

Quality of Life and Management of Living Resources

Key Action 4 – Environment and Health

THz-BRIDGE

Tera-Hertz radiation in Biological Research, Investigations on Diagnostics and study on potential Genotoxic Effects

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Deliverable D-20

Review of biological effects at THz frequencies, recommendations on exposure conditions

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INTRODUCTION

In this deliverable a review of biological effects on different biological samples after exposure to THz radiation is reported together with recommendations on exposure conditions. In particular human lymphocytes, model membranes and epithelial cultures have been used as biological samples. The different experimental activities were carried out by different partners according to Table 1.

Table 1

Involvement of THz-BRIDGE partners in the evaluation of biological effects after exposure to THz-radiation

Human lymphocytes	Membrane	Epithelial cultures	DNA basis
ENE A	ENE A	UNOTT	NHRF
ICE mB at CNR-IREA	ICE mB at CNR-INMM		ENE A
TAU	-----		-----

EVALUATION OF BIOLOGICAL EFFECTS IN VITRO AFTER EXPOSURE OF HUMAN LYMPHOCYTES TO THz RADIATION

Significant assays were developed on the basis of the expertise of **ICE mB** and **TAU** and of the spectroscopic information provided by **USTUTT**, **UFRANK**, **TVL**, **ALU-FR** and **ENE A**. Whole blood samples and blood-cell cultures were exposed to radiation at selected frequencies in the range from 90 to 150 GHz by ENE A and TAU respectively. In parallel with the irradiation experiments, the modeling of the interaction of THz radiation with water, serum and blood constituents was developed by **ICE mB** and **ENE A** taking into account the diffusion and absorption characteristics. It has been shown that, in the low frequency range, the measured absorption coefficient is well explained by a model consisting of a mixture of cylindrical particles (the erythrocytes) dispersed in water.

The irradiation of whole blood has been studied by **ICE mB**-IREA at frequencies in the range from 120 to 130 GHz by varying the average power and the pulse repetition rate in order to evaluate the induction of micronuclei and cell cycle kinetics on peripheral blood lymphocytes. Three different exposure set-up were developed by ENE A as discussed in the Deliverables D-6 and D-17

Whole blood samples were exposed for 20 minutes at an average power ranging from 0.6 to 5 mW. The pulse repetition rates were in the range 2 to 7 Hz. The various exposure conditions are summarized in Table 2. In four experiments, an aliquot of exposed/sham-exposed whole blood was analyzed in the leukocytes population, by means of alkaline Comet assay, in order to verify the presence of any direct DNA damage soon after THz exposure.

Detailed results are reported in Deliverable D-17.

Table 2- Exposure conditions adopted for 20 min. THz exposure

Exposure condition	Frequency [GHz]	Pulse repetition rate [Hz]	Average power [mW]	Delivered energy [J]	Mean electric Field [V/cm]	Peak electric field (4µs) [V/cm]	Peak electric field (5ps) [kV/m]	Irradiation set up	Biological target
1	120	2	1	1.20	0.19	67	170	1	MN, CBPI
2	130	2	0.6	0.72	0.15	50	130	1	MN, CBPI
3		5	3.5	4.20	0.35	78	193	1	MN, CBPI, Comet
4		7	5.0	6.20	0.42	78	193	1	MN, CBPI, Comet
5		7	1.9	2.28	1.52	287	703	2 A	Comet
6		7	5	6.20	2.45	465	1140	2 B	Comet

The results obtained by ICEmB confirm the absence of both genotoxic effects and influence on cell proliferation in the adopted experimental conditions, when the exposure time does not exceed 20 minutes at an average power in the range from 0.6 to 5 mW. Attempts have been made to investigate effects at higher exposure levels by performing the Comet test on leukocytes separated from the aqueous part of the blood (serum) and directly exposed to THz radiation without the shielding effect of serum. However, the results of the Comet test on 12 healthy donors did not provide a clear answer, probably due to a lack of

In a complementary approach to the experiments performed by ICEmB, TAU employed human lymphocyte cultures as a biological model for studying potential genotoxic effects.

Polystyrene culture flasks containing lymphocytes isolated from peripheral blood at 37 °C were exposed to 100 GHz radiation with different time duration at an average power density of 0.043 mW/cm² yielding an average SAR value of 3.2 mW/gr. Lymphocytes isolated from peripheral blood of five individuals were irradiated for 1, 2 and 24 hours and were harvested by standard cytogenetic procedures 72 hours after the onset of exposure (see Table 3). The cells were stored at -80°C before applying the FISH assay (see Deliverable D-14 for details).

Table 3 – Culture and exposure conditions at TAU

CELLS	CULTURE CONDITIONS	EXPOSURE
Human lymphocytes	Lymphocytes were isolated from human peripheral blood cells by density gradient centrifugation, were stimulated by mitogen (PHA) prior to exposure to 100 GHz at 37 ⁰ C, maintained for 72 hours and then harvested using a standard cytogenetic procedure. Grown in suspension	Exposure to a CW source of 100GHZ. $I_{inc} = 0.05 \text{ mW/cm}^2$ since $I_0 = T \times I_{inc}$ I_0 of 0.043 mW/cm² was calculated for a transmission T of 83% , yielding SAR of 3.2mW/gr. The respective total energy input for exposures of 1, 2, and 24 hours is 3.4 J, 6.8 J and 82 J, respectively

The exposed cells exposed were analyzed for changes in the level of aneuploidy (losses and gains of chromosomes), replication timing (early or late during s-phase) and asynchronous replication (coordination between the homologues) of centromeres of chromosomes 17 and 11. When total

aneuploidy was measured (losses plus gains) with all the shams, a statistically significant increase in aneuploidy was observed following 2 hours of exposure for chromosome 11 as well as for 24 hours of exposure for chromosome 17. Following 24 hours of exposure and 2 hours of exposure there is a statistically not significant tendency for an increase in chromosomal losses and gains of chromosome 11 and 17, respectively. When analyzing the frequency of asynchronous replication in the exposed cultures an elevation of asynchronous replication of CEN11 and CEN17 following 24 hours and 2 hours of exposure respectively was observed.

Both genotoxic and epigenetic effects are induced in lymphocytes following exposure to CW 100 GHz radiation of 0.05 mW/cm² intensity when the exposure period exceeds one hour. Although the reported effects have been observed on cells directly exposed to THz radiation, without the shielding effect of the human body, they occurred at a relatively low intensity when compared to the exposure limits set by the ICNIRP guidelines (1mW/cm² for general public exposure and 5mW/cm² for occupational exposure). More experiments are needed to establish accurate dose-response relationships.

EVALUATION OF BIOLOGICAL EFFECTS IN VITRO AFTER EXPOSURE OF MEMBRANES AND EPITHELIAL CULTURES

Epithelial cell cultures

Following the recommendations of the Mid-Term Review, the effects on dividing cells have been examined at **UNOTT** along with the subsequent ability of the exposed cells to differentiate. This has provided a direct comparison with the data already obtained for the confluent keratinocyte cultures. The new direction has involved the evaluation of effects on the sensory neuronal cell line ND7/23, as has been employed in co-culture experiments in vitro aimed at producing an innervated human corneal model. Subsequent to the THz exposure keratinocyte growth and differentiation has been examined in the presence of these nerve cells, using methods already established. Techniques for co-culture of neural and corneal cells have been adapted to neural and human keratinocytes, bearing in mind the data generated by USTUTT on tissue culture inserts and THz penetration. For the exposure to THz radiation in the 0.1-10 THz range three different sources have been used. They were provide by Teraview Ltd, Cambridge, by at the Institute of Microwave and Photonics, University of Leeds, and by ENEA-Frascati. We briefly recall the characteristics of these sources; the exposure conditions are summarized in the Table 4.

- The THz source at Teraview (Cambridge) is based on the optical excitation of a gallium arsenide wide aperture antenna. A large DC-bias is applied across the device which was excited using a Ti:Sapphire laser (RegA 9000, Coherent Inc, CA) emitting 250 fs pulses centred at a wavelength of 800nm, with a 250kHz repetition rate. This gives a usable frequency range of 0.1 THz to 2.7 THz with an average power of approximately 1mW. The THz-radiation is collected and collimated by an f/1 off-axis parabola and then focused by another parabola on to the sample with a spot size of 130 µm to 3.7 mm. The THz spot was raster scanned over sample for the duration of the exposure.
- The THz system at the University of Leeds (Institute of Microwaves & Photonics) uses a Ti:Sapphire laser impacting on an electro-optic photoconverter to generate THz radiation. The total pulse duration is 20-30ps, although approximately 90% of the THz power is delivered within the first two pico seconds. The average output power for this (unamplified) system is approximately 1µW within the frequency range 0.2 – 3.0 THz. The repetition rate of the THz pulse is approximately 80 MHz.
- The 150 GHz system from ENEA - Frascati Italy is an IMPATT diode, Hughes model Number: 47178H-1000, operating in the frequency Band, 110-170 GHz, with a centre frequency of 150 GHz at a bias current of 250mA with an input voltage of 35V_{DC}. The emitted power is about 3 mW continuous wave (CW).

Table 4 - Exposure conditions of the epithelial and sensory neuronal cells and co-culture models.

CELLS	CULTURE CONDITIONS	EXPOSURE	TRANSPORTATION CONDITIONS
Human primary keratinocytes	Sub confluent rapidly dividing undifferentiated	THz (0.1-3) 0.15-0.45 mJ/cm ² 0.15-0.45 J/cm ² GHz (130) 1.24 J/cm ²	22-28°C 3 Hrs None
Human Primary Keratinocytes	Confluent slowly dividing differentiated	THz (0.1-3) 0.15-0.45 mJ/cm ² 0.15-0.45 J/cm ² GHz (130) 1.24, 9.78 or 14.6 J/cm ²	22-28°C 3 hrs 22-28°C 4 hrs None
Human Primary Keratinocytes	Confluent slowly dividing differentiated stressed with 2.5µg/ml Sodium lauryl sulphate	GHz (130) 4.17 J/cm ²	None
Human Primary Keratinocytes	Air Liquid Interface	THz (0.1-3) 0.15-0.45 J/cm ²	None
Human Primary Keratinocytes plus ND7/23 sensory	Air liquid Interface differentiating	THz (0.1-3) 0.15-0.45 J/cm ²	None
ND7/23 sensory neurones	Undifferentiated with division	THz (0.1-3) 0.15-0.45 J/cm ² GHz (130) 1.24 J/cm ²	22-28°C 3 hrs None
ND7/23 sensory neurones	Differentiated Very limited division	THz (0.1-3) 0.15-0.45 J/cm ² GHz (130) 1.24 or 9.78 J/cm ²	22-28°C 3 hrs. None

Table 5 - Total energy delivered to the cultures in the different culture methods.

Plate type	24 well	24 well inserts	96 well plates
Area	1.78 cm ²	0.79 cm ²	0.28 cm ²
14.6 J	26	11.5	4.09
9.78 J	17.4	7.73	2.74
1.24 J	2.2	0.98	0.35
0.45 J	0.8	0.36	0.13
0.15 J	0.27	0.12	0.042
0.45 mJ	0.0008	0.00036	0.00013
0.15 mJ	0.00027	0.00012	0.000042

No changes compared with the unexposed controls, in terms of cell activity (Resazurin assay) or differentiation (Fluorescein Cadaverine assay), or barrier function in terms of the air liquid interface models (sodium fluorescein assay), was observed with any of the exposure rates employed, which exceed exposure times required to generate images in human patients, using the same apparatus, by at least 5 times. It was also found that no damage in terms of cell activity, measured via oxidative stress, or differentiation was caused to ND7/23 cells or human keratinocytes. This was also true even if the cells were stressed by a surfactant, which could form a residue on the skin of patients. Hence we are reasonable confident that the THz exposure rendered to patients by the Teraview imaging system is not potentially harmful.

Membranes

A very simple membrane model, such as that provided by liposomes, has been used to study the permeability of a simple bilayer in response to THz radiation in absence of interfering reactions. This model consists of liposomes enclosing in their interior a soluble enzyme, such as the carbonic anhydrase (CA). Liposomes are microscopic vesicles in which an aqueous volume is entirely enclosed by a membrane composed of phospholipids, which resemble the lipid portion of cell membranes. During the third year of the project, the **ICEmB** group at **CNR-INMM** in collaboration with **ENEA** analyzed the influence of terahertz radiation ranging from 90 to 150 GHz on the influx rate of the substrate p-nitrophenyl acetate (p-NPA) into carbonic anhydrase (CA)-loaded liposomes. Since in the erythrocyte CA is one of the most abundant proteins after hemoglobin, liposome loaded with CA can be considered as a very simple model of the erythrocyte. ICEmB investigated whether terahertz radiation could affect the permeability of these lipid bilayers under well-controlled irradiation conditions. Different pulse repetition rates were studied.

To carry out the irradiation studies a visible spectrophotometer, to be employed for the kinetic measurements of the CA enzyme, was modified to allow irradiation of the liposomes during kinetics measurements. Several experimental set up were used. Details are given in the Deliverables D9-a and D-11a. The results of the various experiments conducted at different carrier frequency, incident power and modulation conditions are discussed in the deliverable D-18. In Figure 1 the effect of exposures is reported as the increase of enzymatic activity in comparison to the total CA activity, determined after rupture of liposomes by detergent, taken as 100%.

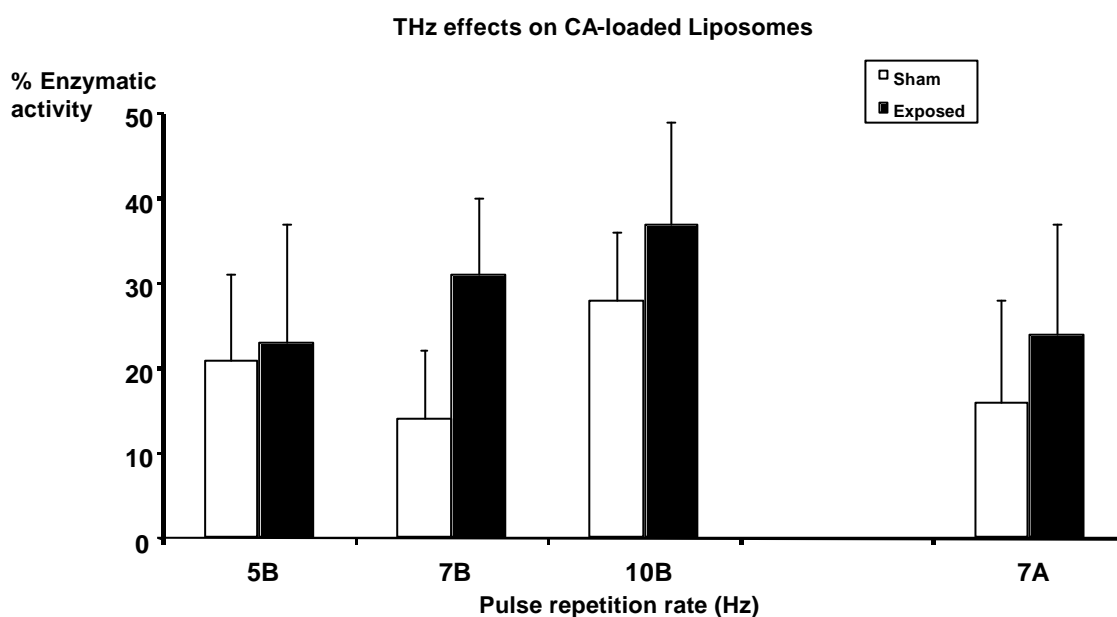


Figure 1) Effect of Thz exposures of CA loaded liposomes reported as the increase of enzymatic activity.

The results indicate that terahertz radiation can affect lipid bilayer permeability. An increase in substrate permeation rate across the liposome bilayer (DPPC:Chol:SA = 5:3:2) was observed over two minutes of 130 GHz irradiation with a pulse repetition rate of 7Hz and a delivered power of about 7.8 mW/cm². To verify the specific role of carrier frequency and pulse repetition rate in eliciting the THz-induced effect, additional experiments have been conducted changing the carrier frequency from 130 GHz to 3 GHz. Continuous wave (CW) irradiation was also performed at a carrier frequency of 150 GHz. No effects were observed in the two latter cases.

EVALUATION OF BIOLOGICAL EFFECTS ON DNA BASES

To evaluate the effects of THz radiation on DNA bases, the group at NHRF developed two different experimental approaches for the preparation of thin films: from vapor deposition at high temperature and from solutions at different temperatures. Regarding the first method, the experimental apparatus consisted from a stainless steel vacuum chamber where a carbon crucible was placed and where temperature could be controlled as accurate as 0.5°C . Films were deposited in background pressure of Argon. Thin films of DNA bases were fabricated on a silicon wafer. Details are reported in Deliverable D-13. The method was improved over the project.

Thin films of DNA bases were also prepared from liquid solutions. The improvement of the method consisted in growing the crystals under vibration free conditions and temperature controllable conditions. With this way crystal samples of dimensions 1mm X 1mm were prepared with good surface uniformity in the z direction. The thickness of the film was measured by first etching the film at 157 nm, and then by measuring its thickness by AFM.

Images of thin films of DNA bases taken after exposing them with the radiation from the ENEA Compact Free Electron Laser operating at 130 GHz, show no evidences of any damage under the exposed conditions, see Fig. 2

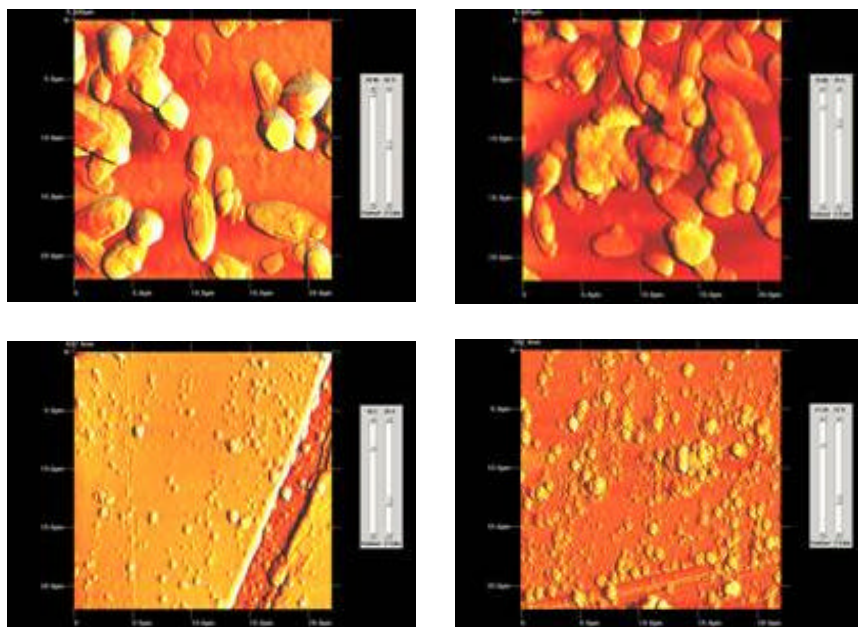


Fig. 2 - AFM images of Adenine, Guanine, and Cytosine irradiated at 2.5 mm. The images show no evidences of any damage under the irradiation conditions

Assessment of the damage of the thin films of DNA bases after exposing them with the radiation from the ENEA Compact Free Electron Laser operating at 130 GHz, shows no evidences of any damage under the exposed conditions. Assessment of damage was done by comparison with irradiated samples at 157 nm where damage of DNA bases takes place even at low energies. The possible damage of DNA bases was also measured by mass spectroscopy at different wavelengths using a pulsed discharged CO_2 laser at $17.6\ \mu\text{m}$, and the output at 21.5, 28.5, and 41.7 nm. From the analysis of the mass spectra, and the number of photons, which were required to break one CN bond, it was found that the damage on the DNA bases under high irradiation conditions is only thermal, with no indication of any photochemical damage