COST EMF - MED (Action BM1309):
European network for innovative uses of EMFs in biomedical applications

STSM Report:

Non-invasive real-time luminescence monitoring of oxidative effects of pulsed electric fields

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Abstract:

Pulsed electric fields have already wide use and further great potential for novel applications in biomedicine and food industry. Although not fully explored, one mechanism of the electric pulses action in biological samples is through the electric field induced oxidation. In this STSM, we have shown that ultra-sensitive detection of endogenous chemiluminescence (developed in Bioelectrodynamics research team in Prague) is able to interrogate the oxidative effects of us-ns electric pulses in biosamples.

A. Purpose of the STSM

The purpose of the STSM was to test whether ultra-sensitive detection of endogenous chemiluminescence (developed in Bioelectrodynamics research team in Prague) is able to interrogate the oxidative effects of intense us-ns electric pulses in biosamples.

Electromagnetic fields with frequency <400 THz cannot directly cause a removal of electron from atom (i.e. cannot cause ion formation and molecule bond breaking), because the photon energy in these fields is smaller than the electron binding/chemical bond energy and thus should not affect chemistry of the biological systems. That is why electromagnetic field with frequency <400 THz is termed as non-ionizing radiation. In contrast, ionizing radiation (electromagnetic fields with frequency >400 THz) are capable of breaking chemical bonds with consequent formation of reactive oxygen species and oxidation (electron removal) of biomolecules, which is primary chemical mechanism of action of ionizing radiation. Intense us-ns pulsed electric fields belong by the definition of their spectral content (<10 GHz) to non-ionizing radiation hence chemical bond disruption and oxidation is not expected from the physical perspective described here.

However, several works showed that the pulsed electric fields cause oxidation in biological and biomolecular systems [1]–[5]. Oxidation of biomolecules very well known to be caused by the electrochemical processes at the interface with the electrodes when the voltage is applied, but it has been also found after delivery of intense electric pulses non-contactly through antenna [6]. Furthermore, electric pulses have been demonstrated to induce biological production of reactive oxygen species which cause

The possibility that electric fields can cause or modulate the biological oxidation processes is potentially of tremendous impact on our understanding of electromagnetic field bioeffects. Oxidative effects of electric pulses therefore have to be carefully monitored both to avoid them when undesired or to utilize and fine-tune them for beneficial effects such as the stimulation of protective antioxidative response of biological system.

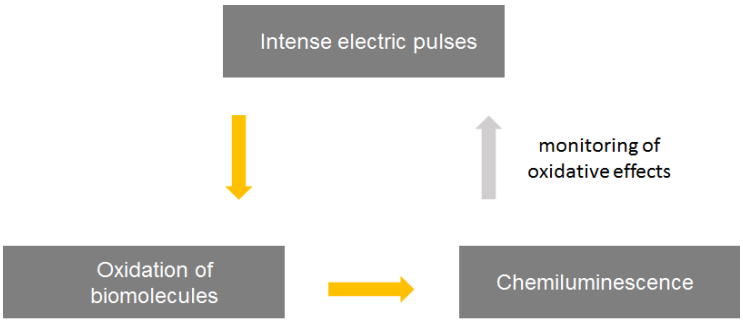

Fig. 1 *Scheme of the principal scientific idea tested during the STSM: Electric pulses induce biomolecule oxidation which in turn causes chemical formation of electron excited states which is manifested as endogenous chemiluminescence. Hence, chemiluminescence can be used to indirectly monitor oxidation of biomolecules in solutions, cells or tissues caused by electric pulses.*

It is known that oxidation of biomolecules gives rise to endogenous chemiluminescence [7]. Endogenous chemiluminescence, often termed as ultra-weak photon emission, is generated from biological systems without any external stimuli or additionally applied external luminophores and that the intensity of this weak endogenous light is modified by application of external stressors [7]. We propose that the endogenous chemiluminescence can be used for monitoring of oxidation induced by electric pulses, as depicted in Fig. 1.

B. Work Description

The work was focused on the effect of electric pulses applied to DC-3F cells on the endogenous chemiluminescence from the cell suspension. At first, luminescence measurement system brought from home lab in Prague (Fig. 2) was set-up in the host lab. Then, we tested the luminescence measurement system

Fig. 2 *Left: Ultra-sensitive luminescence detection system based on selected Hamamatsu H7360-01 photomultiplier and light-tight chamber built in the Institute of Photonics and Electronics, The Czech Academy of Sciences. Right: Combined experimental setup as assembled in France: electric pulse generator (Cliniporator) connected to dark light-tight chamber with luminescence detection.*

compatibility with us-ms pulses delivered by the Cliniporator device and optimized the set-up. Cliniporator Generator enabled max 1 kV, 5 ms, 15 A. Shortest pulse 100 us, In high voltage regime and max 200 V, 50 ms, 15 A In low voltage regime. DC-3F cells (Chinese hamster lung fibroblast line) were cultivated in full media (MDM + 10% FBS + antibiotics (penicillin + streptomycin)) in 5% CO_2, 37 °C and harvested before full confluence by trypsinization and suspended in electroporation buffer (SHM – sucrose/HEPES/Mg+ ions) for the experiment. Electrodes and the sample chamber/holder used for sample treatment is depicted in Fig. 3.

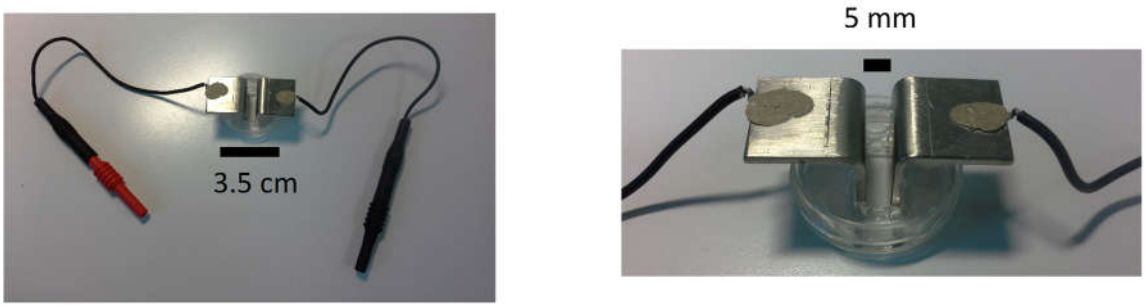

Fig. 3 *The gap between the electrodes used for the experiments was 5 mm. Hence, for example 1kV voltage corresponds to 0.2 MV/m (2 kV/cm) voltage to electrode distance ratio. The sample volume was*

We tested the reproducibility of the basic experiments and answered several research questions focused on the dependence of the luminescence signal on the parameters of us electric pulse treatment (amplitude of the electric field, duration of the pulses, frequency/number of pulses) and sample concentration.

C. Results

In summary, we have found that our detection technique captures changes in the endogenous chemiluminescence from cells samples undergoing electric pulse treatment. Since the mechanisms of origin of the endogenous chemiluminescence are related to oxidation, it is expectable that the changes in chemiluminescence due to electric pulses we observe report the oxidation of the cell sample. To fully confirm this on the mechanistic level, additional tests will have to be performed in future:

- **spin-trapping electron paramagnetic resonance spectroscopy to detect radicals which lead to oxidation**
- **employ antioxidants or transition metals (Cu^{+}, Fe^{2+}) to suppress/increase generation and effect of specific oxidative species**

At first, we show a typical chemiluminescence signal from cell suspension undergoing pulsed electric field treatment is in Fig. 4.

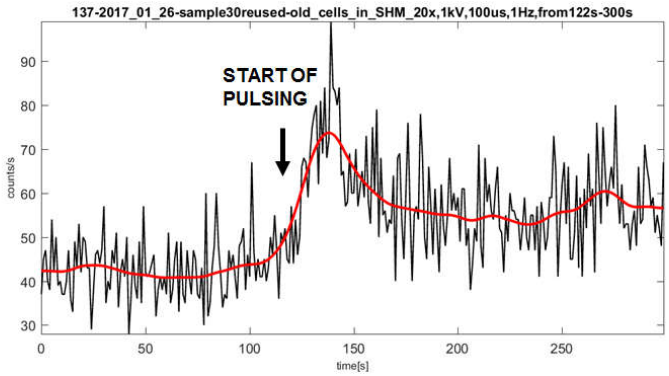

Fig. 4 *Typical chemiluminescence signal from cell suspension undergoing pulsed electric field treatment*

Here comes description of main results formulated as answers to specific research questions.

Question 1 (positive control experiments): Can we detect chemiluminescence signals from cells due to chemical oxidation (Fenton reaction) in electroporation buffer (SHM) ?

In the beginning, we needed to verify if we can detect chemiluminescence due to oxidation with the given cell type and cell medium. Therefore we induced oxidation of cells in SHM by well known chemical oxidation procedure: hydroxyl radical generated by Fenton reaction [8] (0.1 mM Fe_2SO_4 + 100 mM H_2O_2 final concentration) – see Fig. 5 for the result. Since chemiluminescence detection started after 20s from addition of hydrogen peroxide (H_2O_2) to the cell suspension, we cannot detect the burst of chemiluminescence but only decaying part of signal, which is well above the background. This experiment supports the basic argument that the chemiluminescence we detect is due to oxidation of the biological sample

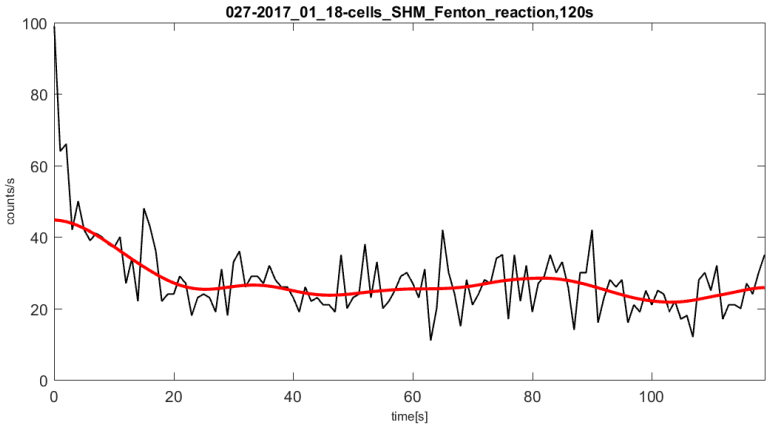

Fig. 5 *Fenton reagents (0.1 mM Fe_2SO_4 + 100 mM H_2O_2 final concentration) added to SHM medium with cells (750 000 cells in 0.6 mL) cause oxidation and increase of chemiluminescence with a decaying trend. Luminescence detection started 20 s after addition of hydrogen peroxide*

Question 2: What are the minimal electric field intensities and cell numbers to observe an effect on chemiluminescence ?

At first, we needed to test what are the parameters of the experiment which will enable us to detectable signals. Based on earlier work, we know that some minimal electric field strength and minimal amount of cells will be needed. From several experiments, we found that detectable luminescence signals were obtained from > 20 or 30 million cells in 0.5-0.6 mL in electroporation buffer while using train of 20x 0.2 MV/m 250 us pulses fired at 1 Hz OR using long low voltage pulses (40 kV/m 5ms) pulses. Therefore we decided to use the settings 20x 0.2 MV/m 250 us pulses fired at 1 Hz rate in the standard experiment.

No signals were reliably detectable for cell numbers less than 10 million (SHM medium, pulsing single 0.2 MV/m pulses (100 us, 500 us or 800 us)) or < 20 million cells (SHM medium, single 0.2 MV/m, 5 ms pulse).

Question 3: What is the dependence of the chemiluminescence intensity on the cell number present in the sample ?

To assess sensitivity of our technique and to find the range of optimal conditions for the experiment in terms of feasibility, we quantified the dependence of the chemiluminescence intensity on the cell number present in the sample. The exposure parameters were fixed as 20x pulses 0.2 MV/m 250 us 1 Hz. The results are in the Fig. 6. It can be seen that at least 20-30 million cells are needed to obtain reasonable signal above the baseline,

confirming the results presented in question 2. Optimal conditions for the experiments have been thus determined as 20x pulses 0.2 MV/m 250 us 1 Hz and 30 million cells in the sample.

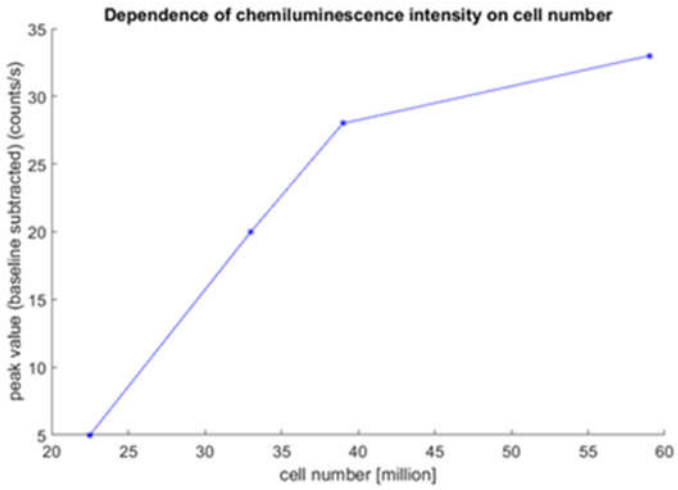

Fig. 6 *Cell number dependence on chemiluminescence intensity from cell suspensions elicited by train of 20x pulses 0.2 MV/m 250 us 1 Hz. Graph is based on the data: 22.5 million cells – experiment 75+76 (first + second pulsing), 33 million cells - experiment 129+130 (first + second pulsing), 39 million cells -*

Question 4: Is there any difference in the treatment with single long pulse vs. several shorter pulses ?

This question is based on the recently published hypothesis [9]. Here it is important to note that only those areas of the cell membranes which are oriented towards the electrodes are porated during the electroporation

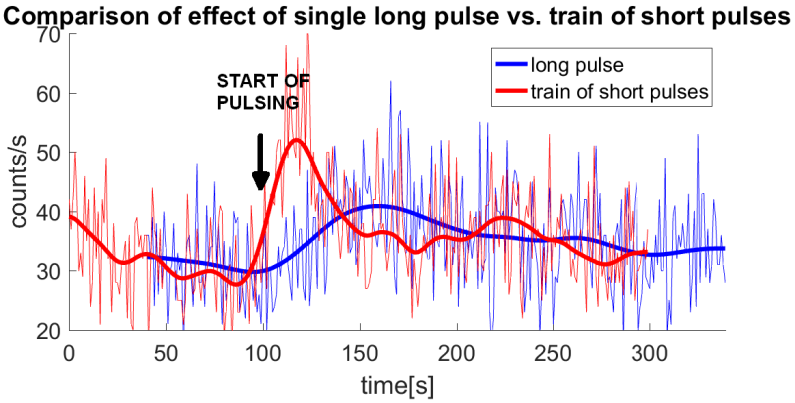

Fig. 7 *Train of pulses (0.2 MV/m 20x 250 us 1Hz - experiment 129) induces stronger response than the single 0.2 MV/m 5 ms pulse (experiment 63).*

process. The hypothesis in [9] is that time gaps between the pulses will cause larger surface of the cells to be electroporated because the lipids in the membrane will have enough time to diffuse along the membrane between the pulses. Therefore we analyzed if there is any difference of response due to single long pulse vs. several shorter pulses with 1s gaps (both cases having the total duration equal). Specifically, we compared single 0.2 MV/m 5 ms pulse vs. 20x 0.2M V/m 250 us pulses. From 5 experiments, we found that the train of 20 pulses seems to elicit a stronger response than a single pulse while both have the same current dose. See sample result in Fig. 7.

Question 5: Is there any "memory" effect of previous pulsing ?

The hypothesis leading to this question is following: we expect that the first pulsing will irreversibly permeate the membrane of some fraction of cells leading to release of biomolecules to the extracellular medium creating thus making these biomolecules available for oxidation at the electrodes. Then, the second pulsing will oxidize these free biomolecules leading to chemiluminescence larger than in the first pulsing when the molecules were not present in the medium. We have demonstrated in few experiments (143, 144+145) that the second pulsing indeed brings about stronger response, the sample result is in Fig. 8.

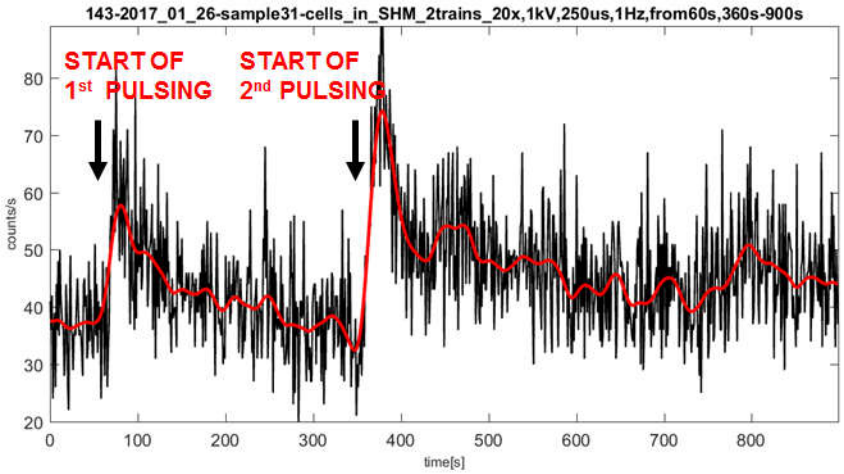

Fig. 8 *The first pulsing primes for stronger response of a following pulsing. Here, 20x pulses 0.2MV/m, 250 us, 1Hz were delivered from 60s and from 360s. Experiment 143, 39 million cells in 0.6 mL SHM medium.*

For future work, we aim to achieve higher sensitivity of the chemiluminescence detection by decreasing the light detector dark count using the cooled (-30 °C) photomultiplier system available in the Bioelectrodynamics research team in Prague. The cooled photomultiplier system is bulkier and could not be transported to France for STSM, in contrast to simple system which was transported and used to get the preliminary data presented in this report. Furthermore, endogenous chemiluminescence seems to have significant spectral content in the red band (>600 nm) [10] where the currently used photomultiplier has rather quantum efficiency. We plan to purchase the photomultiplier with higher sensitivity in the red band and compatibility with our cooling housing. Higher sensitivity will enable detection of chemiluminescence signals from smaller number of cells and/or under influence of weaker/shorter electric pulses.

There are further open questions based on our research. One question is whether the chemiluminescence signal originates from the oxidation at the electrodes or in the larger volume of the sample. This question could be verified by imaging the chemiluminescence with ultra-sensitive cooled EM-CCD camera soon available in Bioelectrodynamics research team in Prague.

In conclusion, we were able to find the experimental settings under which we can detect effect of intense pulsed electric fields on chemiluminescence. According to the plan, the STSM participant familiarized himself with the procedure for electroporation experiments and acquired practical experience with us electric pulse setup. We believe that the results of the STSM and follow-up will help to better understand oxidative component electropermeabilization mechanism to optimize cancer and gene therapy therapeutic protocols in the future.

D. Future collaboration with host institution

During many scientific discussions with several group members, also the social links have been established which warrant future collaboration. We aim to apply for funding for common future collaboration – at first for further research visits from French-Czech bilateral sources and potentially for research projects from European sources in larger consortia.

E. Expected Publications

The first results are planned to be published XXIV International Symposium on Bioelectrochemistry and Bioenergetics, 3-7 July, 2017, Lyon, France. We plan follow-up experiments, which should lead to a common journal paper.

F. Other Comments

STSM in numbers
150 experiments
71 h of lab work, net time
37 h of measurement system net time (*the measurement system which was developed by Bioelectrodynamics team and brought to Institute Gustave-Roussy for the STSM*)
1 billion of DC-3F cells used for experiments

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Confirmation by the host institution of the successful execution of the STSM:
We confirm that Michal Cifra has performed the research work as described above.

Dr. Lluis Mir

Signature

Dr. Michal Cifra

Signature