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FTIR Microspectroscopy in Studies of DNA Damage Induced by Proton Microbeam in Single PC-3 Cells

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In recent years, the Fourier transformed infrared spectroscopy is often applied in studies of biological materials on cellular level. Undoubted advantage of this method is high sensitivity. In presented research the FTIR microspectroscopy was used to analyse the DNA damage in single PC-3 cells (prostate cancer cell line derived from bone metastases) irradiated by counted number of protons. Focused proton microbeam 2 MeV from the Van de Graaff accelerator at the Institute of Nuclear Physics, Polish Academy of Sciences, was used as an irradiation source. Four groups of single cells were irradiated with 1000, 2000, 4000, and 8000 protons per cell, respectively. Following irradiation cells were fixed in 70% ethanol and then analyzed by IR microspectroscopy. Bond analysis of IR spectra served as a base for result analysis. This research has focused on the detection of changes in DNA backbone spectral range (950–1240 cm^{-1}), which could be related to damages such as single and double strand breaks, DNA–DNA, and DNA–protein cross links. Switches and differences in intensity of DNA backbone bands (980–1149 cm^{-1}, 1151–1350 cm^{-1} — symmetric and asymmetric PO^{2-} stretching vibrations, as well as in 1110 cm^{-1} — symmetric stretching of P–O–C band) were observed. Experimental spectra of irradiated and control cells were compared with simulated spectra generated by HyperChem software. The multivariate statistical methods of principal component analysis and hierarchical cluster analysis (Ward's method) were also performed and are discussed.

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1. Introduction

Research of radiation dose-dependent biological effects (such as DNA damage: single and double strand breaks, cross-links and oxidative damages) enhances the understanding of the mechanisms leading to cell death. This may result in the increased effectiveness of radiotherapy, and thus increased chances of total cure of the patient and prevention of the metastases formation. Particularly microbeam facilities are an excellent tool for radiobiological studies due to their ability to deliver precise doses of radiation to selected individual cells in vitro. In the Department of Applied Spectroscopy at the Institute of Nuclear Physics, Polish Academy of Sciences a proton microprobe facility was built and it was adapted to living cells studies as a source targeted irradiation of single cells with the controlled number of protons [1–4].

To detect the DNA damage biochemical methods are generally used. However, they may influence the investigated sample, because of chemical substances used and complex preparation procedures. Most of them are not intended to single cells studies, such as electrophoresis, colorimetric tests: lactate dehydrogenase (LDH), mitochondrial succinate dehydrohenase (MTT). Therefore, the importance of application of complementary technique is evident. From possible methods, the Fourier transformed infrared (FTIR) spectroscopy is well known for its uniqueness as a appriopriate tool in identifying vibrational structure of biological materials.

A growing number of literatures demonstrate the conformational aspects of proteins, nucleic acids and other biomolecules using FTIR. It is known that FTIR is a sensitive tool that can be applied to DNA damage study on chemical bond level thus it has become a potential analytical method in single cells studies [5–7].

In the presented study the human prostate adenocarcinoma derived from bone metastases — PC-3 line was used as a biological material. This cell line is often used as a model in prostate cancer study [8, 9]. The high degree of invasiveness of PC-3 line makes a very interesting research model.

2. Materials and method

2.1. Cell culture and sample preparation

PC-3 cells were cultured in RPMI 1640 medium, supplemented with 10% of FCS (fetal calf serum), 100 U/ml penicillin–streptomycin solution, 10 mM HEPES, 1 mM sodium pyruvate and 4.5 g/l glucose (all compounds delivered by Sigma Aldrich Stenheim, Germany). Cultured cells were grown in 37 °C and an atmosphere of 5% CO_2. To collect cells the 0.5% trypsin in phosphate buffered saline (PBS) was used.

For protons treatment, cells were seeded on specially prepared 35 mm diameter Petri dishes with 10 mm round holes in the central part of the bottom. The Petri dish bottom was covered with the 1.5 μm thick Mylar foil

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(Goodfellow Cambridge Limited, Huntington, UK) using the glue (Master Bond EP 30 med, New York, USA). A population of about 10 000 cells in 10 μl medium were seeded in the central part of the Mylar foil 16–18 h before the experiments. Four hours after the seeding, when cells had adhered to the foil, 2 ml of medium was added.

2.2. Protons source and cells irradiation

The 2 MeV horizontal focused proton microbeam (the external beam of about 16 μm in diameter at the irradiated spot) from the Van de Graaff accelerator was used as an irradiation source. During cell irradiation the beam current was about 0.16 fA, which corresponds to about 1000 protons per second with the 92% targeting accuracy. The silicon surface barrier particle detector (ORTEC B-019-300-150) was used for protons counting. Single cells were irradiated with four doses of protons in the range of 1000–8000 protons per cell and next incubated with medium in 37 °C and the atmosphere of 5% CO_2 for up to 24 h. Not irradiated cells, but processed in the same way, were forming the experimental control group. After irradiation all cells were washed with PBS and fixed in 70% ethanol at 4 °C.

2.3. Analysis under IR microscopy

FTIR spectra of 1–3 different cells (per spectrum) were collected. Spectra were taken in transmission mode with a resolution of 4 cm^{-1}, in spectral range of 600–4000 cm^{-1} for all groups of cells (irradiated and untreated by radiation). As the reference the Mylar foil was used. The extended spectral range was necessary to allow the use of the correction of the Mie scattering which improves the quality of the spectrum. The number of scans was set to 256. In presented study the spectrometer Bruker IFS 66v/S with a mercury cadmium telluride (MCT) detector and the microscope Bruker IRscope II were used. For all groups of cells (control and treated with protons) 20–30 spectra were taken.

As the first step, the baseline correction (second-degree polynomial) was done. Next the correction for the Mie scattering effect was applied. Optimally parameters were used during the corrections [10–12]:

- Number of iterations: 50;
- Mie theory option: resonant Mie scattering (RMieS);
- Lower range for scattering particle diameter: 2 μm;
- Upper range for scattering particle diameter: 12 μm;
- Lower range for average refractive index: 1.1;
- Upper range for average refractive index: 1.5;
- Reference spectrum: Matrigel (measured especially for this experiment).

The spectra of cells control group and irradiated ones (1000, 2000, 4000, and 8000) protons per cell were averaged respectively (Fig. 1). The spectra were analyzed by fitting the bands with Gaussian–Lorentzian curves (Fig. 2). Band fitting was performed in the spectral region of 800–1800 cm^{-1}, because the presented experiment was focused on the detection of changes in DNA backbone spectral range (950–1240 cm^{-1}). The number of peaks was chosen following the literature [13–15], and the intensity of some of them was set to 0. The spectra were analyzed using Omnic software, and the band fitting was carried out by X-ray photoelectron spectroscopy (XPS) peak software.

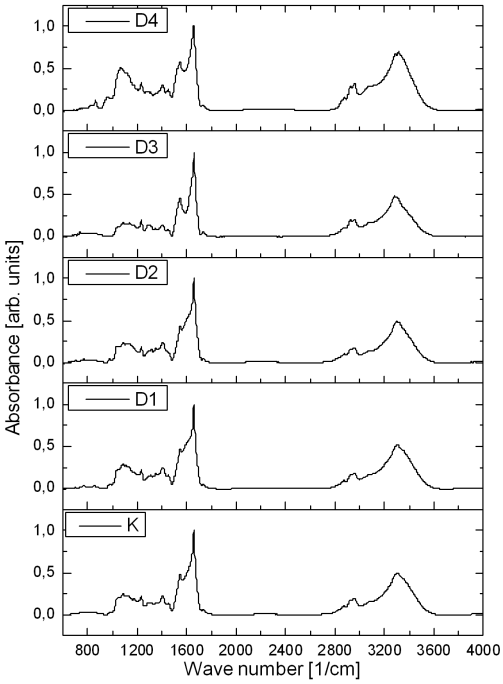

Fig. 1. The averaged spectra of control (K) and cells irradiated with 1000 (D1), 2000 (D2), 4000 (D3), and 8000 (D4) protons per cell, respectively.

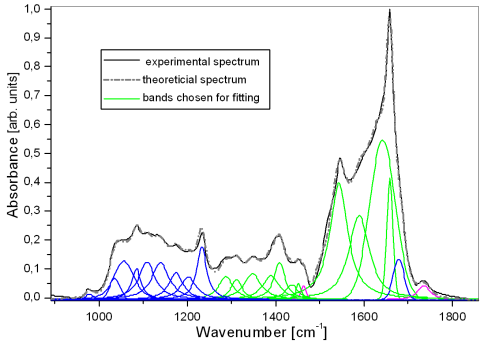

Fig. 2. Fitting analysis in the wave number region of 950–1800 cm^{-1}.

3. Results

3.1. Hierarchical cluster analysis

Spectral range 950–1240 cm^{-1} was analyzed using Ward's algorithm [16]. In presented method, an Euclidean distance produced dendrogram, which illustrates the degree of similarity between the averaged spectra of each cells (control and cells irradiated with 1000, 2000, 4000, 8000 protons per cell) (Fig. 3).

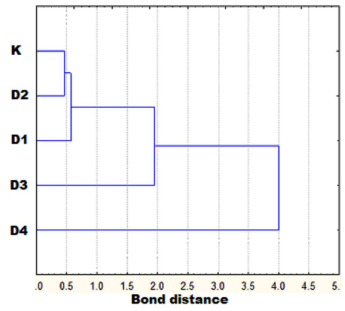

Fig. 3. Degree of similarity between the averaged spectra of each cells: control (K) and cells irradiated with 1000 (D1), 2000 (D2), 4000 (D3), 8000 (D4) protons per cell. Not as expected, the cells irradiated by 2000 are most similar to the control group.

3.2. Principal component analysis

To detect the structure in the relationships between data, principal component analysis (PCA) of the spectral region 950–1240 cm^{-1} was performed using Statistica 8.0 software [17]. Five distinct clusters were evident for five groups of cells spectra (control and irradiated with 1000, 2000, 4000, 8000 protons per cell) (Fig. 4).

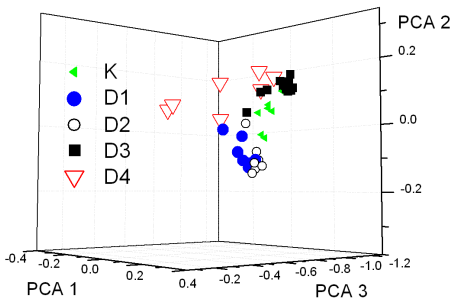

Fig. 4. Five distinct clusters for five groups of cells spectra (control (K) and cells irradiated with 1000 (D1), 2000 (D2), 4000 (D3), 8000 (D4) protons per cell).

3.3. Fitting analysis

The detection of changes in DNA backbone spectral range (950–1240 cm^{-1}), can be related to damages such as single and double strand breaks. Also DNA–DNA and DNA–protein cross links were examined. Differences and switches in intensity of DNA backbone bands were observed in all irradiated cells.

All of the bands are presented in Table. The experimental spectrum of control cells and the averaged theoretical model are presented in Fig. 2. Bands at 980–1149 cm^{-1}, 1151–1350 cm^{-1} are attributed to symmetric and asymmetric PO^{2-} stretching vibrations [2], while at 1110 cm^{-1} to symmetric stretching of P–O–C band and at 970 cm^{-1} — ribose-phosphate skeletal motions [3, 4].

TABLE

DNA infrared bands of cells in spectral range 800–1800 cm^{-1} [12, 13].

Wave number [cm^{-1}]	Assignment [13–15]
Lipids	
1462	CH_3 asymmetric bending
1732	C=O stretching
Proteins	
1294	30% C–N stretching; 30% N–H bending; 10% C=O stretching; 10% O=C–N bending; 20% other (amide III)
1313, 1348	O–H bending (serie)
1380	CH_3 symmetric bending (leucine)
1406	the carboxylate anion and the a-CHa scissors deformation of the carboxylic acid
1426	CO_2 symmetric stretching (glutamic acid)
1451	CH_3 asymmetric bending in valine, benzene ring vibrations (tyrosine)
1546	60% N–H bending; 40% C–N stretching (amide II)
1585–1642	80% C=O stretching; 10% C–N stretching; 10% N–H bending
1650	C=O stretching (aspargine)
Nucleic acids	
970	ribose–phosphate skeletal motions (DNA)
1038	ribose C–O stretching (RNA)
1055	ribose C–O stretching
1086	PO_2 symmetric stretching (DNA)
1105	P–O–C symmetric stretching (DNA)
1144	ribose C–O stretching (RNA)
1175	sugar–phosphate backbone vibrations
1212	C–H ring bending (RNA)
1230	O–P–O asymmetric stretching (DNA)
1680	C=O stretching (RNA)

All changes in spectra of cells irradiated by 1000 and 2000 protons per cell were insignificant, but they were substantial at 4000 and the biggest by 8000 (Fig. 5, Fig. 6). Figures 5 and 6 present the area under DNA backbone bands for all groups of cells (control and treated with different dosages of protons). Figure 5 shows the sum of changes of an intensity of all four peaks together. Figure 6 presents the changes of individual peak separately. The standard deviations were presented at both figures (Fig. 5, Fig. 6).

An area of O–P–O symmetric stretching band increased with the number of protons and an area of P–O–C symmetric stretching band decreased with the

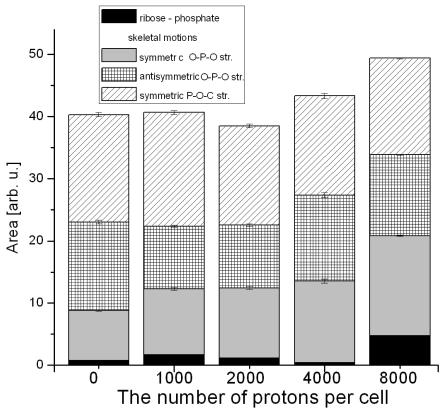

Fig. 5. The total area of DNA backbone bonds in spectra of irradiated and control cells.

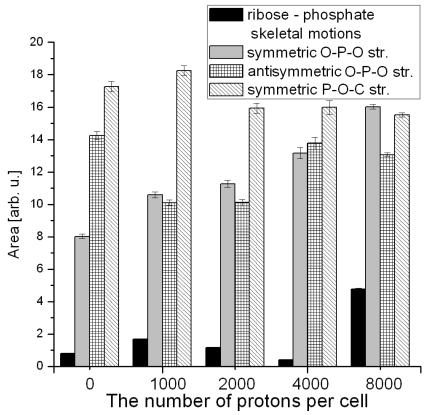

Fig. 6. The individual area of DNA backbone bonds in spectra of irradiated and control cells.

number of protons. It could mean that the probability of DNA strand breaks by destroying C–O bond is higher than O–P one. An increase of the area under the band corresponding to motion bond in phosphate — DNA backbones may be due to destroyed chromosomal structure. Fragmentation of chromosomes could cause increase the number of molecule freedom degrees. The emerging of a new peak at about 830 cm^{-1} in spectra of cells irradiated with 8000 protons per cell was observed (Fig. 1). HyperChem simulations show that the peak could be correlated with single strand break (SSB). PCA analysis shows some clustering between all groups of spectra (control and irradiated with four different doses of protons).

4. Conclusions

Aim of this study was to find if infrared microscopy can be used in radiation DNA damage detection. Statistical analysis shows that there are differences between spectra of irradiated by different dosages of protons and control cells. Fitting analysis allows to follow small changes in spectra. Presented results prove that infrared microspectroscopy could be useful tool in DNA damage study in single cells.

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