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Biological Effects of C_{60} Fullerenes *in vitro* and in a Model System

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With the use of artificial lipid membranes, it is shown that C_{60} fullerenes are capable to penetrate into a lipid bilayer, by locally strengthening its conductivity. C_{60} fullerenes under the UV/VIS and X-ray irradiation do not influence the MTT reduction and the DNA structure in thymocytes. UV/VIS irradiated C_{60} fullerenes give rise to the DNA fragmentation and a decrease of the viability of ascitic Erlich carcinoma cells.

Keywords: ascitic Erlich carcinoma cells; C_{60} fullerene; DNA structure; membrane lipid bilayer; MTT reduction; thymocytes; UV/VIS and X-ray irradiation

INTRODUCTION

The finding of new nanoparticle systems suitable for manipulations at the cell and molecular levels is the urgent problem of bionanotechnology. In this direction, the representatives of a new allotropic form of

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carbon, C_{60} fullerenes, are intensively investigated. The unique physical, chemical, and biological properties [1,2] of these molecules can be exploited in different biological fields. C_{60} fullerenes do not manifest toxic effects in the range of low concentrations [3], have a significant reduction potential, and are able to scavenge active free radicals [4]. Due to a small size and hydrophobicity, the C_{60} molecule can be incorporated into cell membranes [5,6]. Photoexcited C_{60} fullerene is a highly efficient photo-sensitizer, but the kind of active species involved in the biological action of excited C_{60} is still not clear. It is suggested that the direct energy transfer from an excited sensitizer in the triplet state to molecular oxygen generates singlet oxygen [7], while electron transfer reactions between C_{60} in an excited state and radiolytic species derived from irradiated water give C_{60} fullerene radical anion $C_{60}^{\bullet-}$ and superoxide anion $O_2^{\bullet-}$ [8–10].

The application of fullerenes in cancer photo-chemotherapy appears promising. Nevertheless, the mechanisms of the interaction of C_{60} fullerenes with the structural components of cells and the damaging effect of photoexcited C_{60} fullerenes on normal and oncotransformed cells have been scarcely studied. The goal of this work was to investigate the interaction of C_{60} fullerenes with a membrane lipid bilayer in the model system and to assess the effect of C_{60} fullerenes exposed to UV/VIS or X-ray irradiation on the metabolic indices of thymocytes and ascitic Erlich carcinoma (AEC) cells *in vitro*.

MATERIALS AND METHODS

Carbon soot was generated by evaporating spectral carbon rods (Fa. Schunk) in a dc arc at 24 V in a He atmosphere (0.2 bar). The soot was extracted for 6 h in boiling toluene. Undissolved soot particles were eliminated by filtration. The filtrate was then gently warmed under flowing nitrogen to evaporate the solvent. The preparative separation of C_{60} fullerene was performed through flash chromatography on silica gel/activated carbon with toluene as eluent. The C_{60} fullerene fraction has a purity of $>98\%$. For the further separation and analysis, the C_{60} fullerene mixture was dissolved in toluene and fractionated with a preparative high-performance liquid chromatograph (Jasco PU-2086) coupled to a multi-wavelength UV/VIS detector (Jasco UV-2077) and an autosampler. A preparative Cosmosil Buckyprep Packed Column with toluene mobile phase was used. The flow rate amounted to 20 ml/min. The C_{60} fullerene fraction has a purity of $>99.8\%$. A further purification is possible by the sublimation of C_{60} fullerene in a high vacuum.

For the preparation of C_{60} fullerene water solutions, we used a saturated solution of pure C_{60} fullerene (purity $>99.5\%$) in toluene, with the C_{60} fullerene concentration corresponding to the maximum solubility near 2.9 mg/ml, and the same amount of distilled water in an open beaker. The two phases, which are formed, were treated with an ultrasonic bath. The procedure was continued until toluene has evaporated completely, and the water phase became yellow colored. The filtration of the water solution separates the product from undissolved C_{60} fullerene. Different concentrations of C_{60} fullerene in water (from 0.1 to 1.2 mg/ml) are available by this method. The absorption spectra of these products in water and toluene solutions were recorded, indicating that a water-fullerene solution has been formed.

Thymocytes were isolated by the passaging of rat (Wistar line, 120–150 g) thymus through the nylon. The ascite cells were isolated from outbreed mice on 8–12 days after the intraperitoneal transplantation of Ehrlich's adenocarcinoma. The number of cells was calculated in Horyaev's chamber using tripane blue. Cells ($1-3 \times 10^6$/ml) were suspended and incubated in a buffer containing 3 mM Na_2HPO_4, 5 mM KCl, 120 mM NaCl, 10 mM glucose, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4 or in the RPMI medium.

The X-ray irradiation of cell suspensions containing 10^{-5} M C_{60} fullerenes was performed by the use of an X-ray apparatus with the exposure dose of 11.61×10^{-2} C/kg (1.7 Gy).

UV/VIS irradiation of C_{60} fullerene aqueous solutions was performed by the use of a mercury-vapor lamp (24 W) emitting in the wavelength region 320–580 nm with duration 2 min in the glass test-tubes. The concentration of C_{60} fullerenes after the introduction into the cell suspension was 10^{-5} M.

The conductance of a bilayer lipid membrane (BLM) was studied in the joint experiments with Dr. O.Ya. Shaturskyy (Palladin Institute of Biochemistry of the NAS of Ukraine, Kyiv). Planar bilayer membranes were formed by the application of a mixture of phosphatidylcholine and cholesterol (1:1) in heptane across a 0.6-mm-diameter hole in a Teflon cup held within a glass chamber. The internal volume of a Teflon cup was 1 ml, the volume of the outer compartment was 9 ml. The current-voltage characteristics of the transmembrane current were recorded using silver chloride electrodes immersed in a 2 M KCl solution with agar bridges. Electrodes were connected to a high resolution voltage amplifier with a 1-kHz bandwidth. The membrane surface, oriented to the Teflon camera (its polarization potential was defined as zero), was referred as the *trans*-side of the membrane, and the surface, oriented to the outer glass chamber was referred as the *cis*-side. Experiments were curried out at room temperature

(22 to 24°C) in the 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl buffer solution which symmetrically washed the BLM. The polarization potential between the electrodes did not exceed 1.5 mV, the transmembrane currents were monitored at a holding potential of 100 mV using an N307/1 XY recorder (Russia). Fullerenes C_{60} were added to the washing solution at *cis*-side of the BLM.

The MTT (3-[4, 5-dimethylthiazol-2-il]-2,5-diphenyl tetrazolium bromide, "Sigma") test was carried out in the 96-well plates at 37°C [11]. Cells were incubated during 2 h in the RPMI 1640 medium in the presence or absence of C_{60} fullerenes, after that MTT was added and the incubation was continued during 4 h. The mitochondrial dehydrogenase, which is an active component of mitochondrial electron transport chains in viable cells, reduces MTT to a blue formazane product which was determined spectrometrically ($\lambda = 570$ nm).

The DNA fragmentation was estimated by the accumulation of low-molecular DNA fragments, polydesoxyribonucleotides (PDN), in the cells during the 5 h incubation in the presence or absence of C_{60} fullerenes. Incubated cells were lysed in a buffer (10 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.5% Triton X-100), and lysate was centrifugated (15000 g, 20 min). The total DNA content and the PDN content in the supernatant was determined by the Burton reaction [12].

The statistical processing of experimental results was conducted with the use of a "Statistica" program.

RESULTS AND DISCUSSIONS

The interaction of C_{60} fullerenes with the BLM and their influence on the membrane conductivity were investigated in the wide range of C_{60} fullerene concentrations $(10^{-7} - 10^{-5})$ M. The planar lipid bilayer was titrated with different concentrations of the water-soluble C_{60} fullerene at different values of holding transmembrane potentials. After the addition of C_{60} fullerenes to the *cis*-side of the BLM, the membrane conductivity was changed depending on the concentration of C_{60} fulerenes. In the presence of C_{60} fullerenes in low concentrations (10^{-7} and 10^{-6} M), the effect was insignificant. But, with increase in the concentration of C_{60} fullerenes to 10^{-5} M, the BLM conductivity was considerably increased (Fig. 1). The conductance change was not dependent on the applied potential (50–200) mV, so the potential does not play a profound role in the process of C_{60} fullerene-induced conductivity of the BLM. It is suggested that C_{60} fullerenes are build into the BLM, formed ion channels in the BLM, and, as a result, the BLM conductivity is locally increased to the value of 600 pS.

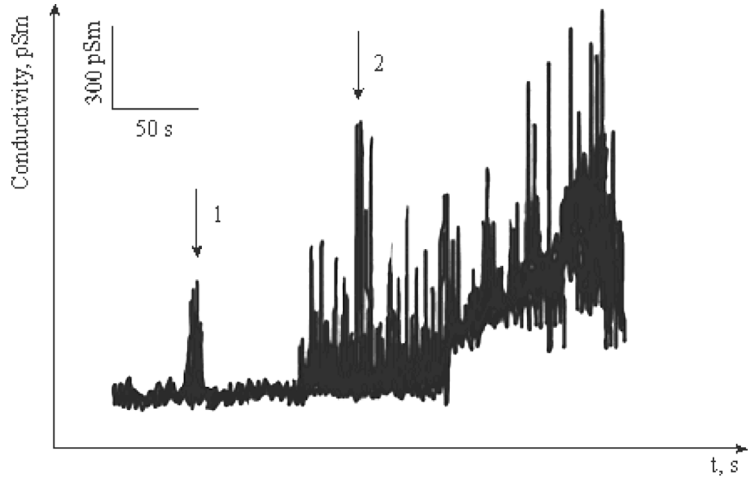

FIGURE 1 Increase in the transmembrane conductance achieved after the addition of C_{60} fullerenes from the *cis*-side of the BLM: $1-10^{-6}$ M; $2-10^{-5}$ M.

The experiments, in which the BLM was further perfused with a washing solution after the application of C_{60} fullerenes at the *cis*-side of the membrane, were carried out to estimate the reversibility of the C_{60} fullerene binding with the lipid bilayer. After a steady increase in the transmembrane current in the presence of 10^{-5} M C_{60} fullerene was achieved, the *cis*-side was washed by 80 ml of a 100 mM NaCl solution. The data obtained show that the absorbed C_{60} fullerenes are retained in the lipid bilayer for a comparatively long period of time (6 min), and then the membrane conductance is instantly restored to the initial level, which was registered prior to the addition of C_{60} fullerenes (Fig. 2). This suggests the reversible binding of water-soluble C_{60} fullerene with the BLM.

Taking into account that the C_{60} fullerene aqueous solution is a hydrophobic colloidal-dispersed system, which contains both the separate molecules of hydroxylated C_{60} fullerene and fullerene aggregates [13], a reversible increase of the lipid bilayer conductance may result from the unspecific partition of a hydrophobic C_{60} fullerene sphere in the matrix of the lipid bilayer.

The lipophilic nature of C_{60} fullerene contributes to its penetration into the cell membrane. In experiments with fluorescent probes incorporated into the bilayer of phosphatidylcholine liposomes, it was shown that C_{60} fullerene is released from the water-soluble complex C_{60}-polyvinylpyrrolidon during the interaction with the membrane of

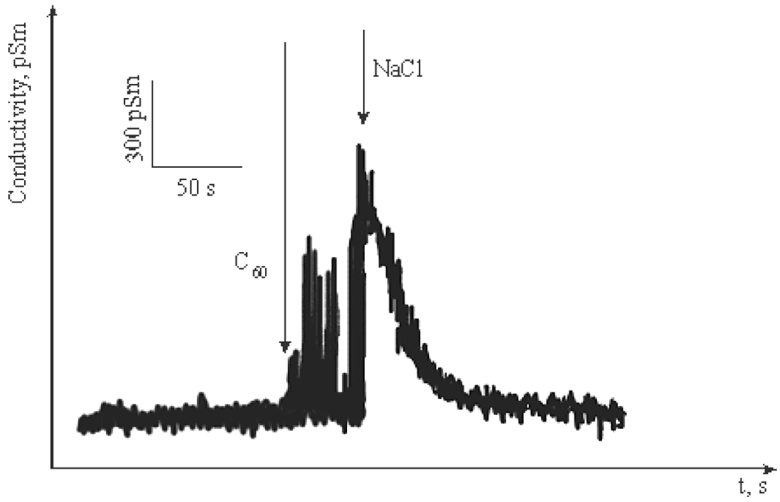

FIGURE 2 Restoration of transmembrane conductance after the wash-out of C_{60} fullerenes from the side of addition.

liposomes and freely penetrates into the membrane [5]. Fluorescent microscopic studies of the subcellular distribution of the water-soluble fullerene derivative $C_{61}(COOH)_2$ with the use of monoclonal antibodies against C_{60} fullerene showed that this compound is capable to penetrate through the plasma membrane inside the cell, where it binds preferably with mitochondria [6].

To estimate the activity of the mitochondrial electron transport chain as the index of cell viability during the incubation, the MTT test was used. MTT was added to the probes in 1 h after the incubation of cell suspensions in the presence of photoexcited C_{60} fullerenes or after the incubation of C_{60} fullerene-containing X-irradiated cell suspensions, and then the rate of MTT reduction was measured.

The rate of MTT reduction by oncotransformed AEC cells was higher than that by thymocytes (Fig. 3). When the cells of both types were incubated in the presence of C_{60} fullerenes, which were not exposed to the photoexcitation or X-ray irradiation, the cell viability was not changed in comparison with that of the control cells incubated in the absence of C_{60} fullerenes (Fig. 3).

When photoexcited C_{60} fullerenes were added to the thymocyte suspension or when the C_{60} fullerene-containing thymocyte suspension was exposed to X-ray irradiation, no changes in the cell viability were observed. But if UV/VIS-irradiated C_{60} fullerenes were added to the suspension of AEC cells, the rate of MTT reduction was decreased

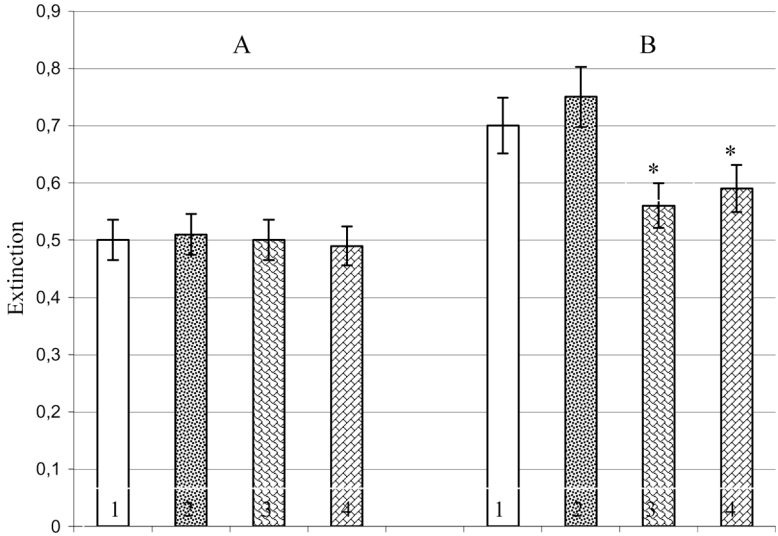

FIGURE 3 MTT reduction in thymocytes (A) and AEC cells (B) incubated: 1 – without additives; 2 – in the presence of C_{60} fullerenes; 3 – in the presence of the UV/VIS-irradiated C_{60} fullerenes; 4 – after the X-ray irradiation in the presence of C_{60} fullerenes ($^{*}p \leq 0.05$ in comparison with control).

almost by 1.5 times (Fig. 3B). The less change of the AEC cell viability was observed when AEC cells were X-ray-irradiated in the presence of C_{60} fullerenes: the MTT test index was decreased by a factor of 1.2.

The data on the influence of C_{60} fullerenes on the accumulation of DNA desoxynucleotide fragments in cells are presented in Figure 4. The level of PDN accumulated during the cells incubation in the absence of additives was taken for 100%.

No essential intensification of the DNA fragmentation in thymocytes in the presence of UV/VIS-excited C_{60} fullerenes or after the X-ray irradiation of these cells in the presence of C_{60} fullerenes was observed. But, the PDN content was increased by 2.5 times in AEC cells, when photoexcited C_{60} fullerenes were added to the incubation medium, and by 1.8 times, when the AEC cell suspension was exposed to the X-ray irradiation. These effects are supposed to be determined by the direct electron transfer from excited C_{60} fullerenes to DNA bases, as well as by the destructive action of hydroxyl radicals formed concurrently with $C_{60}^{\bullet -}$ anions during radiolysis on the DNA polymer structure. The phenomenon of the nucleotidic chain cleavage by photoexcited fullerenes was directly demonstrated only on the plasmids [14]. The DNA photocleavage acts mainly at the guanine site and is

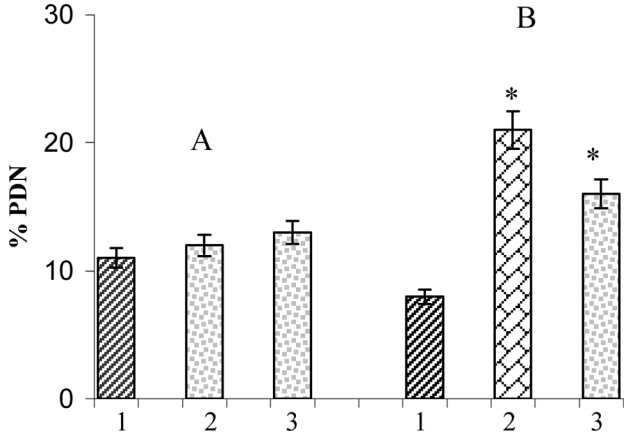

FIGURE 4 DNA fragmentation (in % of PDN from the total DNA content) in thymocytes (A) and AEC cells (B) incubated: 1 – in the presence of C_{60} fullerenes; 2 – in the presence of the UV/VIS-irradiated C_{60} fullerenes; 3 – after the X-ray irradiation in the presence of C_{60} fullerenes ($^{*}p \leq 0.05$ in comparison with control).

assumed to be related to the oxidation of guanosines caused by the generation of singlet oxygen or by the energy transfer from the triplet state of fullerene to bases [15].

But the effects of C_{60} fullerene in biological systems *in vivo* are mediated by the electron transfer between reductants (for example, NADH) [16] and excited C_{60} fullerene which gives fullerene radical anion $C_{60}^{\cdot -}$. The formation of $C_{60}^{\cdot -}$ anion radical is also possible in the reaction of unionized C_{60} with e^- formed by the radiolysis of an aqueous solution under X-ray irradiation [10]. The reaction of $C_{60}^{\cdot -}$ with reduced oxygen species generates superoxide anions $O_2^{\cdot -}$ and more easily diffusing and more active hydroxyl radicals $OH^{\cdot}$ which are capable to damage biological molecules such as DNA, lipids, and proteins [4].

The data obtained demonstrate the damaging effect of irradiated C_{60} fullerenes in cells. This effect is more pronounced after the introduction of photoexcited C_{60} fullerenes into the incubation medium than after the X-ray irradiation of a C_{60} fullerene-containing cell suspension. The deleterious action of photoexcited C_{60} fullerene is specific for oncotransformed AEC cells and is detected both at the level of mitochondrial dehydrogenase systems and the DNA structure. The imbalance of the cell antioxidant potential and the violation of membrane structures under conditions of the hyperproduction of reactive oxygen species (ROS) are assumed to be the initial motive of the

damaging effect. In fact, the direct generation of ROS by C_{60} fullerene in a water solution was detected by the EPR method with the use of spin traps with high affinity to singlet oxygen and superoxide radical-anion [17]. The realization of the mechanisms of the biological activity of C_{60} fullerenes is supposed to be connected with the space-geometric similarity of hydrated C_{60} fullerenes and their fractal clusters [13] to biological structures, in particular, to a clathrate lattice, which always exists in a cell in the hydrated state.

SUMMARY

1) It is shown that, as a result of the building of C_{60} fullerenes into the BLM, the ion channels are formed and the BLM conductivity is locally increased.
2) Introduction of UV/VIS-irradiated C_{60} fullerenes into the medium, as well as the X-ray irradiation of a C_{60} fullerene-containing cell suspension, do not influence the viability of thymocytes during incubation.
3) After the introduction of UV/VIS-irradiated C_{60} fullerenes into the incubation medium of AEC cells, the decrease of MTT reduction and the strengthening of DNA fragmentation are shown.

The revealed effect can be caused by a growth of the electron-transport activity of C_{60} fullerene. So, excited C_{60} fullerenes can accelerate the transfer of electrons from the oxidizing compounds, which affects the rate of the oxidation-reduction processes. With the significant ROS production, the damaging effect of C_{60} fullerenes can be manifested, which is indicated by the above-presented data.

REFERENCES

[1] Dresselhaus, M. S., Dresselhaus, G., & Eklund, P. C. (1996). *Science of Fullerenes and Carbon Nanotubes*, Academic Press: NY.
[2] Wilson, S. R. (2000). *Fullerenes: Chemistry, Physics and Technology*, Wiley: NY.
[3] Prylutska, S. V., Grynyuk, I. I., Golub, O. A., & Matyshevska, O. P. (2006). *DAN Ukr.*, *1*, 163. (in Ukrainian).
[4] Kamat, J. P., Devasagayam, T. P., Priyadarsini, K. I., & Mohan, H. (2000). *Toxicology*, *155*, 55.
[5] Piotrovsky, L. B., Dumpis, M. A., Poznyakova, L. N., Kiselev, O. I., Kozeletskaya, K. N., Eropkin, M. Yu., & Monaenkov, A. O. (2000). *Mol. Mat.*, *13*, 41.
[6] Foley, S., Crowley, C., Smaihi, M., Bon, C., Erlanger, B. F., Seta, P., & Larroque, C. (2002). *Biochem. Biophys. Res. Commun.*, 294, 116.
[7] Hamano, T., Okuda, K., Mashino, T., Hirobe, M., Arakane, K., Ryu, A., Mashiko, S., & Nagano, T. (1997). *Chem. Commun.*, *21*, 22.
[8] Guldi, D. M. & Asmus, K. D. (1999). *Radiat. Phys. and Chem.*, *56*, 449.

[9] Yamakoshi, Y. N., Yagami, T., Fukuhara, K., Sueyoshi, S., & Miyata, N. (1993). *J. Chem. Soc. Chem.*, *Commun.*, *59*, 517.
[10] Lu, C. Y., Yao, S. D., Lin, W. Z., Wang, W. F., Lin, N. Y., Tong, Y. P., & Rong, T. W. (1998). *Radiat. Phys. and Chem.*, *53*, 137.
[11] Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., & Mitchell, J. B. (1987). *Cancer Res.*, *47*, 936.
[12] Burton, K. (1956). *Biochem.*, *62*, 315.
[13] Scharff, P., Risch, K., Carta-Abelmann, L., Dmytruk, I. M., Bilyi, M. M., Golub, O. A., Khavryuchenko, A. V., Buzaneva, E. V., Aksenov, V. L., Avdeev, M. V., Prylutskyy, Yu. I., & Durov, S. S. (2004). *Carbon*, *42*, 1203.
[14] Tokuyama, H., Yamago, S., Nakamura, E., Shiraki, T., & Sugiura, Yu. (1993). *J. Am. Chem. Soc.*, *115*, 7918.
[15] Prat, F., Hou, C.-C., & Foote, C. (1997). *J. Am. Chem. Soc.*, *119*, 5051.
[16] Tabata, Y. & Ikada, Y. (1999). *Pure Appl. Chem.*, *71*, 2047.
[17] Burlaka, A. P., Sidorik, E. P., Prylutska, S. V., Matyshevska, O. P., Golub, A. A., Prylutskyy, Yu. I., & Scharff, P. (2004). *Experimental Oncology*, *26*, 326.