Quality of Life and Management of Living Resources
Key Action 4 – Environment and Health

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

THz-BRIDGE
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Final Report

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1. INTRODUCTION
This report presents the research activity and the results of the THz-BRIDGE project funded in the EU “Quality of Life” programme – Key Action 4 Environment and Health. The aim of the project has been to investigate the interaction of Terahertz (THz) radiation with biological systems. THz radiation covers the frequency range between 100 GHz and 20 THz (i.e. a wavelength between 3 mm and 15 µm), which spans the spectral interval between the microwave- and the infrared regions of the electromagnetic spectrum. Due to the rapidly increasing applications of THz radiation in biology and biomedicine a proactive approach has been adopted rather than reactive research in the study of any induced effect. The project followed a streamline of increasing complexity from bio-molecules to cells, e.g. membranes, chromosomal and DNA integrity.

The objectives of the THz-BRIDGE project are:
- To provide a spectroscopic database for selected enzymes, proteins, biological membranes and cells in the frequency range from 100 GHz to 20 THz (the so-called “THz gap”) under irradiation conditions that preserve the integrity and functionality of the biological samples.
- To identify, from the above data, critical frequencies, which might induce damages on biological systems, and to determine the spectral regions for optimal contrast in imaging applications.
- To assess risk of potential damage to biological activity, both functional and morphological, due to the exposure of membranes, cells, and DNA to pulsed and CW THz radiation.

At present, a number of laboratory-scale THz sources, like electronic tubes, free-electron lasers, and pulsed solid-state THz sources, are in use at research institutes, raising the issue of potential exposure of specialised personnel and users. Biomedical imaging devices based on such sources, have also entered the market during the project life time. In this respect the project has provided a timely assessment of potential hazards and health effects at specific occupational sites of research laboratories and industries, where THz radiation sources are in use or are being developed. THz-BRIDGE has used an interdisciplinary approach to study the effects of the interaction of THz radiation with biological systems forming a consortium that has included the Ente Nazionale per le Nuove tecnologie, l’Energia e l’Ambiente (ENEA), the Forschungszentrum Rossendorf (FZR), Tel-Aviv University (TAU), Stuttgart University (USTUTT), Frankfurt University (UFRANK), the University of Genoa (UGOA-ICEmb), the National Hellenic Research Foundation (NHRF), Teraview Limited - Cambridge (TVL), the University of Nottingham (UNOTT) and the University of Freiburg.

The report is divided in three sections according to the above mentioned objectives:
The Section “Spectroscopy of proteins, enzymes, biological membranes, and selected cells” describes the spectral investigations carried out on a variety of significant biological samples to understand the interaction of far-infrared (FIR) and THz radiation with biological systems on a molecular level, i.e. on the basis of resonant processes with electronic, vibrational, and rotational states of complex biological molecules.

The Section “Evaluation of biological effects in vitro after exposure to THz-radiation” describes the effects of Terahertz radiation on significant biological systems of increasing complexity as a function of incident and absorbed power, wavelength, pulse duration and modulation conditions. The aim is to provide a risk assessment prior the future implementation of THz devices in bio-medical diagnostics. Apart from a limited amount of data in the low-frequency part of the THz region, no information has been available so far on the effect of THz-radiation on biological systems. To study such possible effects, powerful sources of THz radiation, like Free Electron Lasers (FEL), microwave and solid-state sources, with wide and complete control of external parameters, have been used at the partners sites over the frequency range of interest.

The Section “Safety issues of THz radiation” reports the results of a survey on the use of THz radiation conducted during the project lifetime. It addresses safety issues at specific occupational sites, where THz sources are employed or developed. The questionnaire was distributed to a number of research laboratories involved in THz development to collect information on the main radiation parameters, on the exposure conditions (if any) of technical personnel and on the safety measurements or precautions currently adopted.
2. “SPECTROSCOPY OF PROTEINS, ENZYMES, BIOLOGICAL MEMBRANES, AND SELECTED CELLS”

Techniques such as X-ray crystallography, 2-D NMR spectroscopy, and high-resolution electron microscopy deliver static, frozen pictures of proteins, enzymes, and biological membranes. Information on the function and how it is related to the structure, however, requires spectroscopic techniques that probe structural properties and allow high temporal resolution. Among the variety of spectroscopic techniques, Infrared (IR) spectroscopy has probably the best access to minute structural details, in the order of fractions of a bond dimension. The use of infrared spectroscopy for the study of biological systems has greatly advanced due to the high sensitivity and rapid data acquisition provided by Fourier-Transform infrared (FT-IR) spectrometers [Mäntele, 1996]. In the mid-IR spectral range, in particular in the region from 2000-1000 cm\(^{-1}\), FT-IR spectroscopy can monitor alterations at individual bonds even in large protein complexes, thus allowing structural and conformational changes in the course of a biological reaction to be monitored in high detail and in real time, and reaction mechanisms to be elucidated. Yet, the presently available frequency range of FT-IR techniques, typically 4000 – 200 cm\(^{-1}\) does by far not cover the full range of functionally relevant modes of enzymes and proteins, which may extend down to 10 cm\(^{-1}\). This region, which lies between the mid-infrared and the microwave part of the spectrum, is the domain of THz spectroscopy.

In solid materials THz spectroscopy can provide information about the collective modes of the lattice structure. This makes the technique very sensitive to both the crystalline conformation and polymorphic form of the material in the solid phase, and to high frequency molecular rotations in gases. The liquid phase spectroscopic information is a complex mixture of rotational and transitional modes. In THz Spectroscopy conventional infrared techniques can be utilised together with some new ones that derive from a proper exploitation of the features of laser pumped THz sources. The lack of continuous wave (CW) sources in the THz Spectroscopy region restricts the use of conventional Fourier Transform Spectroscopy to the long wavelength part of the THz spectrum (20-60 GHz) where Backward Wave Oscillators (BWO) are available with sufficient power. At higher frequencies pulsed THz emitters allow the use a coherent detection mechanism with which it is possible to perform a Time Domain Spectroscopy (TDS) [Grischkowsky, 1990].

2.1 Materials and methods

The first research issue addressed in THz-BRIDGE has been the extension of current spectroscopic techniques of biological systems from the Infrared (IR) into the Terahertz (THz) region. Spectroscopic investigations are crucial for a clear definition of the external parameters to be kept under control in irradiation studies. The materials most commonly used for the attachment and growth of cell culture in vitro are made from Polyethylene (PE), Teflon (PTFE), Polystyrene (PS) and other non-specified polymeric materials such as Thermonox™ (ICN Pharmaceuticals, Inc). Biological samples are usually placed in cuvettes, Petri dishes, culture cells, made of such materials, which are designed for visual inspection or
optical measurements in the visible and are not necessarily transparent to THz radiation. Prior to using them in spectroscopic measurements and irradiation experiments it is important to ascertain their transparency to THz radiation. Further to this, THz spectroscopy of biological samples has to cope with the absorbance of water, which has an optical absorption coefficient between 100 and 1000 cm$^{-1}$ [Palik,1985] (see Fig. 2.1). Consequently, the path length for transmission spectra is limited to 10-20 µm in the high frequency part of the THz range, and can be extended to 50-100 µm below 1 THz.

On the basis of the expertise gained at the University of Frankfurt on the spectroscopy of proteins and enzymes diluted in a thin layer of aqueous solution, a “model cell” was developed with the typical design shown in Fig. 2.2.

The cell has a circular symmetry, 20 to 40 mm in diameter. The sample, diluted in an aqueous solution, is placed between two plates separated by a Mylar spacer. Due to the absorption of water at the frequencies of interest, only a small thickness of the spacer, typically in the range 5 to 100 µm is allowed. A draught is milled in the bottom plate to allow for the flow of the aqueous solution when the top plate is pressed against the Mylar spacer, without inducing any strain on the sample. The bottom and top plates are made of a suitable material, as discussed below, which is transparent in the frequency range of interest. The typical thickness of the plates is of the order of 1 mm. One of the limits of “soft” plastic materials in the construction...
of such a cell is their mechanical stability, which often prevents the production of thin aqueous samples with high reproducibility in thickness.

A variety of materials were investigated, starting with commercial plastic-ware from NUNC, Corning and Falcon. Among the most widely used materials in biological labs, polystyrene was found to be very useful at long wavelengths. Polystyrene exhibits excellent optical properties in the wavelength range between 200 µm and 3 mm (frequency range: 1.5 THz to 100 GHz). Specific measurements were carried out by measuring both the transmission and the complex dielectric constant over a wide spectral range as it is shown in Fig.2.3. Further spectroscopic investigations have been performed on other materials, like ZnTe, which can be used as a windows of the spectroscopic cell in the high frequency range between 500 and 10000 cm⁻¹ (15 – 300 THz). Other measurements involved Thermonox™ (Fig. 2.4), Polyethylene, Polypropylene, Teflon, LiTaO3 and Silicon. Details of such measurements have been organized in a Spectroscopic Database made available to the scientific community. (www.frascati.enea.it/THz-BRIDGE/public/spectra/searchdb.htm)

The transmission properties of a range of tissue culture plastic-ware and insert membranes used by the group at the University of Nottingham were also measured. The results are discussed in Deliverable D-5. Some of
the insert membranes are highly transparent in the THz range, which make them suitable to use. The Millipore membrane PICM 01250 has the highest transmission being approximately 0.9 in the frequency range from 50 to 400 cm$^{-1}$.

At the University of Stuttgart, optical transmission and reflection measurements are performed using two FTIR spectrometers (Bruker OFS 113v, Biorad) in the range from 5 to 15000 cm$^{-1}$ and a Mach-Zehnder interferometer in the range from 2 to 40 cm$^{-1}$. The analysis of biological samples has also required the modification of the Bruker OFS 113v spectrometer. Due to the large absorption of water vapour in air, FIR spectrometers are usually operated in vacuum. Since biological samples cannot be exposed to vacuum, modifications are needed to solve the problem. The one realised at the University of Stuttgart is a setup where the bottom of the sample holder serves as a window [Matei, 2003]. To allow the quick replacement of samples, a lock is used, which allows to separate the sample holder from the spectrometer. The lock can be evacuated separately and ventilated with dry nitrogen (see Fig. 2.5). A flexible bellows is used in order to ensure that the sample can be aligned with respect to the beam. This is particular important for small samples.

![Fig. 2.5 - Coupling of sample holder to the vacuum spectrometer](image)

The Mach-Zehnder interferometer used at the University of Stuttgart (see Fig. 2.6) is equipped with a series of tunable monochromatic Backward Wave Oscillators (BWO) continuous wave (CW) sources to measure the change in power and phase upon transmission through the sample, which allows to calculate the complex dielectric constant of the material.
The TeraView Ltd group uses a pulsed spectrometer as indicated in Fig. 2.7. The THz radiation is generated by a non-linear antenna excited by a Ti:Sapphire femto-second infrared laser. The THz pulse has a very broad band allowing an accurate spectroscopic analysis. The coherent detection performed through electro-optic sampling of the transmitted pulse allows to obtain the spectral scan from a FT of the time scan of the pulse.

A portable version of the spectrometer has been realized. The instrument is totally self contained with no user adjustments of the optics required. The sample chamber is designed to be similar to traditional Fourier transform infrared FTIR spectrometers, accommodating standard solid, liquid and gas sampling.

An infrared assay for the biomedical analysis of blood samples by mid-IR spectroscopy has been developed at the University of Frankfurt. Attenuated total reflection spectroscopy (ATR) was found to be the optimum
sample interface for full blood and blood serum samples. With ATR crystals cut at the appropriate length and trapezoidal angles, between 7 and 9 reflections can be obtained for sample quantities below 5-10 µL, which allow medical applications with blood squeezed from a finger as usual in blood glucose testing. A diamond-covered ATR plate has been identified as the most successful ATR sample interface, only needing approximately 5 µL of sample volume (see Fig. 2.8).

Currently this technique is being developed for clinical point-of-care (POCT) testing, where important blood parameters could be determined immediately from the patient in order to allow immediate and adequate treatment and therapy. Many parameters of medical relevance can be determined qualitatively and quantitatively from blood samples by vibrational spectroscopy, with the exception of ion concentrations. At present, we aim to determine blood glucose, cholesterol, urea, total protein, triglycerides and creatinine, while other substances can be detected as well. The present determination uses clinical samples with normal values for the parameter under investigation and some pathological variation. For the absolute determination of value of the respective parameters, we rely on the standard clinical chemistry with enzymatic and colorimetric determination. These standards, however, are only of limited precision. Cross-correlation diagrams for the analytes blood glucose, urea, total protein and cholesterol have shown an excellent correlation between the values determined from an evaluation matrix developed for the analysis of the IR spectra between 1200 cm\(^{-1}\) and 800 cm\(^{-1}\), with some extra wavelength ranges above 1500 cm\(^{-1}\). For some of the analytes such as glucose and total protein, the precision is already sufficient for clinical use, even assuming the standard clinical determination to be without error.

2.2 Results

Blood is probably the most important tissue investigated in the THz-BRIDGE project due to the role it covers in all biological processes of human life. Due to this importance the spectroscopic analysis of the blood has been performed over a wide spectral range. In the infrared spectral range from 2500 cm\(^{-1}\) to < 1000 cm\(^{-1}\), several regions can be identified as specific for blood constituents. The spectral regions identified for
the analysis of different parameters were reviewed and partly corrected. At present, the following spectral ranges have been agreed as most useful for the respective parameters:

<table>
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<th>Analyte in full blood or blood serum</th>
<th>Spectral range [cm⁻¹]</th>
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<tbody>
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<td>glucose</td>
<td>1200 - 975</td>
</tr>
<tr>
<td>cholesterol</td>
<td>3000 - 2800</td>
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<tr>
<td></td>
<td>1800 - 1700</td>
</tr>
<tr>
<td></td>
<td>1400 - 1100</td>
</tr>
<tr>
<td>triglycerides</td>
<td>3050 - 2750</td>
</tr>
<tr>
<td></td>
<td>1800 - 1700</td>
</tr>
<tr>
<td>urea</td>
<td>1800 - 1200</td>
</tr>
<tr>
<td>total protein</td>
<td>1700 - 1300</td>
</tr>
<tr>
<td>creatinine</td>
<td>2500 - 2200</td>
</tr>
<tr>
<td></td>
<td>1200 - 750</td>
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Absorption and diffusion measurements on blood constituents at 120 GHz have been performed at ENEA [Giovenale, 2003]. At these frequencies, the macroscopic structure of the sample components must be taken into account for a proper modelling of the interaction. Whole blood can be considered as a colloidal dispersion of blood-cells into the plasma. The penetration depth of the Compact FEL radiation through various biological samples has been measured at 120 GHz to determine the most efficient way to obtain a uniform exposure during the irradiation of whole blood. Absorption measurements have been carried out on whole human blood, serum, plasma, water, saline solution, culture medium etc. The results of the measurements, corrected for the “meniscus-effect” that alters the effective sample thickness, are reported in Fig. 2.9.

From the data, the following values of the attenuation coefficient at 120 GHz are obtained:

- Culture medium (not shown in the graph): $\alpha = 83 \text{ cm}^{-1}$
- Saline Solution: $\alpha = 79 \text{ cm}^{-1}$
- Whole blood: $\alpha = 75 \text{ cm}^{-1}$
- Serum: $\alpha = 71 \text{ cm}^{-1}$
Such values are close to the absorption coefficient of water at room temperature calculated from values listed in [Palik, 1985].

The major blood components were investigated in the range 50 – 700 cm\(^{-1}\) at the University of Stuttgart by FT spectroscopy. Standardized samples were purchased from Sigma-Aldrich (human plasma, clotted whole blood, hemoglobin standards in human plasma and red blood cells group AB). The recorded spectra of whole blood essentially showed characteristics due to the presence of water. Water absorption also dominates the recorded spectra of plasma, red blood cells in plasma, serum and hemoglobin solutions. Additional measurements were performed on a series of proteins important for the blood function: fibrinogen, gamma-globulin and albumin, in crystalline form, purchased from Fluka.

A further THz extension of spectroscopic measurements on blood has been done at the University of Freiburg using the TDS technique [Jepsen, 2004]. The absorption coefficient and the index of refraction of the most important components of human blood, namely red blood cells and white blood cells have been determined and compared to those of water in the frequency range 0.1 – 3 THz. The measurements were performed in transmission geometry in a standard THz-TDS system. The sample cell consisted of two 6-mm thick TPX windows, separated by a nominally 70 \(\mu m\) thick spacer. This thickness was chosen to ensure a useful transmission of the THz pulse, while minimizing the effect of multiple reflections of the THz pulse within the sample volume. The preparation method is a standard procedure based on drawing 40-50 ml blood from a donor into an EDTA-treated tube, followed by centrifugation (typically 2000 RPM for 15-30 minutes). Subsequently the three distinct phases were separated with a micropipette. The white blood cell solution was re-centrifuged to allow removal of the small amount of red blood cells left over from the separation process. Finally the cell concentration was estimated by counting in a modified Neubauer chamber. The total water concentration in the solutions was determined by the weight loss after freeze-drying of portions of the samples. The concentrations and water contents in the used samples were typically:

- Red blood cell solution: \(C = 1.3\times10^9\) cells/ml 55-58 % water
- White blood cell solution: \(C = 10^6\) cells/ml 80-85 % water

In Figures 2.10 and 2.11 the absorption coefficient and the refractive index of the withe and red cells respectively measured over the frequency range 0.1 – 3.5 THz are reported.
The samples of red and white blood cells contained a significant amount of water. An inspection of the dielectric spectra of the samples shows a striking resemblance with the dielectric properties of pure water. It can therefore at this point be assumed that the major absorber in human blood, as well as probably all other types of body fluids, is water.

A different approach to a spectroscopic analysis of blood makes use of dried human hemoglobin. The dried human hemoglobin has been mixed with a 50/50 mixture weight:weight with polyethylene. The mixture was compressed in a mechanical press to a load of 2-tons in a 13-mm disc. The THz absorption spectrum at room temperature is shown in Fig. 2.12. As can be seen, there is a general broad increase in absorption over the range 0.5 to 2.5 THz.
With the same procedure the THz spectrum of uric acid, reported in Fig. 2.13, has been obtained. In this spectrum we observe a number of very sharp features, most notably at 47.6 and 79.1 cm\(^{-1}\). These vibrations could be due to a general umbrella motion in the ring structure of the molecule. It will be interesting to undertake some local-mode calculations to confirm the assignment of these bands.

The temperature dependent terahertz spectra of L-glutamic acid was also measured [Taday, 2003]. Glutamic acid plays a crucial role in nitrogen metabolism in biological systems. It plays an active role in parental nutrition; studies have shown that human milk contains 1.2% protein of which 20% is bound glutamic acid. The molecule is also known to be a neurotransmitter for the central nervous system and is a major degradation product in tumor cells.
As suggested by several authors the contrast mechanism for biomedical imaging using terahertz radiation is still not fully understood. However, it is believed that it is a change of water content in tumour is the primary mechanism. Teraview has made extensive use of its TPI™ spectra1000 spectrometer to study the properties aqueous solutions of ionic salts. More recently the terahertz absorption spectrum of bovine serum albumin in solution has also been investigated. Figure 2.14 shows the spectrum of pure water and the spectra over a range of different concentrations (125 mM, 500 mM, 2 M and 4M) of sodium chloride (NaCl). A physiological concentration of salt in the human body is approximately 150 mM. Since there is no modification to the terahertz absorption spectrum until non-physiological concentrations of salt are reached we can speculate that NaCl does not contribute as a possible contrast agent in terahertz imaging.

Fig. 2.14: Terahertz absorption spectra of different concentrations of water and sodium chloride (NaCl).

Terahertz transmission measurements of healthy human skin have also been obtained [Woodward, 2003]. The subcutaneous tissue and dermis on ex vivo skin samples were removed to obtain skin samples of the epidermis. The absorption coefficient and the refractive index in Fig. 2.15 are the mean value taken from 15 samples of healthy skin of thickness 200-300µm. The “standard error” (s/n) is plotted in the error bars and corresponds to a percentage error of 3% for the absorption coefficient and of 2% for the refractive index. The refractive index from the transmission data is higher than that of water, which is not expected. The absorption coefficient of skin at 1-THz is also slightly lower than water, not surprising since skin also contains other materials such as collagen and protein.
In order to provide a spectroscopic data base for the major components of biological macro-molecules, a systematic analysis of the spectral features of amino acids has been performed. Following the difficulties encountered in performing spectroscopy in aqueous solution, the samples prepared for the present investigations are amino acids in pressed pellets of polyethylene. In most of the following figures two spectra are displayed (black and red line respectively) to show the reproducibility of the results. The two spectra were indeed measured in different days and with different pellets, prepared in the same way, all the samples were measured at least twice. The spectra of 18 amino acids have been recorded. The two that are missing are arginine and cysteine, that require special conditions for measurements, being toxic for humans. In this final report only two of the 18 amino acids are reported as a reference, the complete study is available in the Deliverable 15.

The smallest of the amino acids is the glycine; it has the simplest spectra (Fig. 2.16). Being the simplest amino acid, a special attention was directed to this molecule, in the idea that understanding the smallest structure is a first step in understanding those more complicated [Suzuki, 1963], [Tsuboi, 1958]. There are only four strong peaks in the range 200 – 650 cm$^{-1}$. More lines are observed under 200 cm$^{-1}$; these are considered to be hydrogen bonds modes overlapped with lattice modes and modes from the side chain. The lines over 200 cm$^{-1}$ are, in general, from specific groups without significant overlapping.

The strong peak at 607 cm$^{-1}$ is due to CO$_2$ symmetrical bending. The absorption peak at 502 cm$^{-1}$ is also strong and represents a CO$_2$ rocking. At 357 cm$^{-1}$ is a strong peak due to the CCN bending. Another mode of CCN group appears at 135 cm$^{-1}$. This peak is weak and is an overlapping of CCN torsion, NH$_3$ bending, and hydrogen bond (H•••O) stretching. The weak peak at 170 cm$^{-1}$ and the strong peak at 200 cm$^{-1}$ have the same origin: hydrogen bond deformations, CCN deformations, and intra-molecular vibrations.
The vibrational spectra of Phenylanine and Tyrosine are presented in Fig. 2.17. Both amino acids have a benzyl group in the side chain. The only difference between them is that Tyrosine has one hydroxyl group attached to the benzyl. The structural difference between the two is small, but not the same can be said about the difference in the vibrational spectra. According to [Grace, 2002], the benzene rings should be prevalent in the spectra, and the ring substituents could be treated as point masses. Watching the Fig. 15, we assume that this is a prediction for other region than FIR: the two amino acids do not share too many lines. There is no evidence that most of the lines are due to the benzene ring. There is a prominent peak in Phenylanine, at ~365 cm$^{-1}$, that has as correspondent in Tyrosine three peaks of lower intensity, at 310, 335, and 377 cm$^{-1}$. The assignment is the following: ring OH torsion at 302 cm$^{-1}$, CH in plane bend at 325 cm$^{-1}$, and NCC bend at 398 cm$^{-1}$.

Fig. 2.16: Terahertz absorption spectra of glycine.
Another feature that is common for both amino acids is the peak around 200 cm\(^{-1}\). For Phenylalanine, this peak is situated a few wavenumbers under 200 cm\(^{-1}\), while for Tyrosine is at a few wavenumbers above 200 cm\(^{-1}\). As is already known, in this region of frequencies vibrate the hydrogen bonds and the CCN bonds (deformation).

2.3 Discussion

As a conclusion on the spectroscopic properties of amino-acids we report the statistical distribution of the resonances of all the molecules (see Fig. 2.18). Each red bar represents 20 wavenumbers. The final conclusion is that amino acids have enough common features, like the CCN or CO\(_2\) deformation modes, to show that they belong to a class. On the other hand there are surprisingly many features that make them distinct from each other. In far infrared amino acids behave as individuals. But this individuality is lost when amino acids are introduced in polymers.

One of the limits in analyzing biological samples prepared in forms that can be easily handled, such as in pressed polyethylene powder matrix or in KBr pellets (the latter at least for the 1000 cm\(^{-1}\) to 250 cm\(^{-1}\) range), is that such sample forms do not always mimic in a significant way the real biological systems.
All above measurements, together with all other data collected during the three years of the project have been inserted in a spectroscopic database and made available to the scientific community through the public web-page: [www.frascati.enea.it/THz-BRIDGE/public/spectra/searchdb.htm](http://www.frascati.enea.it/THz-BRIDGE/public/spectra/searchdb.htm). The organization of the database, its main characteristic and search functions are described in the Deliverable D-16.

Fig. 2.18. The distribution of absorption frequency in amino acids.

A variety of techniques and biological assays have been employed within THz-BRIDGE to clarify any potential hazard induced by electromagnetic radiation in the THz region. In this section we present the studies related to the interaction of THz radiation with three important biological systems: human lymphocytes, a model of cell membrane and epithelial cell cultures.

Different THz sources have been employed for irradiation studies, including a Backward-wave oscillator operating at 100 GHz, a Compact Free Electron Laser operating in the frequency range between 90 and 150 GHz, a solid state IMPATT diode operating at 150 GHz and a laser driven solid state source operating in the range between 0.3 and 3 THz. The main characteristics of these sources and the relevant exposure set-up are presented in the sections describing the individual irradiation experiments.

3.1 Evaluation of genotoxic effects on human peripheral blood leukocytes following in vitro exposure to THz radiation.

The induction of genotoxic effects is one of the most interesting aspects in the study of the interaction of THz radiation with biological systems, due to the close correlation between DNA damage and cancer occurrence [Juutilainen, 1997]. In this part of the work we investigated the induction of genotoxic effects in human peripheral blood leukocytes, following 20 min. in vitro exposures, to THz radiation as a function of average, peak power and amplitude modulation, by adopting different irradiation set up. Whole blood leukocytes have been chosen since they are well-known biological system, playing a key role in the defense mechanisms. Moreover they are easily obtainable by venipuncture and have been largely used as a biological model to study the potential genotoxic effects of electromagnetic radiation [Tice, 2002]. Following THz exposure, performed in G0 phase in order to prevent any interference related to possible cell cycle dependencies, the cytogenetic evaluation was carried out by applying the cytokinesis-block micronucleus (CBMN) technique and the single cell gel electrophoresis assay (comet assay).

The cytokinesis-block micronucleus (CBMN) assay is a very sensitive and simple indicator of chromosome damage; it evaluates long-lived DNA damage in the population of T lymphocytes, selectively stimulated post-exposure to divide by PHA. Micronuclei (MN) arise from either acentric chromosomal fragments (structural chromosomal damage) or lagging chromosomes (numerical chromosomal damage) that fail to be incorporated into daughter nuclei during cytokinesis [Heddle, 1993]. Cytochalasin-B prevents cytokinesis without interfering with cell division, which allows for the selective scoring for micronuclei in proliferating cells that have divided once post-exposure [Fenech, 2000]. Moreover, the CBMN assay allows us to obtain information on cell cycle progression by calculating the cytokinesis block proliferation index (CBPI) [Surralles, 1995]. The comet assay, can reveal the DNA damage on the whole leukocytes population soon after the exposure, since it does not require cell division to reveal the damage. The alkaline version of the
comet assay, we applied in this study, is a sensitive technique for the detection of DNA strand breaks, alkali labile sites, cross-linking and incomplete excision repair sites in individual eukaryotic cells [Singh, 1988].

3.1.1 Materials and Methods

3.1.1.1 The ENEA Compact Free Electron Laser

The Compact Free Electron Laser operating at the ENEA Research Center in Frascati, utilizes a microtron as electron beam source at energies between 2.3 and 5 MeV [Gallerano, 1998]. For the experiments reported in this work, a permanent magnet undulator with 8 periods of 2.5 cm is used to generate coherent radiation in the frequency range between 90 and 150 GHz. The Compact FEL produces a "train" of micropulses of about 50 ps duration, with 330 ps spacing between adjacent pulses. The overall duration of the train (macropulse) is 4 µs. Macropulses can be generated up to a maximum repetition frequency of 20 Hz. Means are provided for adjustment and control of the peak and average power levels, as well as for frequency tuning. The typical output power is about 1.5 kW in 4 µs pulses at frequencies in the range 120-140 GHz. The FEL spectrum consists of several emission lines, spaced at 3 GHz intervals corresponding to the period of the driving radio-frequency. The envelope of the emission lines shows a typical relative bandwidth of the FEL around 7%. The FEL radiation is transported to a dedicated user room by means of a special mm-wave transmission line composed of an evacuated copper light pipe with 25 mm clear aperture and appropriate delivery optics.

3.1.1.2 Irradiation set up and procedure

In the user room, two different irradiation set up (IS1 and IS2) have been specifically realized to match the biological requirements. The irradiation set up IS1, shown in Figure 3.1, makes use of a specially designed THz delivery systems (TDS) to expose 2 ml of whole blood in polystyrene Petri dishes with 52 mm internal diameter (Falcon P/N 35.3004). The TDS is made of aluminum and is built to provide a uniform irradiation of the whole blood samples.

The value of the absorption coefficient measured for whole blood at 120 GHz shows that less than 1% of the incident radiation penetrates through 1mm thickness. This sets a severe condition on the minimum irradiation area to be used in the genotoxicity tests, which require a minimum useful whole blood volume of 2ml. An
The upper limit to the irradiation area is set by the maximum feasible expansion of the THz beam needed to get a sufficiently uniform power density at the sample surface. A compromise between these two conditions was found for a diameter of the Petri dish of 52 mm.

The THz radiation is monitored by a pyroelectric detector that measures the incident power and allows to calculate the total energy delivered in a given irradiation time taking into account the macropulse repetition frequency. In order to correctly evaluate the radiation absorbed in the sample, the presence of a “meniscus” at the blood-air interface, due to the surface tension of the liquid, was also taken into account. The presence of such a meniscus reduces the effective thickness of the sample at the center of the Petri dish, thus changing both the absorption and the irradiation conditions. A system was developed to measure the meniscus effect, making use of a two dimensional scanning system equipped with a needle to “map” the liquid surface. Results of such a scan allowed the necessary corrections to be performed [Giovenale, 2003].

The irradiation set up IS2 (Fig. 3.2) was realized to obtain the optimal focusing of THz radiation on human leukocytes. In set-up 2-A and 2-B leukocytes separated from the aqueous part of the blood (serum) are directly exposed to THz radiation. This results in an higher power impinging on the cells respect to the leukocytes immersed in the serum. In setup 2-A a centrifugated blood sample, composed of a leukocyte layer on top of red blood cells, is prepared in an Eppendorf tube, which is placed inside a metallic cone and then exposed to THz radiation with the cap of the tube kept closed. In the set-up 2-B the radiation is focused by means of the metallic cone to the top of the Eppendorf tube, which is put inside a PVC holder. This holder allows the tube to be kept open avoiding the reflection and diffusion of THz radiation by the cap of the Eppendorf. In the setup 2-A we have to take into account that the effective power impinging on the sample is reduced by a fraction equal to the ratio between the sample area and the area of the cross-section of the cone itself. In the set-up 2-B the whole power entering the cone also reaches the sample surface.

![Fig. 3.2 – Irradiation set-up IS2](image)
For both irradiation set up IS1 and IS2, sham-exposed samples were also established and left in the user area throughout the exposure period in absence of THz irradiation. Such samples were used as control since, in preliminary experiments, we demonstrated that environmental conditions in the user room did not influence the MN induction, as shown by comparing the conventional control cultures (established with neither exposed nor sham exposed blood) and the sham exposed ones.

3.1.1.3 Exposure conditions

The exposure duration was fixed at 20 min. in all experiments and, different exposure conditions were tested, by adopting the irradiation set up IS1 and IS2 described above. The exposure conditions are summarized in Table 3.1 where the biological target investigated is also reported.

<table>
<thead>
<tr>
<th>Exposure condition</th>
<th>Frequency [GHz]</th>
<th>Pulse repetition rate [Hz]</th>
<th>Average power [mW]</th>
<th>Delivered energy [J]</th>
<th>Mean electric Field [V/cm]</th>
<th>Peak electric field (4µs) [V/cm]</th>
<th>Peak electric field (5ps) [V/cm]</th>
<th>Irradiation set up</th>
<th>Biological target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120</td>
<td>2</td>
<td>1.0</td>
<td>1.20</td>
<td>0.19</td>
<td>67</td>
<td>170</td>
<td>1</td>
<td>MN, CBPI</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>2</td>
<td>0.6</td>
<td>0.72</td>
<td>0.15</td>
<td>50</td>
<td>130</td>
<td>1</td>
<td>MN, CBPI</td>
</tr>
<tr>
<td>3</td>
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<td>5</td>
<td>3.5</td>
<td>4.20</td>
<td>0.35</td>
<td>78</td>
<td>193</td>
<td>1</td>
<td>MN, CBPI, Comet</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>7</td>
<td>5.0</td>
<td>6.20</td>
<td>0.42</td>
<td>78</td>
<td>193</td>
<td>1</td>
<td>MN, CBPI, Comet</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>7</td>
<td>1.9</td>
<td>2.28</td>
<td>1.52</td>
<td>287</td>
<td>703</td>
<td>2 A</td>
<td>Comet</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>7</td>
<td>5.0</td>
<td>6.20</td>
<td>2.45</td>
<td>465</td>
<td>1140</td>
<td>2 B</td>
<td>Comet</td>
</tr>
</tbody>
</table>

3.1.1.4 Biological procedure

Chemicals

RPMI 1640 medium, Foetal Bovine Serum (FBS), L-Glutamine and Phytoemagglutinin (PHA) were from Gibco (Milan, Italy). Cytochalasin-B and Ethidium Bromide were from Sigma (St. Louis, MO). Dimethyl Sulphoxide (DMSO), Methanol and Giemsa were from Baker (Deventer, The Netherlands).

Blood collection

10 ml whole blood samples were collected, at the ENEA Occupational Health Unit, by venipuncture in heparinized vacutainers (Becton Dickinson) from 31 healthy subjects with informed consent. Blood donors were healthy, anonymous, aged between 30 and 50 years and had not been exposed to chemicals, drugs or therapeutic irradiation in the last 6 months before blood sampling. For each exposure condition, blood from at least three donors has been employed, in order to take into account interindividual variability.
Cytokinesis block micronucleus technique

Following exposure/sham-exposure, whole blood cultures were established from each blood sample in TPP (Switzerland) plastic flasks (25 cm$^2$ growth area) by adding 0.8 ml whole blood to 9.2 ml of RPMI medium supplemented with 15 % heat-inactivated fetal calf serum, 2 mM L-Glutamine and 100 µl of Phytohemagglutinin as mitogen. In order to block cytokinesis, 44 hours after PHA stimulation cytochalasin-B (2 mg/ml in DMSO) was added in culture flasks to give a final concentration of 6 µg/ml. 72 h after PHA stimulation, cells were collected and processed for slide preparation as described elsewhere [Zeni, 2003]. After fixation (80% methanol in aqueous solution for 10 min) and staining (5% Giemsa in phosphate buffer, pH = 6.8 for 10 min) coded slides were scored blindly by the same observer, and MN were counted in binucleated cytokinesis-blocked cells with a light microscope at 1000 x magnification following the criteria summarized by Fenech [Fenech, 2000]. For each subject/treatment 1000 binucleated cells were examined and the frequency of MN was calculated as the ratio between the number of cytokinesis blocked (CB) cells containing MN and the total number of CB cells scored, expressed as percentage. On the same slides cell proliferation was, also evaluated by scoring the number of nuclei in 500 cells, and determining the Cytokinesis-block proliferation index (CBPI). It measures the mean number of cell cycles per cell and, according to Surrallles et al [Surrallles, 1995] is defined as follows: CBPI = [(M1 + 2M2 + 3(M3 + M4)) / N where M1 to M4 indicate the number of cells with 1 to 4 nuclei respectively, and N the total number of cells scored.

Alkaline comet assay

Following exposure/sham-exposure, the standard alkaline SCGE, or Comet assay, was performed according to the method developed by Singh and co-workers [Singh, 1988] with minor modifications, and for each sample two replicate slides were set up. Slides were stained, just before analysis, with 60 µl of ethidium bromide (12 µg/ml) and, images of 100 randomly selected cells (50 from each of two replicate slides) were analysed from each sample, by means of a computerized Image Analysis System (Delta Sistemi, Rome, Italy). This system acquires images, computed the integrated intensity profile for each cell, estimate the comet cell components, head and tail, and evaluate a range of derived parameters. The results have been expressed as both Tail Moment and Comet Tail Factor %. Tail moment is defined as the % of DNA in the tail x tail length [Ashby, 1995]. After classifying 100 DNA spots into five categories, corresponding to the amount of DNA in the tail, according to Anderson et al. [Anderson, 1993], the Comet Tail Factor was calculated, according to Ivancsits et al., as follows: tail factor (%) = [AF_A + BF_B + CF_C + DF_D + EF_E] / 100 where A is the number of cells classified to group A, F_A the average of group A; B is the number of cells classified to group B, F_B the average of group B; C is the number of cells classified to group C, F_C the average of group C; D is the number of cells classified to group D, F_D the average of group D; E is the number of cells classified to group E, F_E the average of group E [Ivancsits, 2002].
Data analysis
In order to compare each exposed sample with its sham-exposed control, two-tailed paired Student’s t test was applied for MN frequency, CBPI, tail factor % and tail moment. Differences are considered to be significant at P<0.05.

3.1.2 Results and Discussion
The effect of 20 min exposures at a pulse repetition rate of 2 Hz, with average power delivered on the samples of 1 and 0.6 mW for 120 and 130 GHz respectively (exposure conditions 1 and 2 listed in table 1), are depicted in Figs. 3.3 and 3.4 for MN frequency and CBPI respectively. In particular, results are reported for control, sham-exposed and exposed samples, as mean ± SD of 6 and 3 healthy subjects for 120 and 130 GHz respectively. It appears that the environmental conditions at FEL laboratory do not influence the MN induction at both frequencies, as shown by comparing control and sham-exposed cultures (P= 0.380 and 0.260 for 120 and 130 GHz respectively). By performing the same comparison, cell cycle kinetics also resulted unaffected (P= 0.724 and 0.500 for 120 and 130 GHz respectively). Concerning the induction of genotoxic effects, the results obtained indicate that the exposure conditions adopted do not affect micronucleus formation, as shown by comparing sham-exposed cultures with exposed ones (P= 0.182 and 0.430 for 120 and 130 GHz respectively). CBPI also resulted unaffected by the exposure, since P values of 0.133 and 0.50 were obtained for 120 and 130 GHz respectively.

When the attention has been focused on the frequency of 130 GHz, and the exposure condition 3, listed in Table 3.1, has been applied, the results, obtained from 5 healthy donors, are depicted in Figs. 3.5 and 3.6.
particular, in Fig. 3.5, results are reported for MN frequency and CBPI in exposed and sham-exposed cultures. No effect has been found for both the biological parameters investigated, as assessed from the comparison between exposed cultures and sham-exposed ones, where P value of 0.122 and 0.159 have been found for MN and CBPI respectively.

Figure 3.5 – MN frequency and cytokinesis block proliferation index in human peripheral blood lymphocyte cultures from sham-exposed and exposed samples at 130 GHz (exposure condition 3). Data are presented as mean ± SD of 5 donors.

In Fig. 3.6, the results of the comet assay are reported as tail factor % and tail moment. Again no differences have been detected between exposed cultures and sham-exposed ones (P values of 0.29 and 0.16 for tail factor % and tail moment respectively). Similar results have been obtained when the exposure condition 4, listed in Table 3.1, has been applied, even if, with respect to exposure condition 1, the pulse repetition rate was raised to 7 Hz, causing an increase of the average power up to 5 mW and a corresponding increase of the average electric field to 42 V/m. In fact no effect has been induced in exposed samples with respect to sham exposed ones, in terms of MN frequency (P=0.95) and CBPI (P=0.074) as shown in Fig. 3.7, and in terms of tail factor % (P=0.96) and tail moment (P=0.75) as shown in Fig. 3.8.

Figure 3.6 – Tail factor % and Tail moment in sham-exposed (SH) and 130 GHz exposed (EXP) human peripheral blood leukocytes (exposure condition 3). Data are presented as mean ± SE of 5 donors.

Figure 3.7 - MN frequency and cytokinesis block proliferation index in human peripheral blood lymphocyte cultures from sham-exposed and exposed samples at 130 GHz (exposure condition 4). Data are presented as mean ± SD of 5 donors.
When the investigation was focused on the Comet assay, and the experimental set up was modified in order to obtain a higher power impinging on the cells (irradiation set up IS2), a statistically significant increase was observed in the exposed samples with respect to sham-exposed ones, both in terms of comet tail factor % (P=0.013) and tail moment (P=0.05) for 5 subjects exposed by adopting the irradiation set up IS2-A. This result was not confirmed when the same experimental condition is tested on 2 more subjects. Such findings indicate that the positive result remains unclear and has to be attribute to not reproducible exposure condition in the case of irradiation set up IS2-A. In fact, when blood from 5 donors was exposed by adopting the irradiation set up IS2-B and the exposure condition 6, despite the higher energy delivered to the sample (6.20 J instead of 2.28 J), the results obtained, indicate the absence of effects both in terms of tail factor % and tail moment.

3.1.3 Conclusions
Overall, the findings of present investigations have demonstrated that, 20 min THz exposures of whole blood samples, at 120 and 130 GHz, in different exposure conditions, do not induce neither direct DNA damage in human leukocytes, nor long lived damage in human lymphocytes. These results suggest that THz exposure, in our experimental conditions, cannot produce genotoxic effects by directly causing DNA damage.
3.2 Evaluation of genotoxic effects on lymphocyte cultures following in vitro exposure to THz radiation

Since genotoxic effects are central in the risk assessment of human exposure to ionizing and non-ionizing electromagnetic radiation, the group at the University of Tel-Aviv employed human lymphocyte cultures as a biological model for studying potential genotoxic effects, due to the fact that lymphocytes, which play a key role in the immune system have served always as a sensitive cellular system towards external insults.

3.2.1 Materials and methods

The exposure to CW 100GHz radiation was carried out in a specially designed exposure system, which allowed to irradiate the cells inside an incubator measuring internally: 48 cm (height) x 58 cm (width) maintaining a temperature of 37°C (see Fig.3.9). The internal surface of the incubator is covered with special microwave absorbing material to avoid reflections.

Exposure Parameters

<table>
<thead>
<tr>
<th>CW 100GHz</th>
</tr>
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<tbody>
<tr>
<td>$I_{inc} = 0.05 \text{ mW/cm}^2$</td>
</tr>
</tbody>
</table>

For calculated Trans. of 83%:

| $I_a = 0.043 \text{ mW/cm}^2$ |

yielding SAR of 3.2mW/gr

ICNIRP guidelines

| 1mW/cm² for general public exposure |
| 5mW/cm² for occupational exposure |

Fig. 3.9 - Exposure system
Since the penetration depth of radiation at 100GHz into a water suspension of lymphocytes is very short (~0.13 mm), the most efficient way to illuminate uniformly the lymphocyte cells lying, due to gravity, at the bottom of the culture flasks was by illuminating them from the bottom. Plastic containers with a base cross-section 4.5 x 2 cm² were used. Considering the size of the liquid container the cross-section of the radiation beam was expanded in order to obtain a uniform illumination of the sample in the horizontal dimensions. A Gaussian beam with spot diameter of about 12 cm provided a reasonable uniformity over the sample surface. The power density of the 100 GHz radiation at the bottom of the flask was 0.05mW/cm² which corresponds to specific absorption rate of 3.2mW/gr (where the international guidelines limit exposure to a value of 1mW/cm² for the general population and to 5mW/cm² for occupational exposure). Lymphocytes isolated from peripheral blood of three individuals were irradiated for 1, 2 and 24 hours and were harvested by common cytogenetic procedures 69 to 72 hours after the onset of exposure.

The genetic and epigenetic markers for genomic instability were the changes in the levels of aneuploidy and replication synchrony, respectively. These parameters were evaluated by interphase FISH based cytogenetics. We scanned slides of nuclei, derived from the exposed and appropriate control cultures, hybridized with probes specific for the centromeric regions of chromosomes 11 (orange labeled; Vysis, USA) and 17 (green labeled; Vysis, USA) using the Metafer platform for semi-automatic interphase FISH scoring. Cells were scored automatically (see Fig. 3.10); the gallery was then manually corrected by two independent technicians.

Between 700 and 2000 cells were scored for aneuploidy and 600-1000 nuclei were scored for replication assays for each culture. The Metafer platform automatically presents the results obtained for the levels of chromosomal gains and losses for each locus plus a correlation between the two loci. The subset of cells which had two hybridization signals for both signals, were manually analyzed for the pattern of replication.

3.2.2 Results
Analysis of the gains of chromosome 11 and 17 shows increased level of gains for 2 hour exposure for both chromosomes when each exposed sample of each time point was compared to its own sham (Fig 311a).
However, when all the shams were grouped into an average one, we could observe increase at 24 hour with a non-significant tendency of an increase for 2 hours (Fig. 3.11b).

**Fig. 3.11a** - The level of gains of chromosomes 17 and 11 following exposure to CW 100 GHz radiation. The row under the figure gives the p value obtained after performing a two tailed Student t-test between exposed and sham for each exposure time. Blue - control and sham samples; Brown – exposed samples.

When total aneuploidy was measured (losses plus gains) with all the shams, we observe a statistically significant increase in aneuploidy 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 unsignificant tendency for an increase in chromosomal losses and gains of chromosome 11 and 17, respectively (Fig. 3.12).
Fig. 3.12: Total aneuploidy (loss and gains) of chromosomes 11 and 17 following exposure to CW 100GHz radiation. The row under the figure gives the p value obtained after performing a two tailed Student t-test between exposed and the average of all shams. White – control; pink – averaged sham; blue – exposed samples.

Of special interest was the comparison of the levels of total aneuploidy observed in interphase with those of metaphase spreads. This is shown in Fig. 3.13.

It can be seen that aneuploidy levels of chromosomes 17 and 11 in metaphase is lower than in interphase. The correlation between aneuploidy levels in interphase vs. metaphase yield R2 values of 0.095 and 0.2 for chromosome 17 and 11, respectively. Thus, no correlation exists between the two in the same cell population. These results may reflect a selection against aneuploid cells from entering metaphase.

When analyzing the frequency of asynchronous replication in the exposed cultures we could detect elevation of asynchronous replication of CEN11 and CEN17 following 24 hours and 2 ours of exposure, respectively (Fig. 3.14). An elevation of asynchronous replication (where p=0.06) was obtained following 2 and 24 hours of exposure for CEN 11 and CEN17, respectively.
Fig. 3.14 - The level of asynchronous replication of centromers 11 and 17 following exposure to CW 100GHz radiation. The row under the figure gives the p value obtained after performing a two tailed Student t-test between exposed and sham for each exposure time. Blue-control and sham samples; Brown – exposed samples.

3.2.3 Conclusions
Both genotoxic and epigenetic effects are induced in lymphocytes following exposure to CW 100 GHz radiation of 0.05 mW/cm$^2$ intensity when the exposure period exceeds one hour. The induced effects seem to saturate already for short exposures. 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/cm$^2$ for general public exposure and 5mW/cm$^2$ for occupational exposure). More experiments are needed to establish accurate dose-response relationships.
3.3 Effects on membrane model systems

Several studies on different types of cells have indicated that radiofrequency and microwave fields exposures may alter membrane structural and functional properties capable of triggering cellular responses [see review by Repacholi, 2001]. The complexity of the issue has induced several research groups to use simple membrane models to improve the understanding of how electromagnetic fields can induce such effects. Liposomes, depicted in Fig. 3.15, have been widely used as a model for cell membranes to mimic their functions and to reconstitute the functional fragments derived from biomembranes [Gregoriadis, 1993; Lasic, 1993]. Liposomes are microscopic vesicles in which an aqueous volume is entirely enclosed by a membrane composed of phospholipids. They form spontaneously when these amphipathic lipids are dispersed in aqueous media. They can be constructed so that they entrap quantities of materials both within their aqueous compartment and within the membrane. By constructing them with natural phospholipids, the liposome membrane forms a bilayer structure as experienced by the lipid portion of cell membranes. In this context, alterations in the lipid membrane permeability induced by 2.45 GHz and millimeter microwave irradiation have been reported [Ramundo-Orlando, 1994; Alekseev, 1995; Ramundo-Orlando, 2004].

![Fig. 3.15 - Schematic representation of a liposome vesicle](image)

The model used in this study consists of liposomes enclosing in their interior a soluble enzyme, such as the carbonic anhydrase (CA). We 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 CA-loaded liposomes. In order to entrap CA inside the liposomes, cationic small unilamellar vesicles containing stearylamine (SA), charged positively in the lipid bilayer, have been developed [Annesini, 1994]. Since CA is one of the most abundant proteins in the erythrocyte after hemoglobin, liposome loaded with CA can be considered as a very simple model of the erythrocyte. We investigated whether terahertz radiation could affect the permeability of these lipid bilayers under well-controlled irradiation conditions. Different pulse repetition rates were studied.

3.3.1 Materials and methods

3.3.1.1 Chemicals

L-$\alpha$-dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), stearylamine (SA), sodium cholate, n-octyl-$\beta$-glucopyranoside, $p$-nitrophenyl acetate ($p$-NPA), and Carbonic Anhydrase (E.C. 4.2.1.1 from bovine erythrocyte, Cat.No. C3934) were purchased from Sigma (St. Louis, MO, USA). Chloroform, sodium chloride, acetonitrile, and Tris(hydroxymethyl)amino methane were purchased from Farmitalia Carlo Erba.
3.3.1.2 Liposome Preparation

Liposome vesicles were prepared by controlled detergent-dialysis (DIAL liposomes) [Weder, 1984] using a Liposomat apparatus (Dianorm Gerate, München, Germany). A chloroform solution (5 ml) containing 50 µmol DPPC, 30 µmol Chol, and 20 µmol SA (5:3:2 molar ratio), and 200 µmol sodium cholate as detergent (lipid/detergent ratio 1:2), was evaporated to dryness in a rotary evaporator under vacuum at 30°C. The suspension was made by adding 4 ml 0.09 M Tris (pH 7.55)-0.081 M NaCl alone or containing 10 mg Carbonic Anhydrase (CA) to prepare empty or loaded liposomes, respectively. Then the liposomes were formed by controlled dialysis of detergent for 18 h at 23°C. The liposomes were washed six times in Tris-saline, followed by centrifugation at 12,000g to remove nonentrapped enzyme molecules. The resulting unilamellar liposomes are stable for at least 3-4 days at storage temperature of 4°C; however, all experiments were carried out as soon as possible, usually 12 h, after the end of the preparation. Liposome DPPC content was determined according to the method of Stewart [1980], cholesterol content was determined with the Farb test (Boehringer Mannheim Kit), and SA content was analyzed by gas chromatography-mass spectrometry, as described elsewhere [Passi, 1991]. Protein content was determined with the Bio-Rad assay using bovine serum albumin as a standard. The CA-loaded liposomes were characterized for size distribution as previously described [Ramundo-Orlando, 1994].

3.3.1.3 Enzyme Activity Measurements

Esterase activity measurements of carbonic anhydrase [Pocker, 1967] were performed in 0.09 M Tris-saline at pH 7.55 using p-NPA as substrate and following, for two minutes, the appearance of reaction product p-nitrophenolate anion at its peak absorbance (λ=400 nm ) on a Cary 50 spectrophotometer.

A typical procedure for a kinetic run was to initiate the reaction by adding 0.1 ml of acetonitrile stock solution of pNPA (2mM final concentration) into 2.9 ml of Tris-saline (pH 7.55) containing 0.06 ml of empty or CA-loaded vesicles suspensions. All spectrophotometric measurements were carried out at a room temperature; however, between kinetic measurements liposome suspensions were placed in an ice bath. The p-NPA hydrolysis rate, expressed as change in absorbance unit at 400 nm per minute (ΔA/min), was computed automatically as the slope of a linear fit to the experimental recorded curves. The increment of absorbance at 400 nm of empty-liposomes was always twofold lower than of CA-loaded liposomes. The total CA activity in liposomes was determined after rupture of liposomes by detergent (Fig. 3.16). Briefly, empty or CA-loaded liposome suspensions (0.5 ml) were incubated for 2 h at 37°C with n-octyl-β-glucopiranoside (0.5 ml) at 1:6 µmol lipid/detergent ratio. After incubation, suspensions were centrifuged at 12,000g to sediment the lipids. The resultant clear supernatants (0.120 ml) were used for kinetic measurements, and determination of protein contents.
3.3.1.4 THz exposure set-up

Prior to the irradiation, transmission measurements have been performed at ENEA either on liposomes or aqueous components to determine their best irradiation geometry. The following absorption coefficient values have been obtained at a frequency of 130 GHz:

- Empty liposomes: \((50 \pm 3) \text{ cm}^{-1}\)
- CA-loaded liposomes: \((55 \pm 3) \text{ cm}^{-1}\)
- Tris-saline buffer: \((43 \pm 8) \text{ cm}^{-1}\)
- Tris-saline plus CA: \((48 \pm 8) \text{ cm}^{-1}\)

An irradiation set-up similar to the one developed for the irradiation of whole blood has been developed at ENEA. A visible spectrophotometer to be employed for the measurements of the CA enzyme has been modified to allow irradiation of the liposomes during kinetics measurements. The experiments were carried out by using two different irradiation set-up referred to as A (already shown in Fig. 3.1) and B (Fig. 3.17):

A) The FEL radiation at the central frequency of 130 GHz is transported through a copper light-pipe of 25 mm diameter and is then fed into a “THz delivery system” (TDS) specifically designed to match the above requirements. The TDS is made of aluminum and is built to provide a uniform irradiation of the liposome samples. The THz beam coming from the light pipe into the TDS is first focused down to a 17.5 mm diameter aperture by means of a conical section and is then let expand by diffraction to about 52 mm diameter to match the required irradiation area. Calculations have been performed in order to minimize internal reflections in the cone, making the beam expansion similar to the free space expansion.

B) In order to irradiate the liposomes and carry out the kinetic measurements in real time, the cell compartment of the spectrophotometer has been modified to deliver the THz beam onto the cuvette containing the liposomes in a final volume of 1.5 ml. In the present experiments we used an irradiation from the side of the cuvette (Fig. 3.17). An electroformed copper horn is used to match the circular cross section.
of the THz beam to the rectangular cross section of the cuvette.

![Image of radiation and sample](image)

*Fig. 3.17 - Setup B: modification of the cell sample into spectrophotometer to allow irradiation from the side of the cuvette.*

To further evaluate the dependence of kinetic measurements on the modulation conditions and carrier frequency two additional exposure systems were set-up at 3 GHz pulsed wave (PW) and 150 GHz continuous wave (CW) referred to as C and D respectively.

**C)** A 3 GHz microwave klystron was used as a source; the radiation is transported into a waveguide by using coaxial cable. Here the radiation is delivered into the sample within an UV spectrophotometer. The klystron produces a train of macropulses of 80 µs duration, with a maximum peak power of 22 W and average power of 5 mW.

**D)** Exposure at 150 GHz CW was also performed by using an IMPATT diode as a source. The diode produced a 150 GHz continuous with an average power of about 3 mW.

### 3.3.1.5 Experimental procedure

We recall the experimental procedures used for the irradiation set-up A and B, respectively:

**A)** Aliquots (0.06) ml of CA-loaded or empty liposomes in a final volume of 0.5 ml Tris-saline (pH 7.55) were placed in a silica cuvette (3 cm$^2$) positioned, at five minutes intervals, in a Teflon grid in the region of maximum uniformity of the field in the TDS of exposure apparatus. Typically five replicate Ca-loaded and empty liposome samples were exposed to THz fields for 60 min at each of the experimental conditions indicated otherwise. Exposure of control samples was performed by maintaining the liposome in the same room, but distant from the apparatus. At the end of exposures, one cuvette was withdrawn, at five-minute intervals, and 2.4 ml Tris-saline (pH 7.55) and 0.1 ml of acetonitrile solution of $p$-NPA (2 mM final concentration) were added and the kinetic measurement were made within 30s after the exposure was stopped. Finally, aliquots of preparations of CA-loaded or empty liposomes, maintained at their optimal
storage temperature of 4°C for 60 min, were removed and assayed for the enzyme activity in order to evaluate the stability of liposome preparations.

B) Aliquots (0.03 ml) of CA-loaded or empty liposomes in a final volume of 1.42 ml final Tris-saline (pH 7.55) were placed in a silica cuvette (3cm$^2$) positioned in the modified cell-beam of spectrophotometer (Fig. 4). The THz radiation is turned on and, after 30s, 0.05 ml of acetonitrile solution of p-NPA (2 mM final concentration) were added and the kinetic measurement were made over 2 minutes of the THZ irradiation. Exposure of sham samples were performed in the spectrophotometer with the THz apparatus turned off.

Irradiation of samples in the set-up C and D were performed under the same experimental conditions as were described above for the set-up B.

3.3.1.6 Statistical Analysis

Data from exposed and sham samples were compared at each frequency. These data were subject to an analysis of variance (ANOVA). If the F value was significant, the unpaired two-tailed Student's t-test was used to compare the exposed and sham samples, the level of significance was accepted at a confidence interval of 95% [GraphPad software].

3.3.2 Results

The enzyme-loaded liposomes, prepared via detergent dialysis method, were nearly 100% unilamellar and homogenous. The mean vesicle diameter of 5:3:2 CA-loaded liposomes was 29 ± 11 nm, respectively. In the first series of experiments we studied the effect of THz radiation at different pulse repetition rates. Table 1 shows the substrate influx rate (expressed as ΔA/min) as a function of the pulse repetition rate and of the average power delivered to the liposome samples, by using either the set-up A or B. After 60 minutes of 130 GHz irradiation at 7 Hz pulse repetition rate in the set-up A, we obtained a slight increase of esterase activity from 0.0024 ± 0.0018 to 0.0034 ± 0.0019. On contrast, by using the set-up B we obtained a significant increase of esterase activity from 0.0027 ± 0.0014 to 0.0058 ± 0.0014 (P= 8.6$^{10} n = 27$) at 7 Hz pulse repetition rate, while there was no significant increase at other frequencies tested. This increment was calculated over three different liposome preparations. By increasing the average intensity of the radiation incident on the sample from 7.8 mW/cm$^2$ to ca 11 mW/cm$^2$, no increment of enzyme activity resulted at 10 Hz pulse repetition rate (Table 3.2).
Table 3.2 - Effects of irradiation at 130 GHz on CA-loaded liposomes at different pulse repetition rates and power delivered to the samples.

<table>
<thead>
<tr>
<th>Setup</th>
<th>T°C</th>
<th>Time of Exposure</th>
<th>Intensity (mW/cm²)</th>
<th>Pulse repetition rate</th>
<th>ΔA/min Sham</th>
<th>ΔA/min Exposed</th>
<th>%CA Enzyme activity sham</th>
<th>%CA Enzyme activity exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20-22</td>
<td>60'</td>
<td>0.16</td>
<td>4µsec/5 Hz</td>
<td>0.0064 ± 0.0015 (n=18)</td>
<td>0.0059 ± 0.0022 (n=20)</td>
<td>34 ± 8</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>A</td>
<td>20-22</td>
<td>60'</td>
<td>0.23</td>
<td>4µsec/7 Hz</td>
<td>0.0024 ± 0.0018 (n=8)</td>
<td>0.0036 ± 0.0019 (n=10) (p=0.100)</td>
<td>16 ± 12</td>
<td>24 ± 13</td>
</tr>
<tr>
<td>B</td>
<td>26-29</td>
<td>2'</td>
<td>5.6</td>
<td>4µsec/5 Hz</td>
<td>0.0025 ± 0.0011 (n=18)</td>
<td>0.0027 ± 0.0018 (n=25)</td>
<td>21 ± 10</td>
<td>23 ± 14</td>
</tr>
<tr>
<td>B</td>
<td>22</td>
<td>2'</td>
<td>7.8</td>
<td>4µsec/7 Hz</td>
<td>0.0027 ± 0.0014 (n=22)</td>
<td>0.0058 ± 0.0014 (n=27)(p= 8.6E-10)</td>
<td>14 ± 8</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>B</td>
<td>29</td>
<td>2'</td>
<td>11.1</td>
<td>4µsec/10 Hz</td>
<td>0.0034 ± 0.0010 (n=7)</td>
<td>0.0046 ± 0.0014 (n=9) (p=0.041)</td>
<td>28 ± 8</td>
<td>37 ± 12</td>
</tr>
</tbody>
</table>

The liposomes showed a basal enzymatic activity of 17 % ± 7 %. Such activity is typical of these cationic liposome preparations due to CA partially located on the external surface of the intact liposomes. The percentage values were calculated by taking as 100 % the total enzymatic activity of CA measured in CA-loaded liposomes after their disruption by detergent as described in Materials and Methods. This latter activity due to CA present on the external surface and inside the liposome was normalized across different liposome preparations (Fig. 3.17).

At 7 Hz pulse repetition rate a significant percentage increase of CA activity from 14 ± 8% to 31 ± 9% (P = 1.72E-09 n = 27) resulted by using the set-up B. Moreover, no difference in the enzyme activity between CA-loaded liposomes maintained at 4°C and sham liposomes was observed, demonstrating a good stability of the liposome preparations (data not shown).
We next studied the effect of 3 GHz irradiation maintaining the same pulse repetition rate of 7 Hz as it was used to irradiate at 130 GHz. The results (Table 3.3) showed no increment of the enzyme activity in liposomes when the carrier frequency of 130 GHz was substituted with 3 GHz.

**Table 3.3. Effect of 3 GHz irradiation on CA-loaded liposomes at 7Hz pulse repetition rate**

<table>
<thead>
<tr>
<th>Setup</th>
<th>Temp. °C</th>
<th>Exposure time (min)</th>
<th>Intensity inc/abs (mW/cm²)</th>
<th>CA activity (ΔA/min) Sham</th>
<th>CA activity (ΔA/min) Exposed</th>
<th>% CA Sham</th>
<th>% CA Exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>19</td>
<td>2</td>
<td>3.7/2.4</td>
<td>0.0024 ± 0.0004 (n=5)</td>
<td>0.0029 ± 0.0008 (n=8) (p=0.254)</td>
<td>35 ± 6</td>
<td>42 ± 12</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>2</td>
<td>19.2/12.5</td>
<td>0.0037 ± 0.0011 (n=5)</td>
<td>0.0032 ± 0.0011 (n=6) (p=0.443)</td>
<td>53 ± 16</td>
<td>46 ± 17</td>
</tr>
</tbody>
</table>

Finally, we checked for effects induced by exposure to 150 GHz CW radiation, by using the set-up D, without any pulse repetition rate. The results (Table 3) showed, also in this case, no increment of enzyme activity in liposomes, indicating that only 130 GHz radiation combined with a pulse repetition rate of 7 Hz elicited the effect on lipid bilayers.
Table 3.4 - Effect of CW 150 GHz irradiation on CA-loaded liposomes

<table>
<thead>
<tr>
<th>Setup</th>
<th>Temp. °C</th>
<th>Exposure time (min)</th>
<th>Power (mW)</th>
<th>CA activity (ΔA/min)</th>
<th>CA activity (ΔA/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Sham</strong></td>
<td><strong>Exposed</strong></td>
</tr>
<tr>
<td>D</td>
<td>19</td>
<td>2</td>
<td>3</td>
<td>0.0038 ± 0.0013</td>
<td>0.0040 ± 0.0006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
</tr>
<tr>
<td>D</td>
<td>19</td>
<td>2</td>
<td>3</td>
<td>0.0033 ± 0.0010</td>
<td>0.0032 ± 0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0035 ± 0.0011</td>
<td>0.0036 ± 0.0007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(n=12)</td>
<td>(n=12)</td>
</tr>
</tbody>
</table>

3.3.3 Discussion
We gave considerable attention to the chemical composition of CA-loaded liposomes and its influence on membrane permeability. The lipid bilayer employed here contains dipalmitoylphosphatidylcholine, positively charged lipid (i.e., stearylamine), and cholesterol. In biological systems, the presence of both Chol and SA regulates membrane fluidity and the main phase-transition temperature [Demel, 1976; López-Garcia, 1993]. However, we used a cholesterol concentration of 30 mol % in the liposome bilayer, which has a marginal effect on the position of the main phase-transition temperature (Tt) of DPPC-liposomes [New, 1990]. The presence of stearylamine in the bilayer induces a slight increase in the Tt of mixed-lipid samples (e.g., DPPC/SA) as reported in the literature [López-Garcia, 1993]. Moreover, DPPC-liposomes undergo a phase transition, in which the lipid bilayer passes from a tightly ordered "gel" phase to a liquid-crystal phase, at approximately 42°C [Papahadjopoulos, 1973]. Thus, under our experimental conditions ranging from 23°C to 29°C, the CA-loaded liposomes retain typical permeation rates of solute across lipid bilayer (i.e., DPPC vesicles) [Cevc, 1993]. In addition, positively charged lipids (e.g., SA), have been recognized as biological mediators affecting many cellular functions [Jonas, 1989], and a cholesterol/lipids ratio of 30 mol %, as in the CA-loaded liposome, is usually found in plasma and other subcellular membrane [Demel, 1976]. Therefore, the composition of the liposome preparation used throughout this work makes possible a direct comparison to lipid bilayers in natural cell membranes, especially their permeability properties in relation to Terahertz fields effects.

Our results indicate that terahertz fields can affect lipid bilayer permeability; we observed an increase in substrate permeation rate across the liposome bilayer (DPPC:Chol:SA = 5:3:2) over two minutes of exposure to 130 GHz radiation with a pulse repetition rate of 7Hz and an incident intensity of about 7.8 mW/cm². Interestingly, the observed differences in p-PNA hydrolysis rate were significantly higher in setup B than in setup A, indicating that real time exposures might reveal possible reversible effects induced by the terahertz field.

One hypothesis which we have set out to test is that the power delivered to the system could be responsible for the observed effect. In spite of an intrinsic variability of the membrane system due to the use of different
liposome preparations throughout all the experiments, the lack of effect at the highest delivered power (i.e. incident intensity 11 mW/cm$^2$ or 19.2 mW/cm$^2$) excludes the possibility that the mechanism responsible for the increase in influx rate of $p$-PNA merely involves absorption of THz energy.

In an attempt to verify the specific role of the frequency of pulse repetition rates in eliciting the THz-induced effect, we conducted experiments changing the carrier frequency from 130 GHz to 3 GHz. Due to the pulsed nature of the 130 GHz source, we could not evaluate the effect of CW 130 GHz radiation on liposome bilayer. CW irradiation was however performed at a carrier frequency of 150 GHz using a 3 mW IMPATT source. The main reason for studying the behaviour of CA-loaded liposomes in these latter conditions came from the need to validate our previous observations indicating possible resonance effects on these cationic CA-loaded liposomes in the extremely low frequency region at 7 Hz [Ramundo-Orlando, 2000a,b]. The lack of effects on liposomes when the main frequency was 3 GHz or 150 GHz does not exclude a possible resonance at 7 Hz frequency pulse repetition rate in relation to the permeability changes observed.

Taken together, these results clearly indicate that the membrane model used in these studies is a system sensitive to irradiation at 130 GHz with the pulse repetition rate of 7 Hz. Further studies are in progress to clarify the role both of positive charges on the liposome surface and of the CA sticking on the liposome surface in eliciting the observed effects [Ramundo-Orlando, 2000b]. If a primary role of charged lipid (i.e., SA) in the terahertz field interaction with lipid bilayer is confirmed a possible theoretical model can be developed to clarify the biophysical mechanism of this interaction.

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 7 Hz and a delivered power of about 7.8 mW/cm$^2$. 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.
3.4 Effects on epithelial models

In this report we summarize the various irradiation experiments carried out on epithelial cell cultures, both innervated and non-innervated at the University of Nottingham, in conjunction with THz facilities at University of Leeds and Teraview Ltd, and make a final assessment on the effects of THz radiation and their relevance in THz imaging applications.

3.4.1 Materials and methods

3.4.1.1 The Teraview source

(Teraview; Cambridge). In this system THz radiation is generated by optical excitation of a gallium arsenide wide aperture antenna [Arnone 1999]. A large DC-bias is applied across the device which is excited using a Ti:Sapphire laser (RegA 9000, Coherent Inc, CA) emitting 250 fs pulses centered 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 [Cole, 2001]. During the experiments the THz-radiation was 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.

3.4.1.1 The THz system at the University of Leeds

(Institute of Microwaves & Photonics), School of Electronics & Electrical Engineering, University of Leeds, uses a Ti:Sapphire laser impacting on an electro-optic photoconverter to generate THz power. The total pulse duration is 20-30ps, although approximately 90% of the THz power is delivered within the first two picoseconds. The average output power for this (unamplified) system is approximately µW within the frequency range 0.2 – 3.0 THz. The repetition rate of the THZ pulse is approximately 80 MHz. An overview of this type of system is provided by Zhao et al., [Zhao, 2002].

3.4.1.2 The 150 GHz system from ENEA - Frascati Italy.

This source 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).

For this set of experiments the cells were exposed through the base of a 96 well plate, in clusters of 6 wells with 3 medium controls, either at room temperature or 37°C in the CO2 incubator.

THz Impatt Diode available at ENEA
3.4.1.3 Culture, Exposure and Assay Methods

The protocols and SOP’s have been defined in [Page, 1993], [O’Brien, 2000] and [Gray, 1999]. In summary, the keratinocytes are cultured in calcium free Keratinocyte Growth Medium (KGM; Cambrex UK Ltd) with calcium <0.06mM added. The Normal Human Keratinocytes (NHK) were purchased from Cambrex UK Ltd, or isolated in house by the method of Barker and Clothier [Barker, 1997]. The ND7/23 (ATCC (LGC, Teddington, UK.), were cultured in DMEM (Sigma Ltd) with fetal calf serum (Autogen Bioclear Ltd.). For THz exposure and transportation to the THz sources, the medium was replaced with Hanks Balanced Salt Solution (HBSS with calcium and Magnesium; Sigma Ltd.). Since the THz sources are kept cool, a warming mat was used to keep the temperature at 28°C during exposure. For the 3D and innervated models, the inserts were removed from the medium and placed into a sterile environment in a 24 well plate for exposure without medium. For the long term, 2-24 hour exposure to the 150GHz radiation cells were exposed in either HBSS or appropriate medium at 37°C inside the CO₂ incubator at 5% CO₂ and 95% air. The endpoint assays employed were the resazurin assay for cell activity, the fluorescein cadaverine uptake assay for differentiation and on 3D models the fluorescein leakage and resazurin combined assay for barrier integrity. The basic method for conducting and evaluating the effects of THz exposure, the appropriate culture conditions for exposure and transportation, the assays for initial damage, culture differentiation and growth have been worked out and produced reliable results.

To expose the cells to THz radiation, clusters of 4 wells in a 96 well plate are seeded with cells as per layout below.

- The cells were seeded at a density of 1x 10⁵ per ml, 150 µl/well.
- The cells are allowed to attach overnight at 37°C, in a 5% CO₂/ Air atmosphere.
- The cells were then examined to assess the degree of confluence.
- For confluent cultures the confluence needs to be 90% or more when the cells are dividing at a reduced rate. For growing cultures the cells should be no more than 50% confluent at exposure.

Resazurin solution is replaced with warm Hanks Buffered Saline Solution (HBSS; Sigma H8624). The space between the wells is filled with warm HBSS. The 96 well plate is transferred to the warmed insulated box, for transportation (28°C) if the THz source is off site.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure 1</td>
<td>Exposure 2</td>
<td>Exposure 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-exposed control 1.</td>
<td>Non-exposed control 2.</td>
<td></td>
</tr>
</tbody>
</table>

Irradiation scheme of the 96 well plate
Following the Mid term review, there was a re-appraisal of the original experimental approach. One of the reviewers commented on the possible incorporation of evaluation of the effects of THz on neural cells and the antigen presenting Langerhan’s cells in the skin. They also suggested that evaluation of the effects on the Stratum Corneum was not particularly relevant and that the effects of THz on corneal cells could also be questioned. In a project funded from another source, exciting results with innervation of a corneal model have been generated. Hence, in the light of this research, the reviewers comments and the importance of innervation in corneal and keratinocyte epithelial barrier functioning, we modified the agreed outline. Another validation study using 3T3 fibroblasts and human keratinocytes has indicated problems with crystal formation so this was not used, and the non-cytotoxic resazurin assay was employed.

3.4.2 Results

A series of human keratinocyte donor cells at passage 2 grown to confluence have been exposed to two different THz Sources. Both were in the range 1 to 3 THz but had different power outputs allowing the confluent, slowly growing basal keratinocytes to be exposed to 0.15, 0.30 and 0.45 J/cm$^2$ or 0.15, 0.30, 0.45 mJ/cm$^2$.

The effects on cell activity (resazurin reduction 1,2,3) and differentiation of three donors was normal (Fig.1a, 1b and 1c) for the experiments conducted at Teraview and at the University of Leeds respectively. The keratinocytes would differentiate in the normal way, responding to the differentiation trigger of raise calcium ion concentrations (Fig 1c). The effects of THz on dividing human keratinocyte basal and stem cells, revealed that little immediate damage occurs, following exposure to 1mW over 30 minutes, i.e. 0.45 J/cm$^2$, in 4 wells (total area 1.7 x 1.7cm). These sensitive exponentially growing keratinocytes and the neural cells showed marked adverse reactions to the transportation and reduced temperature during exposure. Despite this “stress” there was no added adverse effects due to the THz radiation received (see table 1 and 2).
Therefore the THz source, from Teraview Ltd., was employed in the FRAME Alternatives Laboratory to examine the effects on dividing keratinocytes and on neural cells and our *in vitro* 3D innervated keratinocyte co-cultures. This provided a direct comparison with the data already obtained for the confluent keratinocyte cultures. The THz radiation is directed not to the neural cell bodies of the co-cultures but to the human keratinocytes, since this is the case *in vivo*. Subsequent to the THz exposure keratinocyte growth and differentiation are examined in the presence of these nerve cells. Details of the co-culture techniques are limited since this is funded from another grant and not yet fully published, i.e. it is partly confidential to that project.

![Figure 1a. Effects of 1-3THz radiation upon human primary keratinocyte activity (Cambs)](image1a)

![Figure 1b. Effects of 1-3THz radiation upon human primary keratinocyte activity (Leeds)](image1b)
Table 1. The exposure conditions of the epithelial and sensory neuronal cells and co-culture models.

<table>
<thead>
<tr>
<th>CELLS</th>
<th>CULTURE CONDITIONS</th>
<th>EXPOSURE</th>
<th>TRANSPORTATION CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human primary keratinocytes</td>
<td>Sub confluent rapidly dividing undifferentiated</td>
<td>THz (0.1-3) 0.15-0.45 mJ/cm²</td>
<td>22-28°C 3 Hrs None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15-0.45 J/cm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GHz (130) 1.24 J/cm²</td>
<td></td>
</tr>
<tr>
<td>Human Primary Keratinocytes</td>
<td>Confluent slowly dividing differentiated</td>
<td>THz (0.1-3) 0.15-0.45 mJ/cm²</td>
<td>22-28°C 3 hrs None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15-0.45 J/cm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GHz (130) 1.24 J/cm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Confluent slowly dividing stressed with 2.5µg/ml Sodium lauryl sulphate</td>
<td>GHz (130) 4.17 J/cm²</td>
<td>None</td>
</tr>
<tr>
<td>Human Primary Keratinocytes</td>
<td>Air Liquid Interface</td>
<td>THz (0.1-3) 0.15-0.45 J/cm²</td>
<td>None</td>
</tr>
<tr>
<td>Human Primary Keratinocytes plus ND7/23 sensory</td>
<td>Air liquid Interface differentiating</td>
<td>THz (0.1-3) 0.15-0.45 J/cm²</td>
<td>None</td>
</tr>
<tr>
<td>ND7/23 sensory neurones</td>
<td>Undifferentiated with division</td>
<td>THz (0.1-3) 0.15-0.45 J/cm²</td>
<td>22-28°C 3 hrs None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GHz (130) 1.24 J/cm²</td>
<td></td>
</tr>
<tr>
<td>ND7/23 sensory neurones</td>
<td>Differentiated Very limited division</td>
<td>THz (0.1-3) 0.15-0.45 J/cm²</td>
<td>22-28°C 3 hrs None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GHz (130) 1.24 or 9.78 J/cm²</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Total energy delivered to the cultures in the different culture methods.

<table>
<thead>
<tr>
<th>Plate type</th>
<th>24 well</th>
<th>24 well inserts</th>
<th>96 well plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.6 J</td>
<td>26</td>
<td>11.5</td>
<td>4.09</td>
</tr>
<tr>
<td>9.78 J</td>
<td>17.4</td>
<td>7.73</td>
<td>2.74</td>
</tr>
<tr>
<td>1.24 J</td>
<td>2.2</td>
<td>0.98</td>
<td>0.35</td>
</tr>
<tr>
<td>0.45 J</td>
<td>0.8</td>
<td>0.36</td>
<td>0.13</td>
</tr>
<tr>
<td>0.15 J</td>
<td>0.27</td>
<td>0.12</td>
<td>0.042</td>
</tr>
<tr>
<td>0.45 mJ</td>
<td>0.0008</td>
<td>0.00036</td>
<td>0.00013</td>
</tr>
<tr>
<td>0.15 mJ</td>
<td>0.00027</td>
<td>0.00012</td>
<td>0.000042</td>
</tr>
</tbody>
</table>
3.4.2.1 Culture Activity as measured via the Resazurin Assay

It was found that the activity of the ND7/23 and dividing keratinocytes exposed did not significantly change from the pre-exposed activity level as was true for both the unexposed cultures, figure 2a & 2b).

Using a standard resorufin in resazurin curve it was possible to convert the activity into ng/ml resorufin produced. It was also possible to demonstrate that the co-cultures were of higher overall activity (not significantly) than the keratinocytes alone, but this was (Figure 2c). Hence the majority of the cultures activity was due to the predominant cell type, the keratinocytes. As has been observed in other experiments with ND7/23 cells the stimulation towards differentiation results in a decline in their cell activity. Hence by day 6 it was noted that the co-cultures gave the same conversion capacity as the NHK cells alone (Figure 2c).
Differentiation capacity measured via FC incorporation

Since the keratinocytes were grown at the air liquid interface [Gray, 1999, Khammo, 2002], the medium following exposure was replaced with modified Greens medium containing 20µM fluorescein cadaverine (3) and no serum. This was placed into the well only. Following 3 days, 6 and 8 days in culture, post THz exposure, the cells were washed in HBSS, to remove un-incorporated fluorescein cadaverine and then the FC uptake was measured in the spectrofluorimeter with an excitation of 485nm and emission of 530nm (Fig.3). The cultures were replaced in the same medium at day 3 and 6. At day 3 and 6 there were indications of a marginal increase in FC incorporation by the NHK cells alone, and the co-cultures (Figure 4). By day 9 there was a marked decline in the total FC uptake in the NHK alone cultures, not seen in the co-cultures, indicating that during the resazurin assay and washings some of the differentiated NHK cells were removed, since they were not well adhered to the apical surface of the culture. Exposure to the THz, either in the presence or absence of the neural cells, did not alter the capacity of the NHK cells to differentiate. The presence of the neural cells resulted in a retention of the differentiated NHK cells, or the ND7/23 cells increased their FC incorporation between day 6 and day 9 to compensate for the loss of the differentiated NHK cells. As with the resazurin assay, the FC uptake can be quantified using a FC standard curve. The corresponding experiments were conducted on dividing keratinocytes and neural cells alone and the total differentiation status measured at days 8 and 9 (Figure 5).
3.4.2.2 Effect of THz exposure on barrier function

Whilst it is possible for the NHK cells to retain apparent normal cell activity and differentiation capacity it is also possible that changes to the barrier function via changes in the cell – cell adhesion molecules could have been induced.

The loss or degradation of the barrier function could lead to penetration of exogenous toxic molecules that may result in basal keratinocyte damage and or dermal inflammatory reactions. Hence the barrier function was monitored following THz exposure. It is proposed that the neural cells can play an important role in the repair of barrier function following injury, particularly with regard to the corneal epithelial barrier.

The Fluorescein leakage (FL) assay was performed prior to THz exposure to confirm the confluent nature of the keratinocyte cultures and the presence of the epithelial barrier (Figure 6). The resistance to passage of the fluorescein (sodium) was more than 80%. There was no immediate effects noted as a result of the THz radiation, the post exposure barrier being as effective as the pre-exposure, i.e. below 20% leakage (Figure 6). By day 3-4 as the cells differentiated the barrier function improved so that 90% was prevented from crossing.
the epithelium (Figure 6). On the subsequent days the barrier function deteriorated such that by day 9, 40% of the fluorescein was able to leak across. There was a marked increase between day 6-7 and 9, with a doubling in the leakage capacity. Despite this, the THz exposure to either the NHK cells alone or to the co-cultures, did not result in a significant alteration to the barrier function at any of the days (Figure 6). The presence or absence of the ND7/23 cells did not significantly affect the barrier function over the 9 days. There does appear to be an inhibition of the deterioration of the barrier function at day 9 due in the presence of the neural cells.

3.4.2.3 Effects of 60 mins. exposure to THz radiation from the Teraview mobile source

Following the initial experiments with the Teraview mobile system, the two replicate cultures of NHK cultures and two co-cultures were exposed for 60 mins in the absence of medium on the inserts.

Despite the concerns over the possible effects of lack of medium and nutrients the cells did survive and remained active (Figure 7). A marked decline was noted with the ND7/23 cells between day 6 and 9 (Figures 9). This was associated with a loss of the barrier function (Figure 8). Despite this the change could be ascribed to the stress of being out of the medium for a protracted period since there was not difference with the unexposed controls.
3.4.2.4 Effects of THz on total cell culture protein levels

In an effort to equate the quantifying of the resorufin production and FC incorporation an attempt to measure the total protein level of the cell cultures on the inserts was made. The Kenacid blue total protein assay (Knox et al., 1986) had been used and shown to be clearly related to total cell number. Also this assay had been successfully employed after assays for cell viability since the work of Riddell et al, (1986). To quantify the protein a protein standard was prepared using BSA as had been employed when extrapolating from BSA protein attached per well, to cells attached on 96 and 24 well plates. In this case polycarbonate inserts had been used, so a similar approach of treating inserts with known concentrations of BSA was adopted to generate a standard curve. From the curve, as with the assay on the cells themselves, it became apparent that there were considerable difficulties with obtaining a linear relationship due to high residual Kenacid blue dye in the insert (Figure 10). An optical density of less than 0.4 at 570nm was difficult to obtain at protein concentrations below 44 µg/ml (Figure 10, indicated by the red lines).

3.4.2.5 Standard curves for Fluorescein Cadaverine and Resorufin

Standard curves were obtained for the fluorescein cadaverine by reading dilution of the stock in HBSS at 485nm excitation and 530nm emission (Figure 10). For the resorufin, since this was read in the presence of resazurin, the resorufin was diluted in 5µg/ml resazurin solution in HBSS, to give the standard curve (Figure 10).
3.4.2.6  Effects of 130 GHz exposure on human dividing keratinocytes and dividing ND7/23 cells

It is evident that whilst the conditions under which the cultures were exposed did affect the subsequent cell activity, and that the keratinocytes were more resistant to potentially adverse conditions (c.f. figures 11 & 12), even 16 hrs continuous exposed resulted in no discernible adverse reaction, in terms of cell activity. This was even true when the keratinocytes were stressed by exposure to sodium lauryl sulphate (Figure 13) for 24hours after exposure to the GHz, then returned to medium to stimulate differentiation. The cultures employed were grown in 96 well plates and were sub-confluent when exposed, thus in a growth phase.

3.4.2.7  Effects of 130 GHz radiation on differentiation capacity

As we suspected, by using the most sensitive of cell populations, namely the dividing human keratinocytes and the ND7/23 cells in these series of experiments, we found that they do not differentiate to any significant level during the 9 day duration of the experiment. Thus whilst it was noted that the general culture activity declines, this is NOT correlated with an increase in fluorescein cadaverine uptake. Thus the GHz radiation even when the cells were exposed for 24hrs were not stimulated to differentiate, i.e. go into premature programme cells death. It is not possible because of the low level of differentiation to say that differentiation
was inhibited.

![Figure 12. Effects of 130GHz on activity of Human keratinocyte cultures under different conditions](image1)

![Figure 13. Effects of 130GHz on activity of SLS challenged Human keratinocyte cultures](image2)

The neural cells, as shown by the cell activity assay, were reduced in number and hence the amount of fluorescein cadaverine taken up by the cultures was low, again showing that there was no stimulus of the transglutaminases responsible for fluorescein cadaverine incorporation in to the neural cells.

3.4.2.8 Effects of 130 GHz radiation on total protein

In the case of the GHz radiation, the cells were grown in the wells of a 96 well plate and thus the Kenacid Blue protein assay could be conducted. The results showed that there were very few ND7/23 cells left at the end of the treatments, so that only one example is presented (Figure 14).
The exposure to the GHz, either over a short or long term in the absence or presence of surfactant stress did not affect the final protein levels. The protein assay also confirms that the 2.5µg/ml sodium lauryl sulphate did cause marginal stress with a loss of cells and therefore an approximate 50% reduction in total protein in the surfactant treated cultures.

3.4.3 Conclusions
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), were observed with any of the exposure conditions. 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 the surfactant, sodium lauryl sulphate, which could form a residue on the skin of patients, prior to GHz exposure. Hence we are reasonably confident that the THz/GHz exposure rendered to potential patients by the Teraview imaging system is not potentially harmful at least upon one exposure and probably a few exposures.
3.5 Evaluation of biological effects on DNA bases

To evaluate the effects of THz radiation on DNA bases, two different experimental approaches for the preparation of thin films, from vapor deposition at high temperature and from solutions at different temperatures, were developed at NHRF. Regarding the first method, the experimental apparatus consisted in a stainless steel vacuum chamber where a carbon crucible was placed and were 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. 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. In 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 to 130 GHz radiation from the ENEA Compact Free Electron Laser, show no evidences of any damage after 20 min. irradiation at an average intensity of about 5 mW/cm², as shown in Fig. 1.

![AFM images of Adenine, Guanine, and Cytosine irradiated at 2.5 mm. The images show no evidences of any damage under the irradiation conditions](image)

Fig. 1 - 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 damage was done by comparison with irradiated samples at 157 nm were 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 CO₂ laser at 17.6 µm, 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 than the damage on the DNA bases under high irradiation conditions is only thermal, with no indication of any photochemical damage.
4. “SAFETY ISSUES OF THZ RADIATION”

We briefly recall here the results of a questionnaire distributed to collect information on the main radiation parameters of THz sources, which are currently in use at various laboratories worldwide. The questionnaire collected information concerning:

- The main radiation parameters of THz sources (e.g. frequency range, power level, modulation)
- Biological and potential biomedical applications of THz sources
- Exposure conditions (if any) of technical personnel
- Safety measurements or precautions currently adopted

The questionnaire also requested information about any risk perceived in using THz-radiation.

The questionnaire was designed by the THz-BRIDGE consortium members and was delivered to a list of about 150 research groups in occasion of several conferences. The questionnaire was also put in an interactive form on the project web-page. Compiled forms have been collected over an 24-month period and the statistical basis has been continuously updated. The survey was also designed to look at safety issues at specific occupational sites. These were defined as sites where THz sources are employed or in development. Due to the early stage of THz technology these are to be mainly found in Universities or Research facilities.

The questionnaire collected data of currently used terahertz sources for the first time. As of February 2004, 24 groups have returned a completed questionnaire providing information on 34 different THz systems. 37% of the responses came from the European Union; the remaining contributions came from the United States, Japan and Russia. A break down of the THz sources is shown in table and figure 1. The THz-BRIDGE consortium estimate currently that this is only twenty per cent of the active terahertz community. It is planned to continue to update the statistical basis with support from the EMF-NET Coordination Action.

The questionnaire was designed first to obtain information about the THz source parameters, that is, the central frequency, the average and peak power, the tuning range, the pulse duration and repetition rate. These results are summarised in Table 2. The second section of the questionnaire was concerned with the safety precautions taken by occupational workers and their perception of exposure to THz radiation. The results are summarised in Table 3.

Since, lasers are involved in the generation of most of the THz sources 75% of the respondents comply or are aware of current European or US safety laser safety standards. The remaining respondent did not answer the question. The overall majority of respondents were not concerned with exposure to THz radiation and few took precautions to avoid exposure. Five respondents expressed concerns about exposure. Four were concerned with acute effects and three of chronic effects. These respondents are involved in the research of
exposure of THz radiation to cells. The respondents who did take precaution in the exposure to THz-radiation were mainly from the Free Electron Laser (FEL) community; these groups are more concerned about exposure to x-ray radiation and strict safety regulations apply, which also limit the exposure to THz-radiation. In THz-FEL the radiation is usually transported through sealed light-pipes and the THz beam cannot be intercepted until it reaches the sample under study.

SUMMARY

- 25 groups returned data (1 of these was rejected as they submitted laser parameters rather than THz).
  - Most of these groups are using terahertz radiation generated by short pulse solid-state lasers;
  - They are mostly interested in source development and biomedical applications;
  - Data has been provided on 34 systems
  - Five groups expressed concerns of exposure to THz radiation.
- Mainly FEL groups take precaution against THz radiation in the frame of more severe shielding requirements of the accelerators on which they are based.
- It appears currently that there are no records taken of occupational workers exposure to THz radiation.

Table 1: Breakdown of THz-sources

<table>
<thead>
<tr>
<th>Source Type</th>
<th>Number of Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid state based system</td>
<td>23</td>
</tr>
<tr>
<td>Free Electron Laser</td>
<td>2</td>
</tr>
<tr>
<td>Solid-state multiplier</td>
<td>3</td>
</tr>
<tr>
<td>Semiconductor Laser</td>
<td>2</td>
</tr>
<tr>
<td>Gas Laser</td>
<td>2</td>
</tr>
<tr>
<td>Optical Parameter Oscillator (OPA)</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 1: Chart of distribution of respondents’ laboratory systems. The right-hand pie chart shows the break down of national groups.
### Table 2: Summary of the THz sources parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Categories</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Central Frequency</strong></td>
<td>&lt;300 GHz</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.3 – 5 THz</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>&gt;5 THz</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Variable 0.3-33 THz</td>
<td>2</td>
</tr>
<tr>
<td><strong>Average Power</strong></td>
<td>&lt;0.1 μW</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.1 - 10 μW</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10– 100 μW</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.1 – 10 mW</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 – 100 mW</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>&gt;50 W</td>
<td>2</td>
</tr>
<tr>
<td><strong>Peak Power</strong></td>
<td>&lt;1 mW</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1-100 mW</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.1-10 W</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 W – 2 kW</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;2 kW</td>
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<tr>
<td><strong>Pulse duration</strong></td>
<td>&lt;3 ps</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.3 – 2 ps</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1-10 ns</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>50 ns</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1-5 ms</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.1 - 5 ms</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CW</td>
<td>3</td>
</tr>
<tr>
<td><strong>Repetition rates</strong></td>
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</tr>
<tr>
<td></td>
<td>~250 kHz</td>
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</tr>
<tr>
<td></td>
<td>10-1 kHz</td>
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</tr>
<tr>
<td></td>
<td>1000-10 Hz</td>
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<td></td>
<td>&lt;10 Hz</td>
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<tr>
<td><strong>Laser amplitude modulated</strong></td>
<td>Yes</td>
<td>14</td>
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<tr>
<td></td>
<td>No</td>
<td>15</td>
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Table 3: Concerns and precautions of exposure of THz radiation to occupational worker

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<tr>
<td>ANSI-Z-136-1</td>
<td>8</td>
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<tr>
<td>EN60825-1</td>
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</tr>
<tr>
<td>ENV50166-2</td>
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</tr>
<tr>
<td>No return</td>
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<table>
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<th>Precautions</th>
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<td>8</td>
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<tr>
<td>Apparatus encased in glass</td>
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</tr>
<tr>
<td>Enclosed in box</td>
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</tr>
<tr>
<td>Lead shield</td>
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</tr>
<tr>
<td>No return</td>
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<table>
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<th>Occupational workers exposed to THz-radiation</th>
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</tr>
<tr>
<td>No</td>
<td>11</td>
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<table>
<thead>
<tr>
<th>Do you have any concerns?</th>
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<tr>
<td>No</td>
<td>18</td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
</tr>
<tr>
<td>Acute effects</td>
<td>4</td>
</tr>
<tr>
<td>Chronic effects</td>
<td>3</td>
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<tr>
<td>No returns</td>
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<table>
<thead>
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<th>Number of personnel exposed</th>
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<tbody>
<tr>
<td>1 to 3</td>
<td>7</td>
</tr>
<tr>
<td>4 to 7</td>
<td>5</td>
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</table>

<table>
<thead>
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<tbody>
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<td>Less than 6 minutes</td>
<td>6</td>
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<tr>
<td>6 to 20 minutes</td>
<td>4</td>
</tr>
<tr>
<td>&gt;20 minutes</td>
<td>2</td>
</tr>
<tr>
<td>No return</td>
<td>2</td>
</tr>
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<table>
<thead>
<tr>
<th>Frequency</th>
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<tbody>
<tr>
<td>Several times a day</td>
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<tr>
<td>Several times per week</td>
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</tr>
<tr>
<td>Several times per month</td>
<td>4</td>
</tr>
<tr>
<td>No return</td>
<td>2</td>
</tr>
</tbody>
</table>
5. CONCLUSIONS

Being a first step in the understanding of the interaction of THz radiation with biological systems, the project has achieved its main objectives without any significant deviation from the original plans. The spectroscopic database now includes about hundred spectra of a variety of biological samples that has been made available to the scientific community. A full range of irradiation studies on DNA bases, human lymphocytes, membrane model systems and epithelial cell cultures has been carried out at the various partners’ sites. All partners have benefited from the interdisciplinary approach of the project, as it has also been pointed out by the reviewers at the Final Meeting. The results of the project have been published or submitted for publication by the various research group in a number of papers (see the Dissemination section for details).

The exploration of biological effects induced by THz radiation was aimed at providing information on basic data and safety issues involved in employing THz radiation in medical and technological applications. This involved the employment of different biological targets including DNA bases, model membranes (liposomes), human lymphocytes, primary human keratinocytes and a neural cell line. The THz-induced biological end-points studied were alteration of membrane permeability of liposomes, induction of genotoxicity in lymphocytes as well as studying changes in cell activity, differentiation and barrier function in keratinocytes and neural cells. The results indicate that under various exposure conditions no biological effects could be detected. However, under some specific conditions of exposure, change in membrane permeability of liposomes was detected and an induction of genotoxicity was observed to occur in lymphocytes. These studies suggest that medical imaging employing appropriate exposure parameters is probably harmless at least for single exposures. Moreover, since some effects were observed to be induced by the THz radiation at a relatively low intensity when compared to the limits set by the ICNIRP for exposure, these studies should be extended to establish more accurate dose-response relationships. This is expected to provide in future improved guidelines of exposure.

The studies carried out within the THz-BRIDGE project have shown the importance of an interdisciplinary approach to tackle problems from different and complementary perspectives. The various scientific disciplines involved have also pointed out the necessity of carrying out a variety of biological and biomedical experiments using a variety of THz sources with different characteristics in terms of frequency, average and peak power, modulation conditions, which are usually not all easily available at the same site. Analogously, many of the biological assay of interest in the study of the interaction of THz radiation with biological systems can only be performed at laboratories located in close proximity of the sources. It has emerged from the experience of the THz-BRIDGE project that it is important to have the in-vitro tissue culture facilities close to the radiation sources, and ideally such that they can be delivered whilst the cells are in the CO\textsubscript{2} incubators. This reduces the stress on the living cells in particular on rapidly dividing cells or neural cells due to transportation and temperature fluctuations.
The research effort in the THz field has been steadily growing in the USA, Japan and Europe during the past few years. Several Workshops have recently taken place and are being scheduled in the US through government funding agencies to boost the level of investment in THz science.

**February 2, 2004**: DARPA/MTO, “THz Imaging Focal Plane Array Technology” and DARPA/DSO, “FAST READ” initiatives;

**February 12, 2004**: DOE-NSF-NIH Workshop “Opportunities in THz Science”

**March 16, 2004**: “THz Application Symposium” organized by the Southeastern Universities Research Association (SURA) to focus on medical imaging and homeland security

**August 2, 2004**: Special event in the frame of the 2004 SPIE Annual Meeting in Denver (2-6 August) entitled, “International Workshop on the Emergence of Terahertz Radiation”

The application of THz radiation in biology and biomedicine is gaining an increased interest in the scientific community, as it is shown by dedicated sessions at several conferences and workshops. The Annual Meeting of the Bioelectromagnetics Society (BEMS 2004), which will be held in Washington DC in June 2004, has scheduled for the first time two specific sessions on THz research.

So far Europe has a leading position in the biological and biomedical applications of THz radiation, and should quickly act to maintain such position with a joint synergic effort. As a future perspective for THz research in Europe, the proposal of a Design Study of a new research Facility for Terahertz radiation in Biological, Environmental and Advanced Material studies (THz-BEAM) based in Italy has been recently submitted to the “Research Infrastructures” programme of FP6.

The THz-BRIDGE consortium is currently represented by its coordinator, ENEA, in the Coordination Action (CA) EMF-NET funded under the Priority 8 of FP6, which gathers all projects on EMF funded in the Quality of Life Programme - Key Action 4 - Environment and health of FP5. This CA is aimed at collating and harmonizing the results of the various projects and the indications to regulatory bodies. Although EMF-NET does not provide further research funds, it will provide support in the dissemination of results, as well as in the organization of workshops, specific meetings and in the maintenance of spectroscopic database.
6. EXPLOITATION AND DISSEMINATION OF RESULTS
The main objective of the THz-BRIDGE project was to provide a fundamental understanding of the interaction of THz radiation with biological systems. This has been achieved by delivering a spectroscopic database and a timely risk assessment to the scientific community in view of the implementation of THz technology in the biological and biomedical field. In this respect the project will guide and accompany the technological development of diagnostic devices in the above fields.

To disseminate the results of the project and stimulate further contacts and collaborations in the field the "THz-BRIDGE International Workshop" was held on the Island of Capri - Italy from September 29 to October 2, 2002. Beside the THz-BRIDGE partners, representatives of the European Commission, as well as scientists from an extended “THz community” at European and international level (USA, Canada, Australia, Japan, Korea, NIS and Eastern European Countries) have been invited to attend the THz-BRIDGE Workshop to discuss a broad range of scientific issues in the Terahertz and adjacent Infrared and Microwave spectral regions, from spectroscopic measurements to the effects induced from the electromagnetic field on biological systems, to the technological development of terahertz sources and systems for biomedical applications. Invited speakers and authors of contributed papers at the THz-BRIDGE Workshop have been invited to submit a manuscript of their work for publication on a special issue of the Journal of Biological Physics. The Workshop registered 80 participants from 15 different countries, 35 oral papers (of which 15 invited) and 22 posters. 12 papers were presented by the THz-BRIDGE partners. Detailed information about the Workshop as well as presentations on-line are available through a link on the project web-page to the address: www.frascati.enea.it/THz-BRIDGE/Workshop.

Most of the papers presented at the Workshop were submitted for publication in the Workshop proceedings and were peer reviewed by the Workshop Programme Committee with the assistance of independent experts. The publication of the Workshop Proceedings occurred in June 2003 as a special issue (vol 29, issue 2-3) of the Journal of Biological Physics.

As mentioned before, a future perspective for THz research in Europe is represented by the Design Study of a new research Facility for Terahertz radiation in Biological, Environmental and Advanced Material studies (THz-BEAM) based in Italy with the following objectives:
To develop THz sources and study the interaction of THz radiation with biological systems.
To make such sources available to users of a wide interdisciplinary community at EU and international level.
To gather scientists from different disciplines and have them interacting on the same research site.
To develop THz imaging techniques to be applied in the fields of biomedicine, material science, security and inspection devices.
To train young scientists from inside and outside Europe.
7. Policy Related Benefits

The recent technological breakthrough in the field of Terahertz radiation (T-rays) is triggering new applications in biology and biomedicine. At present, a number of laboratory-scale THz sources, like free-electron lasers, electronic tubes and pulsed solid-state THz sources, are in use at research institutes, while the first commercial systems have just entered the market raising the issue of potential exposure of specialised personnel and users. The interdisciplinary and complementary expertise of the consortium made possible to carry out the research project at European level only and not at national level. Europe is at the heart of THz research world-wide and the THz-BRIDGE project has strengthened this position. The entire THz range from 100 GHz to 20 THz can only be covered by exploiting the potential of THz radiation sources available on a European scale. Analogously, the spectroscopic, biologic, biomedical and imaging expertise in the consortium could only be gathered on a transnational scale, due to the variety of research methodologies and techniques involved.

By providing a spectroscopic database of biological systems in the above frequency range the project has contributed to the EU policy of standardisation. The same applies to the definition of adequate exposure standards for the application of THz imaging in the biological and biomedical field. In particular, regarding the imaging of skin for the early diagnosis of malignant tumors, no adverse effect was observed with any of the exposure rates employed, which exceed exposure times required to generate images in human patients by at least five 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. Hence we are reasonably confident that the THz exposure rendered to patients by imaging systems presently on the market is not potentially harmful.

In summary, THz-BRIDGE has addressed central policy issues such as improvements in the Quality of Life, Health and Safety, and Standardisation issues. The unique expertise required to make this programme a success could only be found in Europe. By performing a basic risk assessment on the interaction of THz radiation with biological systems, the project has also contributed to social objectives of the EU community such as safety requirements at specific occupational sites. In the near future a further contribution to the quality of life will derive from the development of biomedical imaging techniques based on THz radiation. Once the defined exposure standards are met, there is a great promise that such systems may provide sufficient information in diagnostics applications and may prove to be much less invasive than other commonly used techniques such as X-ray imaging. Clearly, biomedical applications will also require adequate measurements to monitor the technical personnel involved.

The application of THz radiation and the realization of THz imaging systems is a rather new technology with a great potential for development in the coming years. Adequate training of young researchers in this field will be an incentive for the creation of new jobs in the Community. On the basis of the experience gained
with the THz-BRIDGE project, plans are in progress to establish an interdisciplinary European New Research Infrastructure called THz-BEAM and based in Italy, specifically devoted to the development of THz sources and their application in biomedicine, material studies, security and telecommunication. Means will be provided to appoint young researchers at this site.

Other outcomes of the project are likely to stimulate the generation of new knowledge in DNA studies, such as an alternative to the polymerase chain reaction test, which would produce less waste products, and new tests which can reduce the time and cost of pre-clinical drug trials. In addition, THz imaging equipment, presently at the early stage of commercial exploitation, will benefit from the rigorous scientific understanding of image contrast mechanism investigated in the project.
8. REFERENCES

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Section 3.2

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Section 3.3

9. **PUBLISHED PAPERS AND PAPERS PRESENTED AT CONFERENCES AND WORKSHOPS**

**Published papers**

A. Doria, G. P. Gallerano, E. Giovenale  
Free electron broad-band THz radiator  

M. R. Scarfi, M. Romanò, R. Di Pietro, O. Zeni  
THz exposure of whole blood for the study of biological effects on human lymphocytes  

Absorption and diffusion measurements of biological samples using a THz Free Electron Laser  

THz radiation studies on biological systems at the ENEA FEL Facility  

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‘Chemical recognition of crystalline compounds from their free induction decay at THz-frequencies’

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- E. Giovenale, “The THz-BRIDGE project” 3rd GR-I Conference, Patras (Greece), September 5-8, 2002

- A. Doria, A. Coniglio
  Near field microscopy devices in the THz spectral region for biological investigations
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