency range from a few mHz up to several kHz in cell suspensions [10]. This permittivity increment is caused mainly by negatively charged cell membranes and is therefore not dominant for eukaryotic cells [11]. The β-dispersion occurs up to several hundred MHz and results from capacitive effects on the cell membranes [12]. Dielectric characteristics from both cells and extracellular DNA are expected in pathologic wound exudate.

3. Sensor concept and parameter extraction

Measuring impedance with impedimetric methods has several advantages. Comparing to other methods, like optical or other electrochemical methods, this method is easy to integrate, has a low power consumption and demands only low-price and biocompatible materials. Direct electric measurement on the body with a low current and the cross-sensitivity to other body tissues also lower the signal quality. Nevertheless, a low current avoids a spreading of the electric field in the surrounding tissue and enables a more local measurement in the wound. Therefore, sensor design is a crucial issue. The integration of the wound sensor in a miniaturized chip enables a connection with a RFID structure. An antenna can easily be integrated in a flat wound cover. Modern wound care products come along with hydrogels and hydrocolloids as absorbing layers [13]. Such materials serve as a passive delivery system. Figure 1a displays how such a sensor can be integrated into the wound cover. To develop an impedimetric wound sensor, a suitable parameter of interest has to be found. Therefore, in a first step, we started with cell cultures of neutrophils in a commercial electrode-well system as shown in Figure 1b. As a model environment we used cultivated human neutrophils and stimulated them to form NETs. Using cultivated cells causes limitations with regard to reliability. However, such cells are easily available and animal tests can be avoided at this stage.
4. Impedance characterization of a wound model

The first step to get impedance spectra from NET-forming neutrophils is the isolation of the cells from human blood. It is done by separating the cell constituents, repeated erythrocyte lysis and centrifugation steps. For the impedance characterization the electrodes (Roche xCelligence®) were coated with poly-L-lysine. For fluorescence verification and for impedance measurements, $5 \times 10^4$ and $2 \times 10^6$ cells were seeded on top, respectively. We used RPMI medium with donor serum as a cultivation medium. For fluorescence verification incubation took 1 hour, else 4 hours. Cells were then stimulated with phorbol 12-myristate 13-acetate (PMA) medium, which was exchanged. A self-made adapter connects the electrodes with an impedance analyzer (ScioSpec ISX-3). Impedance was measured in the range between 100 and 1 MHz using an excitation amplitude of 12 mV. For a microscopic verification experiment we stained the DNA with Sytox Green fluorescent dye and took images at constant exposure time of 500 ms with a Zeiss Axio Vision Camera mounted on the microscope. The NETs were then quantified using a self-written threshold and object labelling algorithm in Matlab.

Stimulation of neutrophils with the chemical stimulant PMA leads to an immediate and drastic impedance rise in the range of 30 to 60% of the starting value as shown in Figure 3a. After changing the cell medium in the negative control culture the impedance stays nearly constant with deviations of less than 5%. The time course strongly depends on cell number as it is shown in Figure 3b. For lower cell numbers, a second maximum occurs at the very beginning of stimulation. We interpret this as a cascade effect of NET formation and the diffusion of the stimulant through the cell layer. For $5 \times 10^4$ cells, the cell layer roughly consists of 1 to 2 cells. For $2 \times 10^6$ cells the neutrophils are in a closed thick layer. Therefore, some neutrophils sense the stimulant later, which results in a continuously rising impedance. For $5 \times 10^4$ cells, the maximum deviation after 1 to 3 hours is more consistent with the literature value of beginning NET formation [3].

Fig. 3: a) Time course of impedance change over frequency for $2 \times 10^6$ stimulated neutrophils (little box: unstimulated control) and b) comparison of impedance changes at 20 kHz for $2 \times 10^6$ and $5 \times 10^4$ neutrophils.
A comparison of these results with fluorescent microscopic images indicates a dependency between the impedance change and the NET area measured in the same wells. Figure 4 displays this correlation. Strong deviations are near the maximum of fluorescence NET area and impedance change, respectively. Before stimulation (time $t = 0$) a NET area is already measured. Microscopic observations confirm the presence of NETs before stimulation. We explain this with the harsh preparation steps for granulocytes which result in a pre-stimulation. This explains the existence of NETs in unstimulated samples which cannot be seen in the impedance deviations. Decomposition is observed in both stimulated and unstimulated samples.

![Fig. 4: Impedance change vs. fluorescent area of NETs. Base values for the relations are the maximum values (99.3 Ohms and 403,044 pixels).](image)

5. Conclusions
NET-formation of neutrophil granulocytes resulted in a significant rise in impedance at around 20 kHz. A comparison with fluorescent evaluation indicates a significant relationship. But, this fluorescent verification has to be enhanced by a higher time resolution and an object recognition. One aim will be to understand the NET-specific dielectric mechanisms which lie behind the deviations in impedance behaviour. This can be achieved by detection of the NET formation phases of different in fluorescent images and a correlation with impedance measurements. To reach a higher specificity for the wound sensor we also concentrate our investigations on other measurands, e.g. pH, temperature and elastase.

References
[1] Gardner SE, Frantz RA and Doebbeling BN 2001 Wound Repair Regen. 9 (3) 178–86
[2] Martin P and Leibovich SJ 2005 Trends in Cell Biology 15 (11) 599–607
[3] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss D, Weinrauch Y and Zychlinsky A 2004 Science 303 (5663) pp 1532–35
[4] Sakamoto M, Fujikado T, Hayakawa R and Wada Y 1980 Biophys. Chem. 11 (3–4) 309–16
[5] Berdat D, Rodriguez ACM, Herrera F, Gijs MAM 2008 Lab. Chip. 8 (2) 302–8
[6] Lillis B, Manning M, Hurley E, Berney H, Duane R, Mathewson A and Sheehan MM 2007 Biosens. and Bioelectron. 22 (7) 1289–95
[7] Molinari RJ, Cole RH and Gibbs JH 1981 Biopolymers 20 (5) 977–90
[8] Tomić S, Babić SD, Vuletić T, Křeč S, Ivanković D, Grgurić L and Podgornik R 2007 Phys. Rev. E 75 (2) 21905
[9] Bone S and Small CA 1995 BBA - Gene Structure and Expression 1260 (1) 85–93
[10] Schwan HP 1957 Adv Biol Med Phys 5 147–209
[11] Davey CL and Kell DB 1995 Bioelectrochemistry of Cells and Tissues 159–207
[12] Schwan HP 1999 Annals of the New York Academy of Sciences 873 (1) 1–12
[13] Lippert H (ed.) 2006 Wundatlas. Kompendium der komplexen Wundbehandlung (Stuttgart Thieme)