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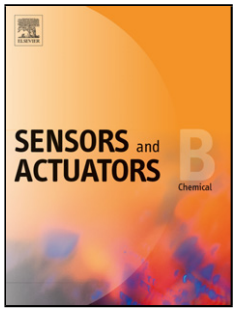

Nanoparticle activated Neutrophils-on-a chip: A label-free capacitive sensor to monitor cells at work

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Nanoparticle activated Neutrophils-on-a chip: A label-free capacitive sensor to monitor cells at work

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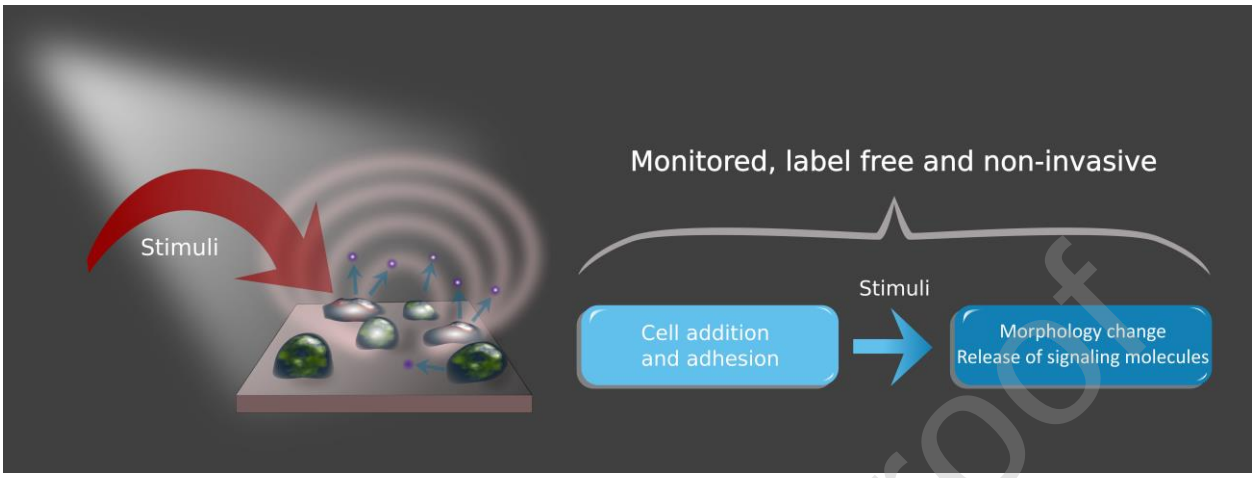
Graphical abstract



"Research highlights

- We present a method with capability to detect and monitor cellular ROS burst processes in time frame of minutes accomplished by using silicon chip technology (CMOS) combined with low temperature co-fired ceramic packaging technology (LTCC).
- The method is label free and gently measures the capacitive response from cells on top of an insulating surface in a weak electromagnetic field
- The capability to detect cell activity in response to exposure of external triggers like nanoparticles is also shown"

Abstract.

Neutrophil granulocytes are the most abundant white blood cells in mammals and vital components of the immune system. They are involved in the early phase of inflammation and in generation of reactive oxygen species. These rapid cell-signaling communicative processes are performed in the time frame of minutes.

In this work, the activity and the response of neutrophil granulocytes are monitored when triggered by cerium-oxide based nanoparticles, using capacitive sensors based on Lab-on-a-chip technology. The chip is designed to monitor activation processes of cells during nanoparticle exposure, which is for the first time recorded on-line as alteration of the capacitance. The complementary metal oxide semiconductor engineering chip design is combined with low temperature co-fired ceramic, LTCC, packaging technology. The method is label free and gently measures cells on top of an insulating surface in a weak electromagnetic field, as compared to commonly used four-point probes and impedance spectroscopy electric measurements where electrodes are in direct contact with the cells.

In summary, this label free method is used to measure, oxidative stress of neutrophil granulocytes in real time, minute by minute and visualize the difference in moderate and high cellular workload during exposure of external triggers. It clearly shows the capability of this method to detect cell response during exposure of external triggers. In this way, an informationally dense non-invasive method is obtained, to monitor cells at work.

KEYWORDS: Complementary Metal Oxide Semiconductor (CMOS), Low Temperature Co-fired Ceramic (LTCC) packaging, Lab-on-a-chip, Neutrophil granulocytes, Capacitive sensor, Cerium oxide nanoparticles.

1. Introduction

Neutrophil granulocytes, also called neutrophils, are known to be one of the most prominent cell types involved in the early phase of inflammation. Generation of reactive oxygen species (ROS) by these cells plays a crucial role in our defense system fighting intruders such as pathogenic microorganisms [1-4]. Upon contact with a foreign material, the cells may try to phagocytose the intruder, leading to an extensive extracellular release of ROS which may lead to tissue damage [5, 6]. This process of frustrated phagocytosis can be harmful for the host and contributes to the ongoing inflammation.

Neutrophils are highly reactive cells and their activation initiated by for example nanoparticles, will release a full set of inflammatory mediators such as myeloperoxidase [4, 7] into the extracellular space (Fig. 1). About 10^{11} neutrophils exit the bone marrow daily under basal conditions [8] and the short half-life in the blood is measured in hours [9].

Monitoring the cell activation during external triggers are of main importance for increased understanding of the mechanistic processes between cells and foreign elements. However, to obtain information on a system, i.e. to view and follow processes without disturbing it by the measurement itself, has been a struggle during scientific development over the years.

The invention of biochips, with focus on the vision of miniaturized laboratories (Lab-on-a-chip (LOC) technology), was first aimed to reduce the cost and to enable bioanalysis to be distributed worldwide [10]. Today the interest is continuously growing, as highlighted in a recent report from the EU commission [11]. Design of immunosensors, such as preclinical immune assays, have earlier been reported and is an important contribution to the field [12]. Electric impedance spectroscopy (EIS) measurements have been performed by amperometric immunosensors to verify presence of antibodies against the bovine leukemia virus protein gp51 on a pre-

immobilized sensor surface with five times higher sensitivity than ELISA [13]. Recently, photoluminescence spectroscopy together with an imaginary capacitor model was used to study the same system now with antigen-antibody complexes adsorbed on TiO_2 nanoparticles deposited on glass [14]. The opportunity to obtain data by non-invasive label free techniques to track and visualize ongoing cellular processes are recently brought to the table. Real-time SPR studies of cancer cells, during drug (daunorubicin) treatment and Au nanoparticle exposure are two such examples [15, 16].

Cell-impedance measurements have been used for almost four decades and big leaps were introduced to this technology by micromachining in the 90s and by microfluidics in the 2000s, which enabled miniaturization of the measurement system [17]. This allowed development of systems for detection and separation of neutrophils from whole blood by using impedance measurements [18, 19]. Electrical impedance is the measure of the voltage to current ratio in an AC/DC circuit. When measuring impedance of cells and their activity, the following three techniques are commonly used; 1) electric cell-substrate impedance sensing (ECIS) [20], 2) impedance flow cytometry (IFC) [21] and 3) EIS [22-24]. When the aim is to study cells adhering onto a substrate ECIS is suggested, see earlier studies on apoptosis [25] and cell proliferation [26]. In the literature, the ECIS is predicted to be combined with other liquid handling models and microfluidics to improve the quality and reproducibility of measured data, to enable drug candidate screening, gene function screening etc. [17]. In these impedance measurements the cells are typically in direct contact with the electrodes.

Techniques such as organic electrochemical transistors are used to measure cells [27]. The low voltages used during these measurements are considered safe for the cells but may induce electrochemical side reactions and possible electrode corrosion, masking the pure cell interaction signal.

Cell patch-clamp techniques are used to study exocytosis dynamics [28-30]. This is done by a specially designed pipette tip that measure capacitance over a small area of the cell membrane giving the opportunity to study exocytosis processes over shorter time periods.

A highly interesting and recently developed technique to study cells adhering onto a substrate is based on capacitive measurements [31-33]. Cell morphology changes and release of charged entities are gently monitored through capacitive measurements utilizing a passivation layer on the electrode i.e. the cells, with surrounding media, are separated from the electrical contacts. When cells are activated, they change shape and the area of adhesion on the sensor surface may increase, resulting in a measurable relative capacitive change.

In this work neutrophils and their activity are investigated, when triggered by nanoparticle exposure. This is monitored using a lab-on-a-CMOS chip, packaged by low temperature co-fired ceramic technology (LTCC) [34], see schematic drawing and photo of the setup (Fig. 2 and 3 a, respectively). The method is purely capacitive, without electrodes in contact with the cells and is based on a CMOS chip with an interdigitated electrode array, connected to the second stage of

individual three-stage ring oscillators. The capacitance change can be interpreted from the change of oscillation frequency. This lab-on-a-CMOS chip technology [10] enable continuous monitoring of cell adhesion and cell activation as a function of time, in a non-invasive manner, through capacitive measurements.

This low-cost technique is thus label free, non-invasive, reusable and can be used to monitor a wide variety of cell types. The combination of these properties gives it high potential for future applications in cell monitoring. The chip can be used in short measurement in time spans of minutes up to days making it suitable for a whole range of experiments and setups.

2. Experimental

2.1 Capacitive sensor chip

The sensor chip was manufactured in a commercial CMOS foundry (X-FAB) utilizing standard 2-poly, 3-metal and 0.35 µm process [35-37]. The sensor chip has 16 pixels in a 4 x 4 array (X, Y pitch 196 µm x 186 µm), each pixel carries one sensor and have a ring oscillator circuit with interdigitated sensing electrode (electrode area of 30 µm x 30 µm) connected to second stage (Fig. 3 a). Pixels are located directly under the uppermost SiO_2/Si_3N_4 passivation layer. Moreover, two identical reference sensors are located on the edge of the chip near the bonding pads. Reference sensors are used to calibrate each sensor and to cancel the temperature fluctuating effects. The chip has reported sensitivity of 590 kHz / fF, resolution of 14.4 aF and yields wide sensing range of 12 fF. The chip is controlled by microcontroller (MicroPython

PYBv1.1) using I^2C communication protocol and the pixels are actuated sequentially giving possibility to map pixel readings individually.

The sensor was packaged using DuPont 951 LTCC material system. Ceramic substrate was manufactured from LTCC tapes using the process recommended by manufacturer. The chip was bonded to the substrate using thermocompression flip-chip process where the gold contacts on the chip were pressed at an elevated temperature to the substrate with corresponding gold traces. Then underfill was applied to cover the electrical contacts and to secure the chip in place and protect the contacts from the corrosive cell media. A glass container with a diameter of 30 mm was attached to hold the liquid sample on top of the sensor (Fig. 3 a). All materials in contact with cells are biocompatible. For more details about the LTCC packaging process, see ref. [27].

2.2 Sensing mechanism

The sensor has a designed oscillation frequency of around 60 MHz in air. The oscillation frequency of the circuit changes when there is a change in dielectric constant or charge at the proximity of the sensor surface. This is why it is possible to detect capacitive changes even when just a few cells are introduced to the sensor surface. Capacitance change is related to the effective permittivity of the sample causing the frequency to increase when effective permittivity is lowered and vice versa.

The frequency output of the ring oscillator is affected by a certain delay introduced by the ring oscillator circuit. The circuit diagram (Fig. 2) and the oscillation frequency without the sensing electrode can be written as in equation (1), where V_{TH} is the threshold voltage of the MOSFET,

I_B is the bias current and C_{L1}, C_{L2} and C_{L3} are the parasitic capacitance of each inverter. More detailed description can be found in ref. [35-37].

$$f = \frac{I_B}{V_{TH}(C_{L1} + C_{L2} + C_{L3})} \quad (1)$$

The effect of the connected sensing capacitor affects the total time that is needed to load the capacitors. This can be expressed as extra parallel capacitance C_{IN}:

$$\tau_2 = \frac{V_{DD}}{I_B}(C_{L2} + 3C_{IN}) \quad (2)$$

The total time can be written as:

$$\tau_{tot} = \tau_1 + \tau_2 + \tau_3 \quad (3)$$

Where the frequency output of each sensor can be calculated using:

$$f = \frac{1}{\tau_{tot}} \quad (4)$$

2.3 Ionic conductivity

The cell media surrounding the cells is changing because of the ions released from the cells, especially when cells are activated the ionic conductivity starts to dominate the change of the sensor output frequency. Ions released to the liquid accumulate on the sensor surface above the electrode and cause capacitance to increase. This can be explained by the Maxwell-Wagner effect [38], which causes increased polarization and thus increasing effective permittivity. It has been shown that permittivity of water rises for dielectric spectroscopy measurements at low frequency (< 200 MHz) when higher contents of ions are present [39].

2.4 Neutrophil isolation

Neutrophils were isolated from venous whole blood donated by healthy, non-medicated volunteers at Linköping University Hospital, a variant of the Böyum method. The blood was anti-coagulated with 10U / ml heparin and allowed to equilibrate to room temperature. Blood was layered on an equal volume of a density gradient (Polymorphprep™), and the tubes were centrifuged at 480 g for 40 min at room temperature in accordance with the manufacturer's instructions. Separated neutrophils were resuspended in PBS and 0,45 % NaCl and centrifuged at 480 g for 10 min at room temperature. Any remaining erythrocytes were removed by brief hypotonic treatment performed twice in ice-cold distilled water for 35 s followed by washing the cells in PBS containing 3,4 % NaCl and HEPES solution (400 g at 4 °C). Isolated neutrophils were resuspended in HEPES solution. The cell concentration was adjusted to 2 x10^6 cells / ml and the isolated neutrophils were kept on ice until use (within 90 minutes). Neutrophils were incubated at 37°C, without carbon dioxide, during experiment.

2.5 Nanoparticles as triggers for cell activation

CeNPs and Gd-CeNPs were prepared using the room temperature wet-chemical synthesis procedure, which was recently reported elsewhere [40]. All solutions used in the synthesis were pumped and purged with nitrogen gas. 0.5 ml of cerium(III)- and gadolinium(III)-acetate was dissolved in 5.48 ml of a 50/50 Milli-Q water and diethylene glycol (DEG) solution. 0.52 ml of 50/50 DEG and 30 % ammonium hydroxide was added dropwise to the solution under constant stirring. Thereafter, the solution was left stirring for 2 h before dialysis.

The nanoparticles were dialyzed (Slide-A Lyzer® MINI Dialysis Devices, 10K MWCO) against MilliQ-water at a minimum ratio of 1:1000 for 24 hours with water exchange twice. After dialysis the nanoparticle solutions were filtered using Acrodisc® 25 mm syringe filter with w/0.1 μm Supor® membrane. The concentrations of cerium and gadolinium in the prepared nanoparticles were measured with Inductively Coupled Plasma – Mass Spectroscopy by ALS Scandinavia AB.

N-Formylmethionyl-leucyl-phenylalanine (fMLP) is a short peptide that mimics / resembles peptide bacteria release that triggers the innate immune system. It is often used as a positive control for activation of neutrophils and other immune cells. [41]

2.6 Microscopy and image analysis

For transmission electron microscopy (TEM) studies nanoparticles were deposited directly onto an amorphous carbon film supported on a copper TEM grid. All measurements were taken with a FEI Tecnai G2 (FEI) operated at 200 kV.

The diameter for the as-prepared nanoparticles were estimated to be under 10 nm (figures in appendix 1.4), in good agreement with the results in a previous paper [40] ,where the Gd-CeNPs were further characterized.

For Scanning Electron Microscopy (SEM) fixation of the cells was performed by adding paraformaldehyde to a final concentration of 4% and left overnight.

After fixation, the cell samples were washed with buffer three times. The samples were dehydrated in an ascending series of ethanol, critical-point-dried, and subsequently sputtered with platina (~1.5 nm). The specimens were examined in a SEM Leo 1550 Gemini, operated at 3 kV.

The microscope used for fluorescence microscopy was a Nikon Eclipse Ti-E connected to a Nikon DS-Fi2 camera. Images were captured using the NIS-elements software (version 4.12.00). Image analysis and enhancement was carried out in Fiji (ImageJ) software [42].

3. Results and Discussion

In this work, we use NPs to trigger neutrophils and study the cell activation with the LTCC packaged CMOS based sensor chip. The LOC principle used in this study is based on the CMOS/LTCC sensor device with the ability to deliver high quality results by means of kinetic response, efficiency and sensitivity for such fast measurements. The principle is shown in figure 2.

This sensor can easily be used as a label free tool to compare the response of settled cells, when exposed to NPs of interest, when for example comparing sets of NPs with controlled parameters such as composition, size, capping etc.

Previously, we have developed several kinds of gadolinium-based nanoparticles (GdNPs) for contrast enhancement in magnetic resonance imaging (MRI) [43-47] . Recently, we developed gadolinium-containing cerium oxide nanoparticles (Gd-CeNPs) [40]. Implementation of gadolinium within the CeNPs enabled contrast enhancement for MRI, which were about 2-3

times more efficient compared to the MRI contrast agent used in the clinic today [40]. In this work a rapid cell response is observed and an enhanced activation level (~500-1000 kHz) is obtained due to activation and changes in cell morphology (Fig. 3).

3.1 Cell adhesion

The capacitive response from an array of 16 sensors are measured sequentially. Each sensor is recorded every 35 second which enables continuous measurements.

Prior to cell adhesion, pure buffer was measured until a baseline could be established. Neutrophils were added to a final concentration of $1*10^6$ cells/ml and the measurement was continued for 20 minutes before it was stopped for a microscope image of the cells on the sensor surface. A typical response curve for cell adhesion is shown in Figure 4.

A small decrease in frequency is observed when cells are added (Fig. 4A) which we address to the tiny difference in ion concentration between pure buffer and buffer plus cells.

The response from all sensors as a function of time is measured sequentially. Initially, the sensors are exposed to HEPES buffer, until a stable signal for all sensors is achieved (time before A). Neutrophils are introduced after 22 minutes. This results in a clear increase of the response by ~500kHz, and an individual response on each sensor correlated to surface coverage, indicating the measurement to be sensitive to the local environment of each sensor. The capacitive change is, due to lower effective permittivity of the cells ($\varepsilon \sim 50$), compared to cell media ($\varepsilon > 80$) [48] in agreement with previous studies [32, 49]. When cells adhere to the sensors

this is seen as frequency shift upwards, which corresponds to the decrease in monitored capacitance.

A microscopy image is obtained when the cells have adhered, which shows that each sensor only has a few cells i.e. about 1-5 cells in its proximity or surface (Fig. 3 b).

3.2 Neutrophil activation

The settlement of the neutrophils on the sensor was confirmed, both registered by a frequency change in the sensor signal and by microscopy. The experiment was started by introducing stimuli in form of CeNPs or Gd-CeNPs. When a small amount of Gd-CeNPs (0.02mg/ml end concentration) were introduced to the chip, no direct frequency change was observed (Fig. 5A). Such small concentration is commonly used as a primer i.e. to upregulate the cells prior to activation. This is performed to sensitize the cells prior to a second dose of nanoparticles. After the second step, with addition of Gd-CeNPs in double dose (B) a clear signal increase was observed corresponding to the fast response of the primed neutrophils to the stimuli. At 30 minutes the sensor signal has reached its peak and starts to decrease. Now the cells have been fully activated and starts to release a diversity of signal molecules, enzymes etc. The signal flattens out after 40 minutes when the cells have finished the release of these molecules.

A proposed mechanism for the increase of signal directly after activation in B is that the activated cells change morphology which affects the signal. The decrease of signal (after 30 minutes) can most probably be explained by release of ions in the solution from the cells.

Another possible explanation may be that the cells bind ions to their membrane making them invisible to the sensor.

In a new experiment cells from another donor was tested to verify that CeNPs (introduced at B, Fig. 6) could activate the neutrophils after a primer dose of Gd-CeNPs (Fig. 6 A). In order to investigate if the cells were activated or if it was another process involved, fMLP, a well-known activator, was added at C. In the first experiment (Fig. 6, right, bottom graph) the primer dose was not enough to activate the cells and no response is observed. After the CeNPs dose was added, the cells were activated and a clear increase of signal, which reaches its peak after 10 minutes was observed (similar as in Fig. 5). fMLP was then added (C) to investigate if the cells are fully activated. A small increase followed by a decrease of the signal was observed for most sensors. The variation in signal increase shown for the sensors can be explained by changes in local ion concentration, which influence how the cells near the sensors react. The signals then continue to decline over time showing that we capture the immediate response of the cells but can also follow the whole process until the end.

In the second experiment with a lower number of cells (Fig. 6 right, top graph) the cells were activated already by the primer concentration of nanoparticles (A), shown by an increase of the signal that quickly reaches a peak followed by a small decrease and then flattens out. When additional nanoparticles were added (B) no change in signal was observed. The fMLP control gave a small observable decline, indicating that most cells were already activated.

Due to the non-invasive nature of our experimental method, we can follow the same individual cells all through the process (Fig. 6, left, A) before B) during and C) after nanoparticle exposure). As shown, we can observe the full response for just a few cells as well as an ensemble of cells. This is very useful when monitoring how cells react to external stimuli in order to

investigate the dynamic processes of cell activation. Microscopy images were taken before and after addition of NPs and fMLP to monitor possible changes of cell attachment/detachment and movement (appendix Fig. S6).

3.3 Reference trials and data analysis

Further characterization of the performance of the CMOS/LTCC sensor was carried out to determine the sensing capabilities. A dilution series of NaCl in water gives the signal vs ion concentration relationship. The results can be explained by the Maxwell-Wagner effect [38] due to the free ions moving towards the electrode and causing polarization, which shows as a decrease in frequency.

The device working in lower frequency range is sensitive to ion strength of the nearby surroundings of each sensing electrode, as clearly shown in Figure 7. The calculated sensitivity, at low concentrations (< 50 mM) is 137 kHz / mM, and higher (150-250 mM) is 11,7 kHz / mM, with a 10,4 kHz resolution giving the possibility to track changes in the 0.1 μM and 1 μM range, respectively.

The HEPES buffer consists of 152 mM of small ions (NaCl, KCl, $MgSO_4$) and 20 mM of larger molecules (HEPES and glucose) and are in good agreement with the reference trial measurements (Data not shown comparing HEPES buffer and saline solutions).

Reference trials with human serum albumin, CeNPs Gd-CeNP, fMLP and hydrogen peroxide, all in HEPES buffer, was carried out with no signal increase compared to pure buffer (data not shown). This concludes that it is the cells reacting to the stimuli that is responsible for the signal

change in the experiments and not the stimuli itself. This strengthens the hypothesis that larger ions do not contribute to the signal, because the relatively large mass prevents them from moving towards the electrodes in response to the alternating electric field.

We calculated the increase of ions in the cell experiments, by using the NaCl reference signal to make a model, for frequency (kHz) vs ionic concentration (mM) (Fig. 7).

From the model the increase in ion concentration after the cells have been activated was calculated (Table 1). Interestingly, the mean value of the signal after activation for both experiments (Fig. 6) are very similar, this may imply that there is a correlation between cell activation and nanoparticle concentration that are less dependent of the total number of cells but more thorough studies are needed to confirm this. The cells are working in a team, few cells will work harder and more cells have to work less for the same number of nanoparticles. When investigating in detail, for the experiment with higher cell amount the standard deviation is significantly higher. This can be explained such that for an excess of cells just a fraction of the cells needs to react towards the stimuli, while in the case with few cells, most cells will be activated. To investigate the non-invasive characteristics of the method we did experiments with two chips in parallel: (1) One chip was used for a full experiment with cells present i.e. capacitive measurement as usual. (2) One chip was used with cells present but resting i.e. no capacitive measurement at all. No significant difference for the two chips with respect to the cell viability was observed (Fig. S7 in appendix).

The results clearly show the potential for future applications where cell kinetics can be studied as a response from a single cell or small groups of cells. The cell response could be measured as a function of nanoparticle concentration or sensor chip surface composition. There is an urgent

need of nanotoxicology studies [50, 51]. The CMOS/LTTC sensor offers high-throughput screening of nanomaterials and possibly nanogranules of drugs, drug delivery systems etc due to the small volumes needed for each sensor (~1µl).

The CMOS/LTCC sensor has been applied earlier to track slow processes such as cell growth over long periods of time monitoring the cell proliferation [52]. In this paper it is shown that the CMOS/LTCC sensor can track rapid responses from neutrophils. These cells are a vital part of the immune system and have fast response to external triggers. This impose strict requirements on the electrical output units. The kinetics by means of change in minutes is proven to be successfully recorded in this work.

4. Conclusion

We herein present a novel Lab-on-a-chip approach based on capacitive measurements of human neutrophil granulocytes and their activation, externally triggered by Ce-oxide based nanoparticles (Ce_2O_3 and Gd alloyed Ce_2O_3 nanoparticles). The cell experiment includes settlement of neutrophil granulocytes on a sensor surface and monitoring sequential activation using external trigger. The 16-sensor array on the chip is individually addressable and supporting microscope pictures reveal that even only a few cells gives a detectable response from that sensor unit.

This is a proof of concept, of an information dense, non-invasive method to monitor cells at work and the capability to also detect fast cell response on exposure to external triggers. The ability to detect the neutrophil response, but not the stimuli, together with the scalability and simple procedure makes it a strong candidate for future drug or toxicity screening. This method clearly shows the capability to detect and monitor cellular activation processes in the time frame

of minutes and this is accomplished by silicon chip technology (CMOS) specially designed for fast and intermediate response, combined with low temperature co-fired ceramic packaging technology (LTCC).

Conflicts of interest

There are no conflicts to declare.

Supporting Information.

More details about packaging, experimental setup and TEM pictures can be found in Appendix.pdf

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

CRediT roles:

Kalle Bunnfors: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Roles/Writing - original draft, Writing - review & editing

Niina Halonen: Methodology, Supervision,

Peter Eriksson: Methodology, Writing - review & editing
Jari Juuti: Funding acquisition, Methodology, Supervision

Anita Lloyd Spetz: Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing

Joni Kilpijärvi: Data curation; Formal analysis, ; Methodology, resources; Software,

Natalia Abrikossova: Data curation, Investigation

Caroline Brommesson: Supervision, Writing - review & editing

Kajsa Uvdal: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Roles/Writing - original draft; Writing - review & editing

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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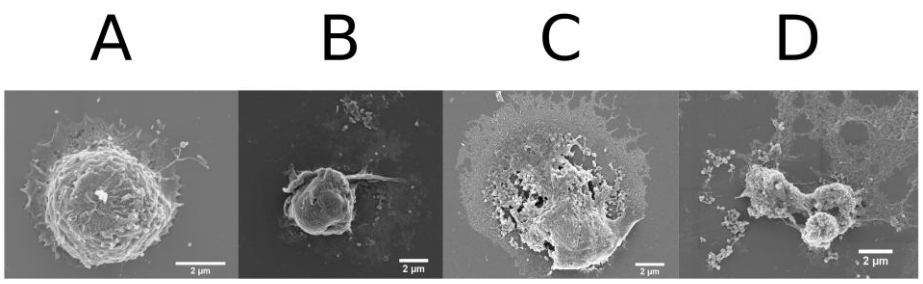

Figure 1. Scanning Electron Microscopy (SEM) of neutrophils on insulator surface (A-B) Neutrophil in initial activation stage (C). Strongly activated neutrophils show irregular reticular structure and (D) globular domains and neutrophil extracellular traps.

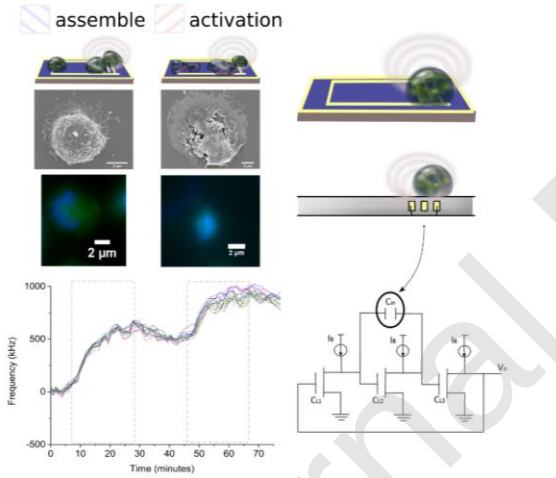

Figure 2. Schematic drawing of the principles how to measure Human neutrophil granulocytes-on-a chip; as a label-free capacitive method to measure oxidative stress. Top left: Fluorescent and SEM images of cells resting (left row) vs activated (right row) Bottom Left: Example of response curve after adding cells and activating them (pink shaded area). Top Right: Overview and cross section of the sensor with a cell in proximity of the capacitive field. Bottom right: Circuit diagram of the three-stage ring oscillator where an interdigitated electrode is located parallel to second stage.

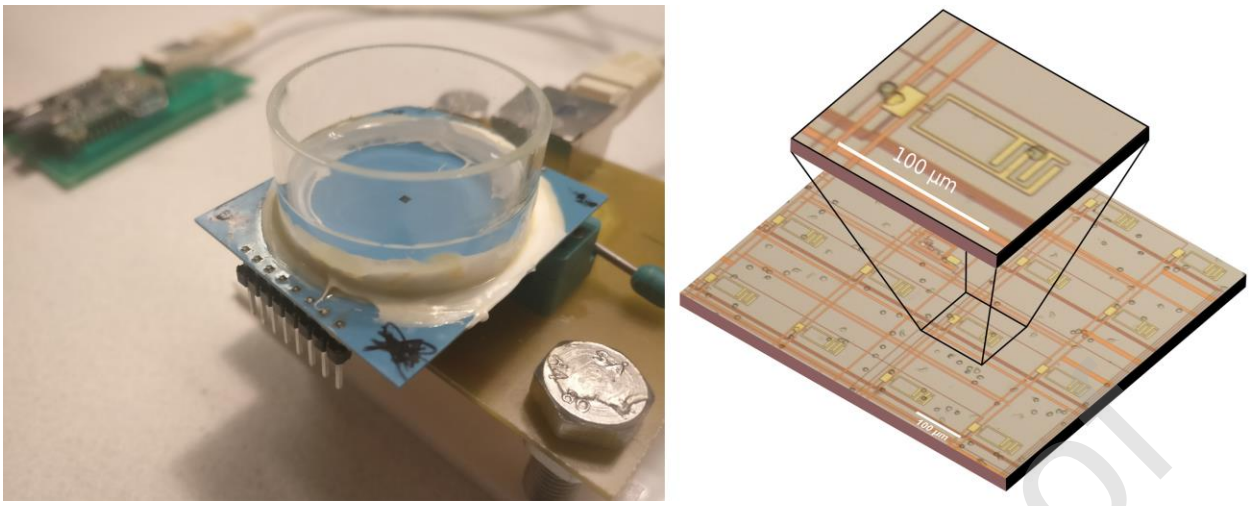

Figure 3. a. Photo showing the experimental setup. Pyboard (green) which is connected to a computer and to the sensor chip. LTCC package (blue) with well, where to apply the medium, and the middle of the well, the sensor chip. b. Microscopy image of the 16 sensors on the part of the chip that is exposed to the medium after cells have been added. A blow up of one of the sensors.

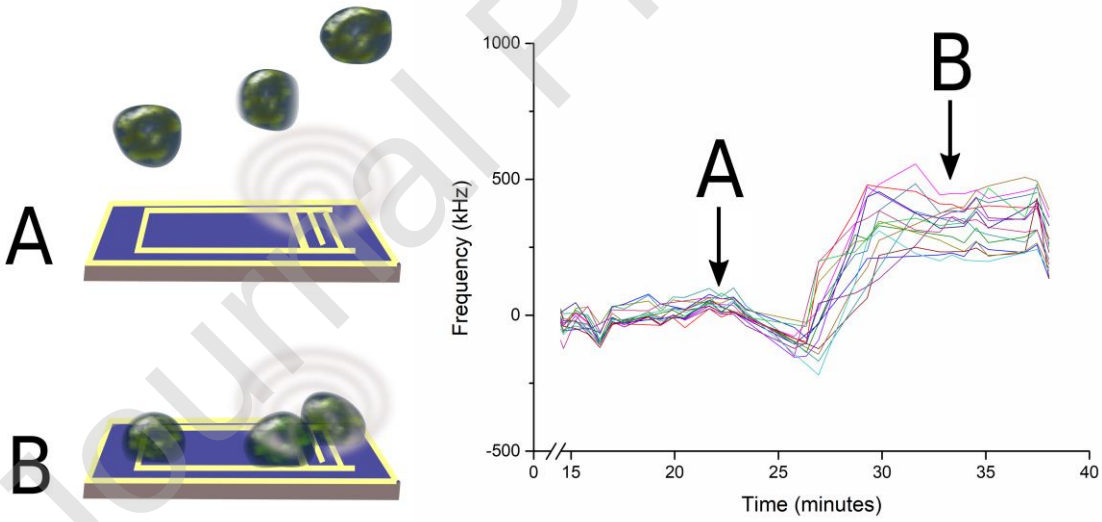

Figure 4. The response of the CMOS/LTCC device: A, cells are added. B, cells have adhered to the surface. The different sensors have different number of cells in proximity to them and therefor split up. The baseline has been normalized to 0 for all sensors.

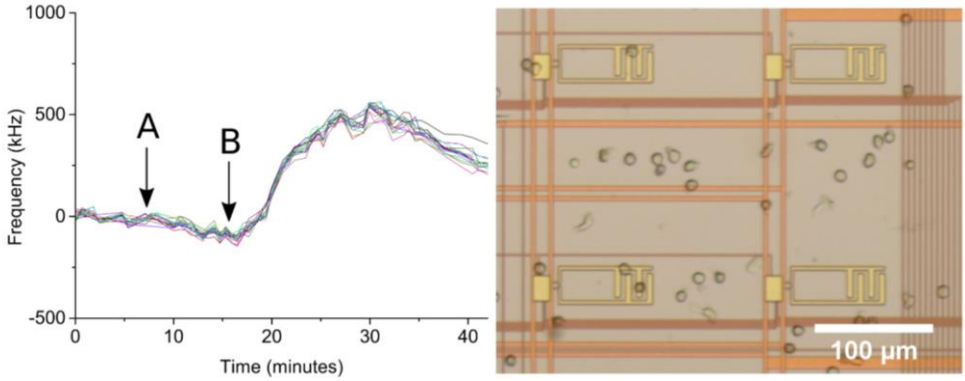

Figure 5. Cells at low concentration on the surface. A, 40µl of Gd-CeNPs is added. B, 80µl of Gd-CeNPs is added.

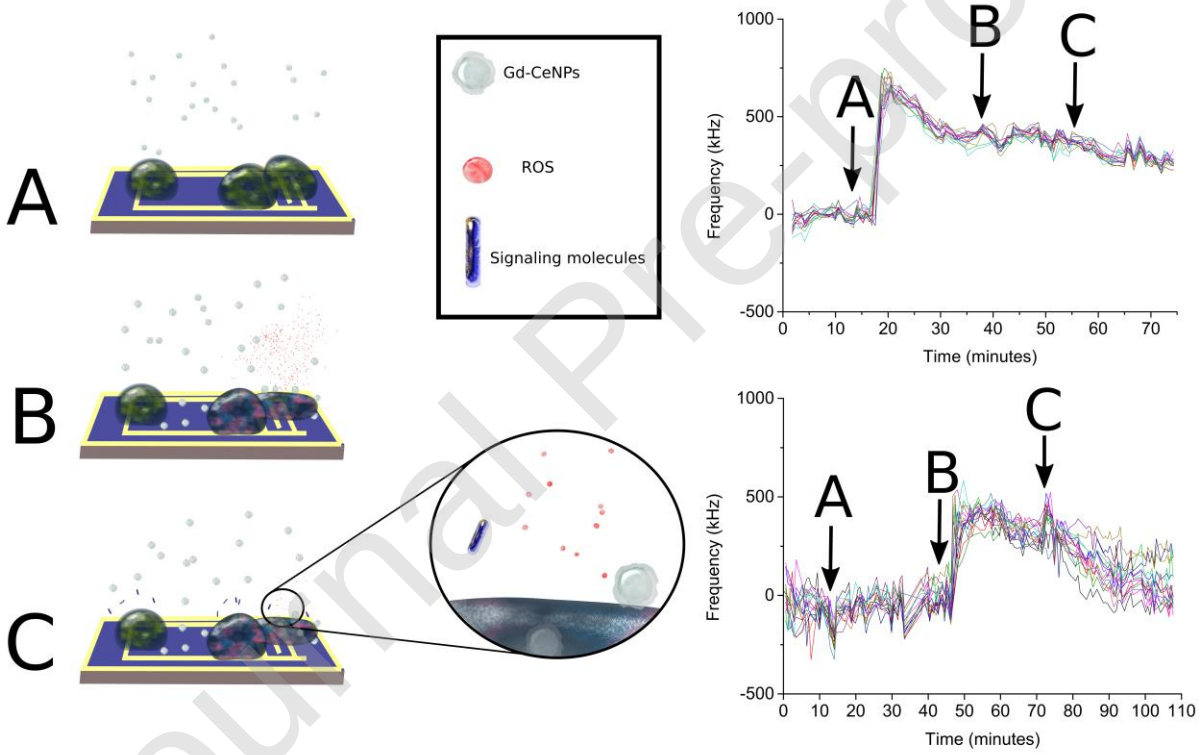

Figure 6. Schematic pictures on: A, cells adhered on surface and addition of nanoparticles. B, cells activated by the nanoparticles. C, cells response slowing down. Experiment with < 3 cells per sensor and > 5 cells per sensor, bottom and top graph respectively with frequency change as a function of time. Addition of stimuli: A, 40µl of Gd-CeNPs is added. B, 80µl of CeNPs is added. C, fMLP is added to a final concentration of 1µM. Cells from a different donor than in Figure 5. (schematic drawings of cells and molecules in A-C are not to scale).

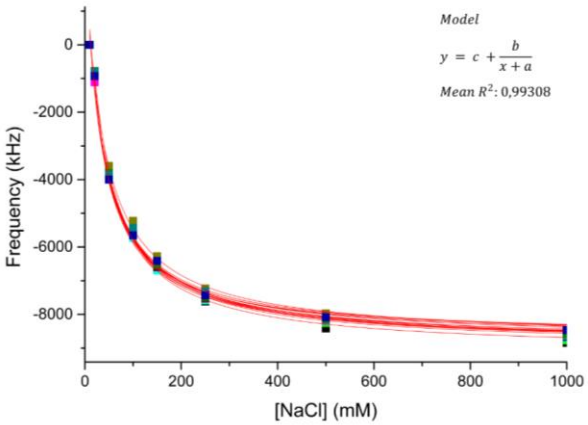

Figure 7. Fitted response curve of various ion concentrations vs frequency for all sensors. All sensors have its own model parameters and the mean R2 value for all sensors is shown.

Table 1. Statistics of calculated increased ion concentrations (mM) after cell activation.

Numbers are increase of ions in mM	Mean	Standard Deviation	Minimum	Maximum
High cell coverage	33,2	12,7	10,8	52,2
Low cell coverage	33,2	6,2	20,6	43,2