A Novel Sample Preparation Concept for Sepsis Diagnostics Using High Frequency Electric Fields

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Abstract— **Despite their high potential to replace the time consuming gold standard blood culture for infection diagnosis from whole blood, molecular biology based techniques often lack sensitivity and specificity due to the high human background in complex biological samples such as blood. Concerning the realization of a point-of-care device for sepsis diagnosis which would enable immediate targeted antimicrobial therapy, new, implementable and scalable sample preparation strategies are urgently needed that aim at reducing the human background. In this paper we report a first proof-of-principle for such a novel concept of selective human blood cell depletion preserving bacterial pathogen integrity by using high frequency electric fields. By thin film electrical passivation of the electrode material, ohmic current through the biological sample, which causes unspecific cell lysis, is drastically reduced. With the developed SiO_2-passivated electrical cell lysis unit (ECLU), cell specific lysis of up to 75.3 % of the total human blood cells could be shown with less than 10 % loss of *E. Coli* viability. Additionally, a small electrode distance of 25 µm reduced the required voltage for selective cell lysis to a range of 8-20 V. The results presented here show great potential for the further development of fully automated and integrated cell selective sample preparation.**

Keywords— **Infection diagnosis, sample preparation, cell specific, cell lysis, electric fields**

I. INTRODUCTION

Bacterial infections are one of the leading causes of death worldwide and the incidence of sepsis has been continuously increasing, tripling the number of sepsis-related deaths over the past 20 years [1]. Currently, the majority of bacterial infection diagnoses are conducted via blood cultures, which can take many hours, typically even days. For bloodstream infections a study carried out by Kumar et al. showed, that already 5 hours after the occurrence of first symptoms of septicaemia the mortality rate rises to 50 % if no appropriate therapy is initiated [2]. With respect to the occurrence of antibiotic resistant bacteria and the need for targeted therapy, this underlines the enormous need for fast and accurate diagnosis. Recently, molecular biology based techniques for pathogen detection have found their way into clinics. With the advantage of detecting pathogens which are difficult to culture, the nowadays used molecular testing kits share important disadvantages with the "gold standard" culture method: they are both very laborious, time- and reagent consuming and often lack sensitivity. To address some of these challenges a lot of research was conducted over the past 5-10 years to incorporate molecular biology based techniques into a fully automated point-of-care (POC) device [3,4,5,6]. Those systems still require the development for full automation and the transfer to whole blood. The biggest pitfall when using whole blood is however low sensitivity (LOD $>>10^2$ bacteria/ml blood containing ~$5x10^9$ eukaryotic cells), primarily caused by the high human background and sub-optimal sample preparation. Thus, different sample preparation strategies to increase sensitivity are currently investigated, concentrating on the separation of eukaryotic and prokaryotic cells previous to bacterial cell lysis e.g. by size exclusion, dielectrophoresis or antibody-coupled beads [7,8]. One commercially successful development using eukaryotic specific chemical and enzymatical cell lysis prior to bacterial lysis is the MolYsis Kit from Molzyme, that has shown to significantly increase the specificity and sensitivity of PCR results [9,10]. However, such multi-step chemical lysis strategies are time-consuming and labor intensive and it is difficult to integrate those methods into a fully automated POC device. In this work we present a novel strategy for specific human cell depletion in a bacteria-spiked blood sample. High electric fields are applied to cause cell lysis as a consequence of an excessive transmembrane potential (TMP), which is cell size and cell-morphology dependent [11,12,13]. We use this dependency to induce different TMPs for different cell types enabling blood cell specific lysis in a heterogeneous sample, while keeping the bacteria to be analyzed viable.

II. MATERIALS AND METHODS

A. Cell Preparation and Handling

Blood was withdrawn from a healthy volunteer and the ethics committee of the city of Vienna approved the collection and experiments. Collection was performed with the K2EDTA Vacutainer system from BD. Blood samples were immediately stored at 4°C and used for a maximum of 5 days. For the experiments, whole blood was diluted 1:100 in 250 mM sucrose in ultrapure water.

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Kanamycin resistant *E. coli* were grown over night in Luria-Bertani (LB) medium with 50 µg/ml Kanamycin sulfate in a shaking incubator at 37°C the day before the experiments. Next day, bacterial cell counts were determined by measuring the absorbance at 600 nm using a NanoDrop. Dilutions were made in 250 mM sucrose in ultrapure water and the final concentration of *E. coli* spike-in in the 1:100 whole blood dilution was adjusted to 2-5x10^2 cells per 15 µl treatment volume. After the experiments, samples with spiked-in *E. coli* were plated on LB Agar with 50 µg/ml Kanamycin sulfate and incubated over night at 37°C.

B. Device Fabrication

For the bare gold electrodes, 5 nm chrome and 200 nm gold were deposited on microscope glass slides using high-vacuum thermal evaporation.

Polymer passivated electrodes were fabricated by spin coating a square-cut printed circuit board (PCB) substrate with AZ6612 photoresist at 5000 rpm for 1 minute followed by a post bake at 125°C for 50 seconds on a hotplate. To ensure uniform thickness of the passivation layer, the outer 2 cm were cut-off after coating.

For the SiO_2 passivated electrodes, P/Bor doped 2" Si-wafers (0,01-0,02 Ohm*cm) with a 50 nm thermal oxide layer were diced to form square electrodes (Active Business Company GmbH). Contacts were created on the backside of the wafer by removing the thermal oxide in hydrofluoric acid. To reduce the semiconductor/metal interface resistance, the silicon surface was mechanically roughened with sand paper prior to the deposition of 5 nm chrome and 200 nm gold by high-vacuum thermal evaporation. The wafers were then mounted on massive aluminum blocks using conductive silver paste.

For all experiments, a 25 µm PEEK foil was used as electrode spacer framing a 20 µl chamber in the middle of the electrode surface.

C. Electroporation and Data Analysis

A function generator (Rigol 4102) connected to a voltage amplifier (Falco WMA-300) was used as a power source. Voltage and current (via a 1 or 0.1 Ohm resistor as indicated in the Figure captions) was monitored with an oscilloscope (Rigol DS1104B) as seen in Figure 1.

For the experiments, 15 µl of a 1:100 blood dilution in 250 mM sucrose in ultrapure water with and without spiked-in *E. coli* were manually pipetted onto the bottom electrode. The PEEK foil and the upper electrode were manually assembled and fixed in position. A high frequency voltage of 1 MHz with varying amplitudes was applied for 3 seconds or 1 minute for the gold or the passivated electrodes, respectively. After treatment, the sample was aspirated from the electrodes. For blood cell lysis analysis, the cell suspension was transferred to a Hemocytometer and bright field images were taken with an inverted microscope equipped with a digital camera. Cell counts were done using ImageJ's automatic particle count [14].

Spike-in experiments were evaluated by colony count.

III. RESULTS

Planar 2D electrodes in a batch design with an electrode gap of 25 µm showed highest practicality due to several reasons. First, planar 2D electrodes are simple to manufacture and guarantee a uniform electric field. Second, the batch design allows convenient electrical parameter evaluation as it reduces influences from fluidic parameters such as flow rate or inhomogeneous field effects at the fluidic in- and outlets. Third, an inter-electrode distance of 25 µm reduces the required voltage to generate the high electric fields needed for cell lysis. Additionally, a low electrode distance reduces possible side effects of multilayer cellular alignments between the electrodes [15].

After evaluation of different electrical parameters, a high frequency alternating current (AC) square wave signal was found to be most efficient for sudden cell rupture. First parameter tests indicated higher lysis efficiency using square wave signals of 1 MHz (data not shown).

A. Non-passivated Electrodes

First experiments with blood samples and spiked-in *E. coli* were performed with planar gold electrodes. Figure 1 shows efficient lysis of human blood cells already at applied voltages of 7 Volts. With the used chamber design, this corresponds to electric field strengths of 2.8 kV/cm. This value is comparable to literature data in respect to erythrocyte cell lysis [16,17]. Surprisingly, also the spiked-in *E. coli* were inactivated at electric field strengths as low as 4 kV/cm resulting in a reduction in the number of colony forming units of more than 60 % compared to the 0 V control. Figure 1 shows the lysis efficiency and the voltage and current characteristics of electrodes in direct contact with the fluid sample. The electrical cell lysis unit (ECLU) comprising of two bare electrodes has RC-characteristic, where the current is in phase with the voltage, which means that the dominant load is due to ohmic losses through the liquid. This high current leads to a drastic temperature increase already after less than 3 seconds that is generated in the ECLU by joule heating.

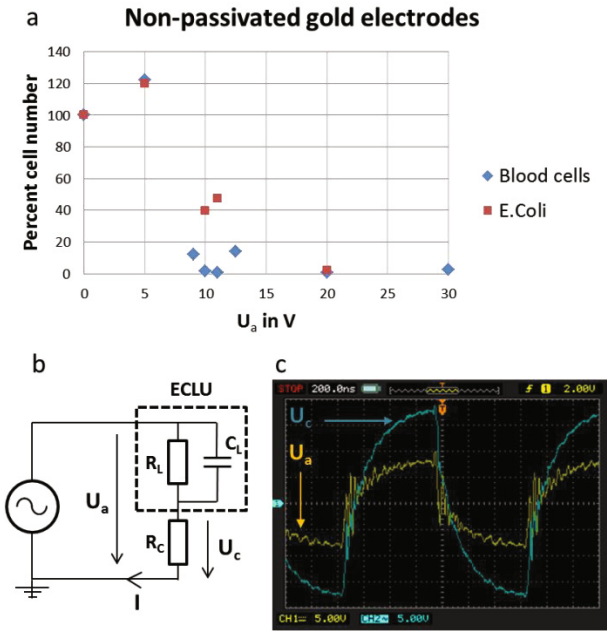

Fig. 1 (a) Non-specific lysis of human blood cells and spiked-in E. coli using bare gold-electrodes. (b) Measuring circuit. ECLU: Electric cell lysis unit, UA: applied voltage, UC: voltage proportional to current, I: current, RC: resistor for current measurement, RL: resistance of lysis cell, CL: capacitance of lysis cell. (c) Voltage and current plot, Uc is proportional to the total current I (voltage at a 1 Ohm resistor in series to the electroporation cell).

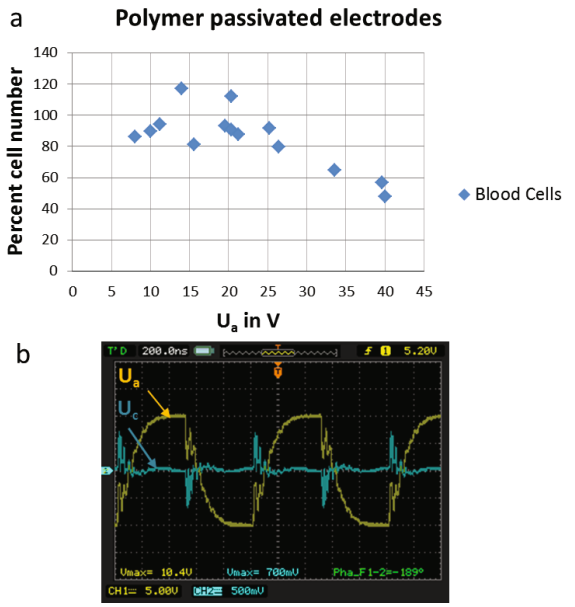

Fig. 2 (a) Voltage-dependent lysis of human blood cells using copper electrodes with a 1 µm polymer passivation. (b) Voltage and current plot, Uc is proportional to the total current I (voltage at a 0.1 Ohm resistor in series to the electroporation cell).

B. *Polymer Passivated Electrodes*

To inhibit joule heating of the sample, the amount of ohmic current passing through the sample was minimized by electrically insulating the electrodes. We used a photo-resist polymer (AZ6612) of 1 µm thickness to cover planar copper electrodes from a standard printed circuit board (PCB). As shown in Figure 2, ohmic current through the sample was significantly reduced as can be seen by the capacitive current characteristic. Voltage dependent reduction in total cell number compared to the 0 V control was observed, however the lysis efficiency of human blood cells was significantly reduced compared to the bare electrodes. Even amplitudes of 40 V resulted in just 52.15 % cell number reduction. This reduction in lysis efficiency can be explained by the capacitive reactance of the coating responsible for a significant voltage drop. The impedance of the liquid and the coating impedances represent a voltage divider where the voltage across the liquid sample and thereby the electric field experienced by the cell is reduced.

C. *SiO_2-Passivated Electrodes*

To increase lysis efficiency at lower voltages, the capacitive reactance of the passivation layers needs to be reduced, which can be achieved by reducing the thickness. The ECLU was therefore designed with heavily p-doped silicon wafers with a 50 nm thin SiO_2 passivation layer. As shown in Figure 3, voltage dependent lysis of blood cells can be observed at lower voltages in comparison to the polymer passivated electrodes. Additionally, as can be seen in the voltage and current plot, ohmic current through the sample is strongly reduced. With this set-up cell-type specific lysis of human blood cells without significantly comprising the spiked in bacteria is demonstrated in the range between 8 and 20 V. The median of the number of total blood cells is reduced to 57 % or 34 %, whereas more than 97 % and 90% of the spiked-in bacteria remained viable at 8 V and 15 V, respectively. The onset of human blood cell lysis at 8 V and the onset of significant viability reduction of *E. Coli* at 30 V correspond to electrical fields strengths of 3.2 kV/cm for human blood cells and of 12 kV/cm for *E. Coli* with the developed ECLU.

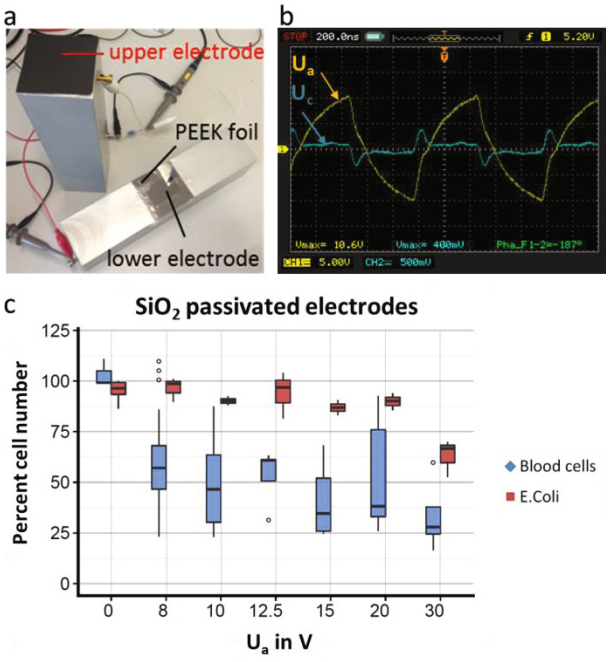

Fig. 3 (a) P-doped silicon electrodes with a 50 nm SiO2 passivation layer mounted on aluminum blocks. (b) Voltage and current plot, Uc is proportional to the total current I (voltage at a 0.1 Ohm resistor in series to the electroporation cell). (c) Voltage-dependent lysis of human blood cells (NTotal=78) in comparison to the survival rate of the spiked-in E. coli (N=3 per Voltage).

IV. Discussion

The apparent increase in cell numbers after applying low voltages in Figure 1 and Figure 2 as well as the high variance in lysis rates in Figure 3 can be explained by the manual withdrawal of the samples from the electrode surface, especially concerning the hydrophilic surface of the SiO_2 set-up. Furthermore, continuous cell lysis after the exposure to the electric field was observed under the microscope for several minutes, which further increased the variance between the samples. The results of the non-passivated gold electrodes indicate that, when using electrodes in ohmic contact with the sample, in addition to the electric field effect, secondary effects are present. Significant heating, as observed after less than 3 seconds as well as pH changes near the electrodes due to electrolysis [18] superimpose pure, cell-size specific electric field lysis, which leads to unspecific lysis. This explains the data gained from the bare gold electrodes indicating inactivation of *E. Coli* at electric field strength values less than half of the published values [19,20]. Hence, for microfluidic applications, as the reduction of the electrode distance to the µm-range drastically reduces the resistance of the inter-electrode space, the influences of secondary effects are further increasing. Surprisingly, however, these effects together with voltage and current characteristics are rarely discussed in the literature concerning cell lysis through electric fields. On the other hand, electrical passivation can significantly reduce these secondary effects to enable cell-specificity, but needs to be optimized regarding its electrical and geometrical design. The data gained from the experiment with AZ6612 polymer passivation demonstrate that the electric field induced in the liquid and therefore experienced by the cells is strongly influenced by the parameters of the coating. As passivation layers are introduced, they act as a voltage divider in combination with the impedance of the liquid and the voltage drop across the passivation layer is proportional to its capacitive reactance. The lower the thickness of the layer or the higher its dielectric constant, the higher the capacitance and the lower the capacitive reactance, resulting in a lower voltage drop in the passivation layer itself. This could be shown by using a 50 nm SiO_2 passivation of highly p-doped silicon as electrode material. In contrast to the 20 times thicker polymer passivation, effective and selective lysis could be observed at lower voltages.

V. Conclusions

To the best of our knowledge this work shows for the first time cell specific lysis in a heterogeneous biological sample through high frequency electric fields with electrically passivated electrodes. This developed concept represents a great potential in the field of sample preparation of complex biological specimens in particular for sepsis diagnostics. In addition, the developed method is specifically suited for POC devices through simple integration, process automation and upscaling. This could enable more sensitive, robust and accelerated patient diagnostics to realize instant targeted antimicrobial therapy to significantly reduce sepsis mortality.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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