SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA15211
STSM title: Investigations on driving mechanisms of lipid vesicles destabilization due to intense electric fields with characteristics of the lightning
STSM start and end date: 15/02/2020 to 18/03/2020
Grantee name: Laura Caramazza

PURPOSE OF THE STSM: (max.200 words)

Combining electromagnetic engineering skills with chemical-technological competences, this STSM was dedicated on investigating the interaction of atmospheric electricity with biological samples in terms of lightning strokes-like signals able to destabilize cellular and subcellular membranes. To study the occurring physico-chemical processes in laboratory, under controlled and reproducible conditions, the electrical trigger was generated as a train of short and intense (MV/m order) pulsed electric fields (PEFs) and it was applied on artificial lipid vesicles, due to the complexity of cells. To this aim, the study was conducted preparing, in a reproducible way, unilamellar vesicles, called liposomes, usually studied as model for both cellular and subcellular membranes. In particular, the electropulsation effects were studied evaluating the macroscopic membrane oxidation, as a result of chemical processes occurring at the molecular level, and it was performed exposing liposomes based on slightly oxidable phospholipids (with only one unsaturation degree) and changing the electrical parameters, for a total of six PEFs exposure conditions . Thus, thanks to this STSM, we identified the characteristics of the PEFs able to enhance the oxidation process for future *in vitro* studies. Finally, other experimental conditions were tested, adding chemical compounds, to validate their efficacy in promoting lipid oxidation.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS (max.500 words)

The first step of this STSM regarded the lipid vesicles preparation using Egg-phosphatidylcholine 80E (Lipoid GmbH, Germany), 80% in weight of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) with one unsaturation degree. According to the thin film hydration method, lipids (40 mg) were dissolved in chloroform ($CHCl_3$) that was poured into a round bottom flask. The organic solvent was evaporated under reduced pressure using a rotavapor until a thin lipid film was formed on the flask bottom and dried under a nitrogen (N_2) flux. The dry lipid film was then hydrated with 5ml of HEPES buffer, previously prepared and characterized (pH=7.4 and σ=0.035S/m). Five consecutive freezing/thawing cycles were performed to ensure a good formation of vesicles. The obtained multilamellar vesicles were downsized to form unilamellar liposomes by sequential extrusion, using polycarbonate membrane filters of decreasing pore size (0.8–0.4–0.2 μm) upon twelve times, obtaining a narrow size distribution. To increase the lipid population homogeneity, the sample was subjected to size exclusion chromatography (SEC) with a Sephadex G-50 column.
The prepared liposomes were characterized for sizing, surface charge and stability, using the NanoBrook 90Plus PALS. Then, liposomes stored at 4°C overnight, were pre-treated at room temperature (23°C) for 30 min and exposed to the external PEFs. In particular, the exposure

system was composed by the Cliniporator (Igea S.p.a., Italy) as pulses generator -usually used in clinics for electrochemotherapy applications- connected to a BioRad device which holds a standard electroporation cuvette with a 1 mm-gap between the electrodes, hosting the lipid suspension. Once the PEFs characteristics had been set on the generator, a train of PEFs was delivered. The duration of each pulse was tuned from micro- to milliseconds, exposing to μsPEFs and msPEFs. Moreover, for each pulse duration, three exposure conditions were tested varying the number of pulses.

The oxidation of phospholipid membranes, due to PEFs application, was estimated for all samples before and after the exposure, by monitoring the UV absorbance in arbitrary units, using a spectrophotometer (Nanodrop2000 spectrophotometer, Thermo Fisher Scientific) and comparing exposed to control samples data. Each measure was performed in triplicate.

Moreover, the oxidation of lipids was analysed using other experimental conditions on both raw POPC lipids and prepared liposomes (to consider the contribution of liposome preparation in the oxidation process):

- adding chemical reagents (H_2O_2, $FeSO_4$ and both H_2O_2, $FeSO_4$), 2:1 dilution, to obtain the maximum lipid oxidation;
- adding PBS buffer and plasma activated PBS (previously prepared using cold plasma) 2:1 dilution, as external source of oxidative species, able to facilitate future interactions with EF;

these obtained samples were kept for 2 h at 60°C.

All the experiments were conducted in triplicate. At the end of each experiment, an aliquot (50 μL) of internal standard (DSPC 2.5 ng/μL methanol) was added to the sample (100 μL), that was successively extracted with 500 μL of chloroform–methanol (2:1, v/v). After the organic solvent evaporation and centrifugation, the dried extract was drawn in 100μL of methanol and stored at 4°C, ready for the analysis by electrospray ionization-tandem mass spectrometry (ESI-MS/MS).

DESCRIPTION OF THE MAIN RESULTS OBTAINED (max.500 words)

After the preparation, liposomes were physico-chemically characterized, obtaining a diameter of about 280nm. The vesicles, stored at 4°C, were stable over a week.

For each PEFs exposure, all samples were pre-treated and, to obtain reliable results, each exposed sample (130μl) was compared with two controls:

- 130μl aliquot placed in an Eppendorf tube (control);
- 130μl aliquot, placed in the exposure system with the generator switched off, to consider possible interactions between liposomes and electrodes (sham).

The PEFs exposure conditions are listed in the table below, with the measured current data:

	Number of pulses	Max Applied Voltage [V]	Frequency [Hz]	Pulse duration	Max Measured Current [A]
μsPEF	20	1000	1	100 [μs]	3
	40				
	80				
msPEF	1	200	2	10 [ms]	0.6
	8				
	20				

Soon after the exposure, samples were analysed in terms of absorbance at 234nm, to investigate the oxidation of lipid membranes. The blank sample is represented by HEPES solution exposed to each PEF condition.

Interesting results were obtained exposing the lipid vesicles to both the three μsPEFs (20, 40 and 80 pulses) conditions and the 20 msPEFs ones, with values that differ from the controls in a statistically significant way ($p < 0.001$ or $p < 0.01$ from t-test evaluation) and obtaining a dose-effect response. Sham and control results are in line each other.
Monitoring the size and zeta potential of liposomes before and after the exposure, contrasting results were obtained: dimension reduction (of about 30nm), together with a decrease of the zeta potential, was obtained after μsPEFs applications; while stable values were carried out with msPEFs.
Moreover, a second analysis of samples exposed to 20 and 40 μsPEFs was performed four days after the treatment to investigate the vesicles stability. To this regards, the samples were kept at 4°C in Eppendorf tubes after the exposures and the changes in the UV spectra waveform were obtained for both exposed and sham samples, evidencing a possible interaction between the suspension and the electrodes for especially 20 μsPEFs application.
For what concern the MS/MS analysis, preliminary investigations on lipid oxidation were performed for the following conditions: 20 and 40 μsPEFs; incubation for 2h at 60°C adding chemical reagents (H_2O_2 only, $FeSO_4$ only, and both H_2O_2, $FeSO_4$), to both POPC raw material and liposome suspension; incubation for 2h at 60°C adding PBS or activated PBS (dilution 2:1) to both raw POPC and liposome suspension.
The MS/MS outcomes reveal that the maximum oxidation could be achieved adding H_2O_2, $FeSO_4$ together and $FeSO_4$ only, for liposome suspensions and these results are in line with the raw POPC samples. However, the MS/MS analysis should be upgraded to a High Resolution MS investigation to both better discriminate the background from the sample signal, and identify all the oxidation products formed in each sample.
These final analyses were decided and all the treated samples were extracted and kept at 4°C for these advanced MS analyses, to clarify from an MS point of view the interaction between biological membrane and lighting strokes-like electric signals, considering chemical factors that could enhance membrane oxidation.
Due to Covid-19 pandemia Force Majeure, the Host Institution had to close the laboratory, thus the final analyses will be performed later by a host institution collaborator.

FUTURE COLLABORATIONS (if applicable) (max.500 words)

Thanks to this STSM (developed in the framework of and supported by COST Action CA15211 – ElectroNET) and thanks to the collaboration with the research group of the Gustave Roussy Institute lead by Prof. Lluis Mir, it was possible to design the next steps of this multidisciplinary project. The investigations performed during this STSM focused on the interaction between lighting strokes-like electric signals with bilayered membrane vesicles, mimicking the cellular and subcellular membrane, in terms of lipid oxidation and vesicles stability. The results show that the application of PEFs (with identified parameters) on lipid vesicles could determine an oxidation effect together with a possible effect in terms of vesicle stability.
Starting from the STSM results, it was decided that the next step would be to continue with investigating the interaction of atmospheric electricity signals with lipid vesicles using a non-linear optical microspectroscopy technique based on a wide field Coherent anti-Stokes Raman scattering (CARS). The Gustave Roussy research group is currently setting up the complete CARS system, which is sensitive to specific vibrational signatures of molecules. The CARS analysis could let us to deeper understand the mechanisms occurring on biological lipid membrane during and after their exposure to PEFs. The results of these future investigations could be matched with multiphysics simulations of lipid vesicles interacting with PEFs, that I am performing using the software Comsol Multiphysics. The numerical simulations could give information on the mechanisms involved from on the micrometer scale and could be useful to predict future exposure conditions that should be experimentally used to study the physico-chemical interaction between atmospheric electricity

signals, and biological objects. Moreover future *in vitro* investigations are considered to study the PEF interaction with cells.

Expected Publications

As mentioned before, the High Resolution MS investigation of the samples collected during my STSM will performed later by a host institution collaborator (due to the interruption of my STSM caused by the Covid-19 pandemia Force Majeure and the Host Institution laboratory closure). With these results and the data here reported, a manuscript will be prepared for publication in a peer reviewed journal.

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

Contact Person of Host Institution: Lluis M. Mir

Signature

Name of researcher:

Ms. Laura Caramazza

Signature

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