Extension of DNA based dosimetry to broad-band UV radiation

Ph.D. Thesis

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1. Introduction

Human activity is leading to a changing radiation environment, including the extension of ultraviolet radiation to shorter wavelengths. Besides the increasing levels of exposure to natural sunlight under an anthropogenically depleted ozone layer, the use of artificial UV sources is becoming more and more widespread (e.g. sunbeds, medical and industrial applications). The adverse effects of ultraviolet radiation are not only dependent on the total incident dose but also on the spectral composition of the radiation. There is an increase in the high-energy ultraviolet radiation (UVB: 280–315 nm) which was not part of the natural environment before, as it was filtered out by the ozone layer of the atmosphere. UVB radiation is also associated with more severe biological hazards than higher wavelengths of UVA (315–400 nm) radiation. In extreme environments, for example in space the extraterrestrial component of solar radiation (UVC: 100–280 nm) also plays an important role.

In order to get an estimation of the biological effects resulting from environmental changes the spectrum of incident radiation has to be weighed by the energy and wavelength dependent spectral sensitivity of the living organisms. This makes the estimation and prediction of adverse biological effects on human health and the biosphere a complex problem. The key to the solution of this problem is the use of biological dosimeters, which are capable of biologically weighing and integrating the absorbed energies at different wavelengths.

Nucleic acids interacting with proteins play a primary role in the radiation damage of living organisms and show increased sensitivity due to maximum absorption in the high-energy ultraviolet region (UVC). Radiation induced alterations of DNA can lead to cell death and the development of skin cancer, which makes UV radiation the most ubiquitous environmental carcinogenic agent. The majority of the biological effects of ultraviolet radiation is initiated by various lesions of the DNA, therefore it seems reasonable to use the DNA itself as a biological dosimeter. The need for the development and application of DNA based biological dosimetry for a broad spectrum of ultraviolet radiation is supported by these facts.

Microorganisms (bacteria, spores, viruses) containing the target molecules of ultraviolet radiation can be used as biological dosimeters. Bacteriophage T7 is an ideal model system for the study of DNA damages, because intact phages T7 can be regarded as a model for the chromosome based on their structure. The comparison of the results obtained on intact phages T7 and on isolated T7 DNA
makes the investigation of DNA–protein interaction possible. The structure of phage T7 is known, the methods of its laboratory application are well established, it is suited for physical and chemical analysis methods, and it also shows biological activity on \textit{E. coli B} host cells.

Ultraviolet radiation not only has a strong effect on the present biosphere but it could have also influenced the formation of life on Earth. Today the surface of interplanetary dust grains is thought to be one of the possible places for the synthesis of simple biomolecules. Here – as on the surface of Earth, unprotected by ozone at that time – the molecules were processed under the effect of strong electromagnetic radiation. The interplanetary transfer of biomolecules or DNA bound to pieces of meteorite material may also had been possible. Today spacecrafts contaminated by microorganisms are a problem for space environment and planetary protection. This makes space survival of DNA and nucleoprotein complexes a current scientific issue.

Exobiological experiments dealing with this problem can be carried out on the EXPOSE unit developed for the International Space Station. One of the experiments supported by the European Space Agency is PUR (Phage and Uracil Response) where our aim is to study the effects of space parameters on phage T7, DNA and uracil thin layers.

2. Main objectives

T7 biological dose was earlier defined by the decrease in plaque forming ability of the phages on \textit{E. coli B} host cells. The development of molecular biology techniques opens new ways in the detection of UV photodamages, which make the determination of the biological dose more simple and less time consuming. In the present work our aim was:

I.1. The development of a polymerase chain reaction (PCR) based technique for the detection of DNA damages;
I.2. To elaborate a method for the quantitative evaluation of the PCR results;
I.3. The optimization of the polymerase chain reaction for fragments to be used for different dose ranges;
I.4. To study the effects of UVB, UVC and polychromatic sources in phage / DNA;
I.5. The validation of phage T7 biological dosimeter;
I.6. To study the role of different DNA conformations in photodamage formation;
I.7. The investigation of the role of DNA–protein interaction in the UV damage of DNA.
DNA lesions caused by ultraviolet radiation and vacuum limit the survival of biological substances in space. The experiments with phage T7 and DNA thin layers on the International Space Station have to be preceded by ground-based preliminary measurements. During Experiment Verification Tests (EVT) we demonstrated the routine use of the new QPCR based method – combined with other techniques – for the detection of DNA damages. In space simulation experiments of our phage / DNA thin layers we examined:

II.1. The quality of the phage and DNA thin layers to be sent to the EXPOSE unit;
II.2. The effects of simulated space vacuum;
II.3. The effects of temperature fluctuation;
II.4. The effects of simulated extraterrestrial radiation (monochromatic UVC and solar simulator);
II.5. The total number of DNA lesions and the distribution of different types of photodamages;
II.6. The effects of combined treatments (vacuum + UVC);
II.7. The shielding effect of layer thickness and the possibilities of taking this effect into account.

3. Methods

T7 bacteriophages were grown on *E. coli* B host cells, purification and concentration of the phages was carried out by CsCl gradient centrifugation and dialysation using the methods developed earlier at the Department of Biophysics and Radiation Biology, Semmelweis University. The protein capsid of the phage contains a double stranded, linear DNA molecule, which is made up of 90% essential genes, therefore every lesion can be considered to be lethal.

The number of active phages in the solutions was determined by infecting *E. coli* B cultures on a boullion-agar plate (Gratia-titration). By definition one unit of T7 dose corresponds to a 1/e decrease in the biological activity, that means in the plaque forming ability of the phages: $H_{T7} = - \ln (N/N_0)$, where $N_0$ and $N$ is the number of active phages before and after treatment, respectively. One unit of T7 dose determined by this standard technique means 1 detected lesion per phage, which equals 2 UV induced lesions before host cell reparation ($\beta \approx 0.5$).
For the determination of DNA damages we developed a quantitative method based on polymerase chain reaction (QPCR). The Taq DNA-polymerase is synthetizing a copy of the existing DNA fragments flanked by primers in each cycle, therefore the amount of DNA is increasing exponentially in the reaction. The copy process is blocked by the (photo)lesions of DNA. The determination of the number of damaged sites in the DNA is possible based on the decrease of the amount of synthetized fragments of treated samples compared to untreated ones.

The QPCR technique is very sensitive and works well only on artifact-free template DNA. Therefore we had to replace the conventional phenol–chloroform DNA preparation technique by a much less aggressive one using 5% sodium-dodecyl-sulfate (SDS) and KCl.

We used on-line databases to find primers for a 555 and a 3826 basepair long fragment of phage T7 DNA. The reaction protocol had to be experimentally optimized for the concentration of primers, DNA, nucleotides, MgCl₂ and AmpliTaq Gold polymerase as well as for the values of cycle number and the temperature and duration of each step.

The result of the PCR was electrophoresed on 2% agarose gels together with 1 kilobase standard or λ Hind III fragments and DNA samples of known concentration. The amount of ethidium-bromide fluorescence over the UV transilluminator is proportional to the amount of DNA present in a given part of the gel. This was quantified by computer analysis of the photographs taken from the gels. In the range of exponential amplification the average number of lesions per strand (s) can be expressed by the Poisson-equation: \( s = -\ln (A/A₀) \), where \( A₀ \) and \( A \) is the amount of PCR product of the control and treated sample, respectively.

With the polymerase chain reaction we were able to determine the number of DNA photoproducts after treatments of given biological doses with five various UV sources covering the UVA–B–C range (Sun, solar simulator, FS20 lamp, TL01 lamp, germicidal lamp). The effective wavelength range of the emission of each source was determined by spectroradiometer measurements.

For the investigation of how protein interaction influences the formation of DNA photolesions we carried out measurements on intact phages, on DNA isolated from phage proteins and on „heated phages” where after a temperature treatment of 65 °C the genetic material was released from the capsid but was still attached to it.
We studied the role of DNA conformation in photoproduct formation in A, B, C and disordered (D) conformation. These states were produced by influencing the hydration of DNA – in solutions by adding ethanol (or heat denaturation), in thin layers by changing the relative humidity and the concentration of NaCl / LiCl.
The work with solutions under space conditions is difficult and we also wanted to examine the effects of dehydration, therefore our research group has developed a method for phage/DNA thin film preparation, where the concentration of phage/DNA in the original NaCl/LiCl solution is determining the thickness of the layer. The particles were precipitated by adding increasing amounts of ethanol to the solution and they were deposited onto quartz plates by centrifugation. Homogeneity and uniform covering of the films was checked by polarization and phase contrast microscopy and by the amount of scattered light determined from empirical fitting of the Rayleigh-Mie formula \(E = k\lambda^{-n}\).

The treatment of the thin layers was carried out in the vacuum tight sample holders developed for the EXPOSE unit at the space simulation facilities of the German Aerospace Center in Cologne and the Austrian Academy of Sciences in Graz. We studied the effect of \(10^{-4}–10^{-6}\) Pa vacuum for one week on open sample holders and the effect of temperature fluctuation following two profiles marked 1V2 (1 week 40 °C, 1 day 60 °C, 1 week 40 °C) and 2V1 (1 hour +20 °C, 1 hour -20 °C, for 1 week). Extraterrestrial radiation was simulated using a germicidal lamp emitting at 254 nm and a solar simulator (SOL 2000) emitting between 200-400 nm.

The „stacking” interaction between the nucleotide bases of DNA on thin layers is reflected in the absorption spectra. After analysis and light scattering correction of the data recorded by a spectrophotometer we dissolved the phage/DNA from the thin layers. We used the QPCR method for the determination of total lesion frequencies in phage/DNA. Double strand breaks were quantified on neutral gels and – after separation of the DNA strands by NaOH – single strand breaks on alkaline gels, using the average fragment lengths determined from the neutral/alkaline gels. We combined these methods with specific enzymatic digestion for the quantification of specific lesions. Endonuclease V. cleaves the DNA strand next to cyclobutane pirimidine dimers (CPDs), endonuclease IV. next to apurinic/apirimidinic sites. The strand breaks were quantified by electrophoresis on 1% agarose gels. DNA–protein cross-links were determined based on the decrease of the amount of DNA isolated by SDS from the irradiated phages.
4. Results and discussion

Based on our results concerning the use of bacteriophage T7 as a biological dosimeter I made the following conclusions:

I.1. I developed a quantitative polymerase chain reaction (QPCR) capable of directly detecting lesions of the phage DNA instead of the indirect method based on the decrease in the plaque forming ability of the phages. After optimization of the PCR parameters the amount of intact template DNA was the only limiting factor of the reaction, therefore the amount of the PCR product was directly proportional to the amount of the template DNA, where the correlation coefficient was 0.9998. Any DNA lesion blocks the synthesis by the Taq polymerase. It was possible to calculate the ratio of damaged DNA molecules based on the decrease of PCR product of treated samples compared to the product of untreated ones (control). The quantitative polymerase chain reaction is capable of detecting practically all lesions, therefore it could be used to study the effects of various kinds of DNA damaging agents, including high-energy ultraviolet radiation, but its application could also be possible in other fields, e.g. for ionizing radiation or genotoxic agents. The reaction is so robust that the purification of T7 DNA is not necessary, whole phages can be amplified as well. The possibility of measurements on intact phages makes the workflow more simple and is favourable for routine application.

I.2. DNA lesion frequencies can be exactly determined by the quantitative evaluation of the PCR. Based on the ratio of the amount of the PCR product of treated and untreated DNAs the proportion of damaged and intact molecules in the sample can be calculated. If we assume a random formation of DNA damages, then lesion frequencies in the population will follow Poisson-distribution. The average lesion frequency in the fragment under examination can be calculated by a Poisson-equation of zero order from the experimentally determined amount of intact DNA molecules, and the total number of DNA lesions can also be calculated from this data. These results were confirmed by the results of the conventional microbiological techniques (decrease in biological activity).
I.3. The exponential amplification of the target DNA fragment in the PCR favours the very sensitive detection of DNA damages. The sensitivity of the method depends on the length of the target sequence. In the case of shorter fragments larger doses are required to produce the same amount of damages. The 3826 bp fragment of the phage T7 genome is \( \approx 7 \) times longer than the 555 bp fragment, and for the same doses inhibition of the Taq polymerase was \( \approx 6.5 \) times greater in the longer fragment. This shows that the number of lesions is directly proportional to the fragment length. The lesion frequencies calculated for the whole genome show a close match in the case of the two fragments. The phage / DNA biological dosimeter can be used for measurements in different dose ranges depending on the length of the amplified sequence in the QPCR. Detection of small doses is possible with the 3826 bp fragment, while the 555 bp fragment is suited for larger ones. The phage / DNA dosimeter can be used as a short-, mid- or long-term dosimeter for monitoring natural sunlight or for the detection of small, middle or large doses of artificial UV sources. Phage T7 DNA and QPCR analysis – unlike other dosimeters – can be used for investigations throughout the total biologically relevant dose range.

I.4. In our experiments we used five different UV sources covering the UVA–B–C range. There was no significant difference in the lesion frequencies determined by polymerase chain reaction in the case of different UV sources for a given biological dose. Earlier with immunodot-blot technique the research group was able to demonstrate a strong wavelength dependency in the formation of two typical photoproducts (CPDs and 6-4 PDs) even though the biological effect of the applied irradiation was the same in all cases. This lead us to the conclusion that the new method has an important advantage: QPCR is able to detect practically all kind of DNA photolesions – independently from the spectral composition of the radiation provoking them.

I.5. I validated the phage T7 biological dosimeter based on QPCR results. In bacteriophage T7 the unit of the biological dose was defined through the decrease in biological activity. A UV dose of \( H^{T7}=8 \) determined by the ratio of the plaque forming ability of treated and control samples corresponds to 16 damages per phage before a host cell reparation of \( \beta=0.5 \). Lesion numbers from QPCR analysis of the two fragments showed a close match
with this data with all five UV radiation sources. From this we can conclude that phage T7 contains a large number of vulnerable sites, a single DNA lesion can lead to inactivation of the phage and the formation of damages is a random process which follows Poisson-distribution.
I.6. The formation of UV photodamages of the genetic material is largely dependent on how the nucleotide bases are situated in the DNA, because this influences the probability of photoproduct formation. Based on QPCR results I observed that there is no significant difference in the lesion numbers between different conformational states (A, B, C and D conformation) of the genetic material. The general conformation of DNA does not play a decisive role in photoproduct formation.

I.7. I studied the effect of phage proteins on DNA photoproduct induction by ultraviolet radiation in intact phage, isolated T7 DNA and „heated phage“. Because of the ability of proteins to stabilize the DNA structure to some extent and to provide at least a small amount of shielding a protective role of proteins was expected. However, for various UV sources the lesion frequencies in intact phages were \(\approx 3.5\) and \(\approx 2.5\) times greater than in isolated DNA and in „heated phages“, respectively. We showed earlier that lesion numbers do not differ significantly in A, B, C and D conformation. Therefore it is likely that the proteins around the DNA can not only protect it, but the pirimidine nucleotide bases near the sites of protein interaction can also become more susceptible for UV lesions. Another possible explanation for increased photodamage is that the dissipation of the absorbed energy along the DNA strand can be blocked by protein binding.

Based on the above results I suggest bacteriophage T7 biological dosimeter and the polymerase chain reaction for its evaluation to be suitable for the study of DNA lesions of different origins, including high-energy UV radiation, ionizing radiation, environmental extremes and genotoxic agents. I demonstrated a practical application of the QPCR method by the analysis of the effects of extraterrestrial parameters on phage and DNA thin layers. As the result of the space simulation measurements preceding the EXPOSE experiments I made the following conclusions:

II.1. The phage and DNA thin films prepared by the centrifugal deposition technique of our research group proved to be suitable for space simulation experiments. The thin films are of good quality and according to spectrophotometry measurements and microscopic pictures they are homogeneously covered. The low amount of scattered light (\(n \approx 1.5–2.8\) instead of the expected \(n \approx 4\)) determined by the empirical fitting of the
Rayleigh-Mie formula can also be explained by the homogene structure of the thin films. The phage and DNA samples are strongly adsorbed to the quartz plates and they are stable even under very low pressure simulating space vacuum.

II.2. Based on the results of vacuum treatment ($10^{-4}-10^{-6}$ Pa) it can be concluded that this kind of extreme parameter is relatively well tolerated by phage and DNA thin films. Vacuum causes dehydration of the samples resulting in conformational alteration of the genetic material – this can be followed by the change in the absorption spectra reflecting „stacking” interactions between nucleotide bases. Both the D conformation at 0% relative humidity and the result of vacuum treatment can be characterized by $\approx 30\%$ hyperchromicity at 260 nm. After equilibration at higher relative humidities the absorption spectra matched the original ones. Our results confirm that strong dehydration causes partial denaturation of the DNA but on thin layers a certain rigid structure is maintained which makes the changes almost perfectly reversible upon rehydration. The amount of permanent damages after vacuum treatment was not significant and constituted mainly of strand breaks and DNA–protein cross-links.

II.3. Our samples maintained their stability during simulated on-board temperature fluctuations expected on the EXPOSE unit. The temperature treatment favoured the denaturation of DNA but did not lead to a significant amount of additional damages.

II.4. After monochromatic UVC (254 nm) irradiation of the phage / DNA thin layers hyperchromicity was observed in the absorption spectra despite of the fact that the dimer photoproducts (CPDs and 6-4 PDs) characteristic for this UV range in solutions cause a decrease in absorbance. This can be explained by the formation of other, denaturing photoproducts (cross-links, strand breaks, spore photoproducts) characteristic mostly for thin layers.

Our results showed a saturation tendency in the total number of lesions determined by QPCR for large doses of UVC (above 30–40 kJ/m$^2$) in the case of both phage and DNA thin layers. This can be explained by a dynamic equilibrium between formation and reversion of photoproducts where the quantum yields for photoreversion are almost two orders of magnitude greater than those of lesion formation. The number of DNA
damages is not increasing above all limits. Assuming the photoinduced reactions are statistical events, during the irradiation the chance for a number of nucleoprotein / DNA molecules to survive the radiation damage can be a reality. On thin layers the relative hypersusceptibility of phages compared to isolated DNA is decreased, probably because the salt crystals present on the thin layers are capable of stabilizing the genetic material to some extent.
II.5. I studied the distribution of some specific types of photodamage after monochromatic UVC (254 nm) and solar simulator (SOL 2000: 200–400 nm) irradiation. In the former case cyclobutane pyrimidine dimers (CPDs) were the major photoproducts (≈61%) but in the presence of other wavelengths (polychromatic UV) this dominance was reduced (≈18%) and other, partly thin layer specific photoproducts were produced in greater amount. This includes DNA–protein cross-links (≈57%) as well as single and double strand breaks (≈11%), the formation of which is favoured by the tightly compacted structure of thin layers. The determined specific lesions gave only ≈85% of all damages, the remaining part can be owed to photolesions not yet studied, where – based on literature data – spore photoproducts might play an important role.

II.6. Simultaneous vacuum treatment and UVC irradiation had a synergistic effect as this was observed by both UV absorption and QPCR. The combined treatment resulted in a greater lesion frequency than expected and the amount of damages can not be simply calculated as the sum of the lesion numbers caused by the components. This draws attention to an important problem of simulation experiments. When simulating real world conditions it is not sufficient to study the effects of isolated parameters, one also has to take into account the possible interactions between them. For example the non-additive nature of high-energy UV radiation (formation-reversion of photoproducts) can not always be neglected. For the sunlight reaching the Earth’s surface the effects of different wavelengths are mostly taken into account additively but under space conditions this simplification can not be justified any more.

II.7. On thicker films of phage / DNA upper layers can act as a shield thus protecting the layers below from UV damage. This protection results in lower lesion frequencies for thicker layers. For example 60 kJ/m² UVC (254 nm) irradiation caused a ≈70% decrease in the amount of the QPCR product for a layer characterized by an OD₂₆₀ of 0.1, however the decrease was only ≈30% for a layer of OD₂₆₀=0.5. The extent of protection is proportional to the layer thickness. Based on the inverse of the initial slope of the QPCR dose–effect curves a correction factor was calculated. This correction factor was used to calculate the average dose throughout the layer in function of the incident dose. Shielding by thicker layers and e.g. mineral material may
result in substantial protection of phage / DNA from extraterrestrial damages.

5. Summary

Human activity is leading to a changing radiation environment, including the extension of ultraviolet radiation to shorter wavelengths. The main target of ultraviolet radiation in the living organisms is the genetic material, therefore it seems reasonable to use the DNA itself as a biological dosimeter. The DNA damage as a first step in the development of skin cancer makes UV radiation the most ubiquitous environmental carcinogenic agent.

The aim of this work was the development and application of a method for the quantitative determination of DNA damages caused by a wide spectrum of UV radiation by polymerase chain reaction (QPCR). QPCR was optimized experimentally for two fragments of bacteriophage T7 DNA. Based on the amount of the PCR product Poisson-equation was used to calculate the average lesion frequency in DNA. The QPCR works also on intact phages and its sensitivity depends on the fragment length used. Routine use of the 555 bp fragment is possible for the determination of large doses, while the 3826 bp fragment is suited for smaller ones. In the case of five various sources of UVA–B–C radiation a good agreement was found between the lesion frequencies determined by QPCR and calculated from biological activity data. This indicates that the QPCR method is capable of detecting practically all UV photoproducts and it can be used for the validation of the phage T7 biological dosimeter. The comparison of total lesion frequencies in intact phage and isolated DNA led to the conclusion that protein binding to DNA can increase its UV sensitivity due to local structural changes, where the protein binding is the decisive factor and not the DNA conformation.

High-energy ultraviolet radiation is also one of the deleterious parameters in the space survival of biomolecules. Our T7 phage / DNA thin layers to be sent to the EXPOSE unit of ISS proved to be suitable for space simulation experiments. The dehydrating effect of vacuum and on-board temperature fluctuation resulted in low phage / DNA lesion frequencies. Interestingly UVC irradiation led to a saturation tendency in lesion formation both in phage T7 and DNA, which can be explained by the photoreversion of dimer photoproducts. A broader spectrum of
extraterrestrial radiation caused a significant amount of other photoproducts than dimers as well. UVC radiation combined with vacuum treatment caused a synergistic effect in phage / DNA damage. However, shielding by thicker layers and e.g. mineral material may result in substantial protection of phage / DNA from extraterrestrial damages.
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ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>6-4 PD</td>
<td>6-4 photodimer</td>
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<td>β</td>
<td>efficiency of host cell repair</td>
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<tr>
<td>CPD</td>
<td>cyclobutane pirimidine dimer</td>
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<td>DNS</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EVT</td>
<td>Experiment Verification Test</td>
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<td>H\textsuperscript{T7}</td>
<td>T7 equivalent dose</td>
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<td>ISS</td>
<td>International Space Station</td>
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<td>OD</td>
<td>optical density</td>
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<tr>
<td>Q / PCR</td>
<td>quantitative / polymerase chain reaction</td>
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<tr>
<td>SDS</td>
<td>sodium-dodecyl-sulfate</td>
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<td>UV</td>
<td>ultraviolet</td>
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7. List of publications

JOURNAL ARTICLES


   \[ \text{IF: 1,314} \]

   \[ \text{IF: 1,929} \]


   \[ \text{IF: 0,548} \]


IF: 1,597


IF: 0,209

PRESENTATIONS AND POSTERS


3. Hegedüs M., Fekete A.: DNS sérülések detektálása polimeráz láncreakcióval, Student Research Conference, Medical and Pharmaceutical Sciences, Semmelweis University, Budapest, 2002


13. Hegedüs M., Módos K., Kovács G., Rontó Gy., Fekete A.: Világűrbeli ultraibolya sugárzás és vákuum hatása T7 baktriofág biológiai dózismerőre, 7th Ph.D. Science Symposium, School of Ph.D. Studies, Semmelweis University, Budapest, 2005


PUBLIC SCIENCE


3. Hegedüs M.: QPCR of T7 DNA in UVC – From where we come and where we go?, Science Essay and Communication Competition of the School of Ph.D. Studies, Semmelweis University and the British Council, 2005, 11-12