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ORIGINAL RESEARCH PAPER

BIOMEDICAL MATERIALS BASED ON FULLERENES♦

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Abstract: Fullerenic soluble derivatives are essential for many biomedical techniques that exploit the chemical and physical properties of these unique structural carbon nanospheres. Their toxicity, demonstrated *in vitro* and *in vivo* is important for the characterization and the limitation of these applications. The photo toxicity of some fullerene molecules has been identified as a future therapeutic tool. The specific objective of the study is the synthesis of C60 derivatives. Starting from the characteristics of fullerene compounds, we tried to study *in vitro* C60 fullerene and some functionalized derivatives (C60 complexes with poly-vinyl pyrrolidone (PVP) and with the oxo-dimer (Fe-O)2TPP), experimental models *in vitro* with normal and tumor cells and investigation of their toxicological profile, in order to identify novel anti-neoplasic therapeutic devices.

Keywords: *biomedical activity, biomedicine, characterization, fullerene materials, synthesis*

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INTRODUCTION

Starting from the characteristics of fullerene compounds, we tried to study in vitro C60 fullerene and some functionalized derivatives (C60 complexes with PVP (poly-vinyl pyrrolidone) and with the oxo-dimer $(Fe\text{-}O)_2TPP$), experimental models *in vitro* with normal and tumor cells and investigation of their toxicological profile, in order to identify novel anti-neoplasic therapeutic devices.
The compounds were characterized using UV-VIS and IR spectroscopy as well as considering their biomedical applications.

EXPERIMENTAL

Materials

Free-base tetraphenylporphyrin (TPP) was synthesized in our lab, after own proper method [1 – 3]. UV-Vis (nm, log ε) in benzonitrile: 415(5.45), 511.5(4.15), 544 (3.82), 589(3.71), 642(3.59).
TPP-PVP-C60 complex have been synthesized in liquid phase by evaporation of a combined solution TPP (99.9%)/PVP and C60 (99.95%) and in toluene : chloroform = 1:1 with a molar ratio 1:6 at 150 °C, as previously reported [3].
For the synthesis of TPPFeCl we have used 0.0270 g $FeCl_3$ (in 50 mL glacial acetic acid), 1 mL benzaldehyde and 0.7 mL pyrol. After the synthesis it was obtained a black-purple precipitate.
Oxo-iron-porphyrin dimmer was obtained as following: after dissolving 0.5 g in 60 mL $CHCl_3$ TPPFeCl (reflux one hour) were added to 50 mL 25% KOH. The complex $(TPP\text{-}Fe)_2\text{-}O$ was separated by column chromatography on alumina and eluted with chloroform.

Apparatus

A M400 Carl Zeiss Jena UV spectrophotometer with a 1 nm slit width, 1 nm step size, 0.3 $nm.s^{-1}$ average scan rate, deuterium lamp, and quartz cell was used to measure the aqueous solution absorbance and the molar absorption spectra for each sample. The spectrophotometer has been connected to an external computer for data processing.
The FT-IR spectra were recorded on a FT-IR GX Perkin Elmer, working domain 4000 - 400 cm^{-1}, Dynascan interferometer, DTGS detector, equipped with ATR (Attenuated Total Reflectance) device.

Cells

K562 lymphoblastic human cell line (supplier ATCC Nr. CCL-243) derived from chronic leukemia [4] was used. Cells were maintained in culture or cryopreserved as stated by the supplier. In all the PDT experimental systems the cell line was used in RPMI1640 complete medium without phenol red in order to avoid the interference with the detection methods.

Biological tests

The loading was performed at various incubation time, cell concentration and photo sensitizer concentration. The following experimental conditions have been established: incubation time 1 – 48 h, the photosensitizers loaded in the 5 – 250 $\mu g.mL^{-1}$ range and the cell suspension in the $(0.05 \div 1.5) \times 10^6$ $cells.mL^{-1}$ concentration range. The loading degree was indirectly measured by spectra registration of cellular supernatants by means of recording absorption spectra for each of them (supernatant and lysate).

Irradiation

Cell suspensions, pre-incubated for 24 h with TPP-C60-PVP, were subjected to irradiation after washing them 3 times in culture medium and resuspended at 2×10^5 $cells.mL^{-1}$ in RPMI1640 culture medium without phenol red or FCS (fetal calf serum). The cell suspensions irradiation was performed with a 450-W Hanovia medium pressure Hg lamp in quartz well equipped with an UV39 filter (436 nm filter). Samples were placed in a quartz cuvette. Exposure times necessary to achieve UV fluences from 0 to 1000 $mJ.cm^{-2}$ at fluence rates of 20, 100 or 200 $mW.cm^{-2}$, were determined from the average irradiance as calculated with a spreadsheet program using the lamp spectrum, solution absorbance, the incident irradiance read from a calibrated radiometer (IL1700, SED 240/W, International Light, Peabody, MA). Light flux was measured in terms of irradiation at wavelengths which correspond to absorbance bands of the sensitizer. After irradiation, the cells were washed again twice in RPMI1640 culture medium without phenol red with FCS for removal of cell debris generated during irradiation.

Cell viability

Cell membrane integrity was assessed using Cytotox96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation) [5], a test that quantifies lactate dehydrogenase (LDH) release by damaged cells. LDH release was presented as LDH index = OD 490 nm of cells in presence of compound/OD 490 nm of control cells. Trypan Blue exclusion test was performed in order to establish the percentage of dead cells. Cell viability results were expressed as index (viability index = cell viability in presence of compound/cell viability of control).

Cell proliferation

The capacity of tumoral cell line to proliferate was assessed as number of metabolically active cells, using the CellTiter 96AQueous One Solution Cell Proliferation kit (Promega Corporation) [6]. The test measures the activity of intracellular dehydrogenases based on tetrazolium salt reduction to a spectrophotometrically quantifiable soluble formazan compound. Results are expressed as index (MTS index = OD 490 nm of cells in presence of compounds/OD 490 nm of control cells).

RESULTS AND DISCUSSION

The electronic absorption spectrum of TPP is dominated by the very strong Soret band located at 428 nm. The symmetrical Soret band suggests an absence of molecular aggregates observed. Also, in the absorption spectrum of TPP, could be observed in the region 500 – 700. By coupling TPP with PVP and C60, the very narrow and symmetrical Soret absorption band in absorption spectrum is bathochromically shifted to 437 nm, suggesting an electronic interaction between the porphyrin and fullerene moieties. The Q bands (from 500 to 700 nm) in this triad also undergoes medium bathochromic shift by about 9 nm [3]. All these experiments have been achieved in $CHCl_3$: toluene (1:1) (Figure 1).
Because in DMSO : water (the solvent used for biological tests in the following ratio: 0.05% - 99.95%), TPP exists in dicationic form, it can interact electrostatically (with the positive charged nitrogen of the pyrrolidone ring) with the negative oxygen atom (lactam carbonyl group) from PVP. Taking into account all these data, we presume that the possible structure of the TPP-PVP-C60 triad is the one presented in Figure 2.

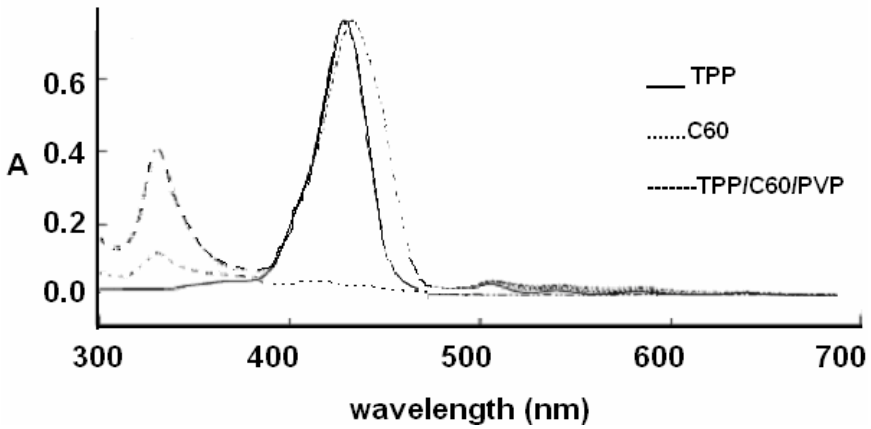

Figure 1. *UV-Vis spectra of C60(……), TPP(____) and TPP-C60-PVP (-----)*

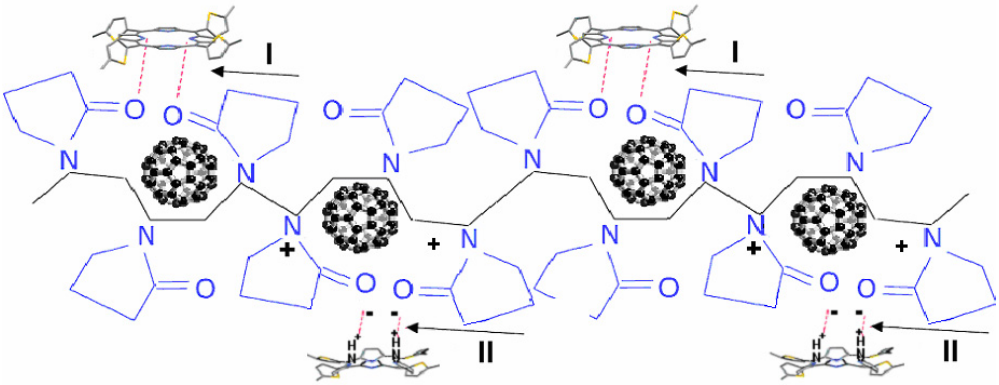

Figure 2. *The structure of TPP/PVP/C60 [3]*

In Figure 3 is presented the FT-IR spectra of the triad, as well as those of the precursors. The proposed structure and the UV-Vis characterization for the complex C60-(TPP-$Fe)_2$-O are presented in Figures 4 and 5.

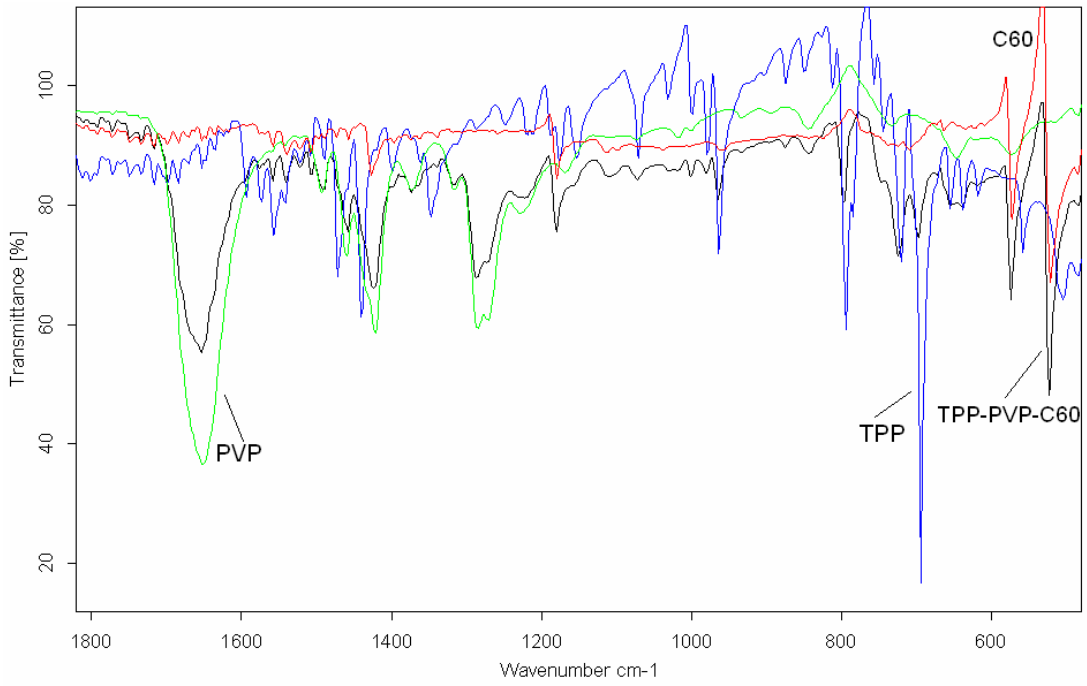

Figure 3. *FT-IR spectra of PVP (green), C60 (red), TPP (blue) and triad (black)[3]*

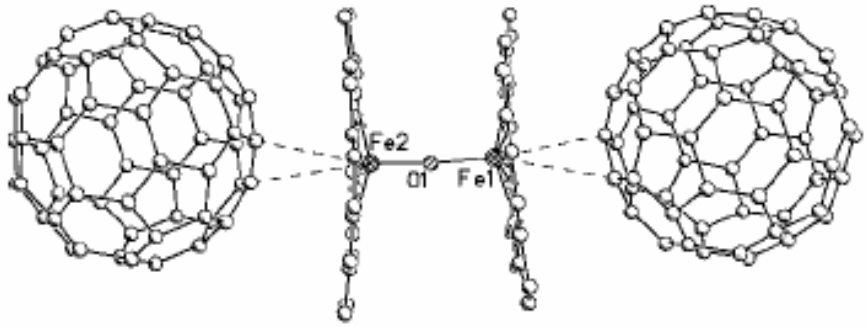

Figure 4. *The proposed structure of C60-(TPP-Fe)$_2$O*

Considering that the long range purpose of the study is the biomedical application of the compound, the *in vitro* cytotoxicity profile is obviously an important start point. In the 0.05 – 50 μM TPP-C60 concentration range, the LDH release from metabolically active K562 cells incubated for 1-6-18 h displayed the same pattern (Figure 6). We have obtained at higher than 5 μM an increase of the LDH release compared to controls while concentrations above 25 μM kill over 50% of the cells. For studying the loading efficiency we have detailed the lower than 5 μM concentration namely 0.25 – 1 μM range, using 1 – 18 h incubation time.

Cells were efficiently loaded with the 0.5 μM concentration, thus for irradiation we have chosen the mentioned concentration.

The actual cell counting showed that after irradiation we recover a mean of 20% when cells were loaded for 18 h compared to an 80% in unloaded and irradiated controls (Figure 7) [3].

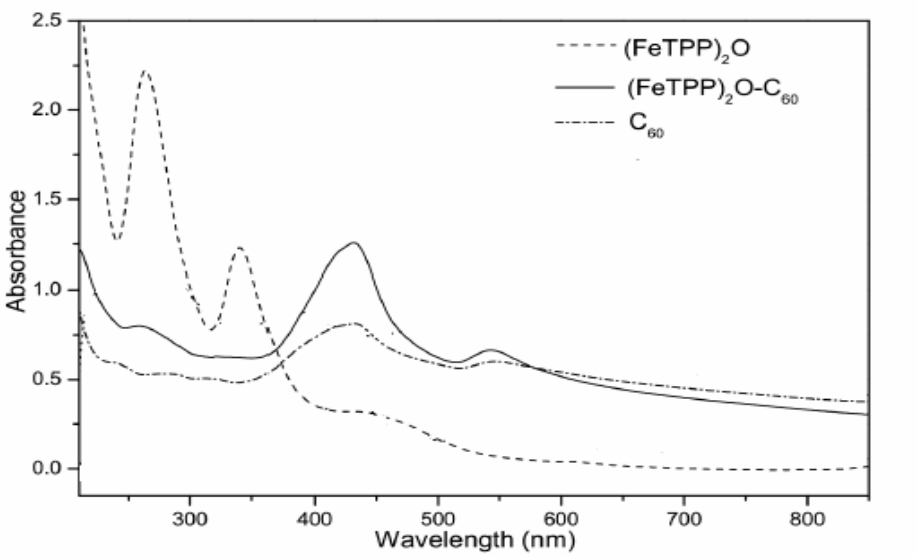

Figure 5. *UV-Vis spectra of C60,(TPP-Fe)$_2$-O and C60-(TPP-Fe)$_2$-O*

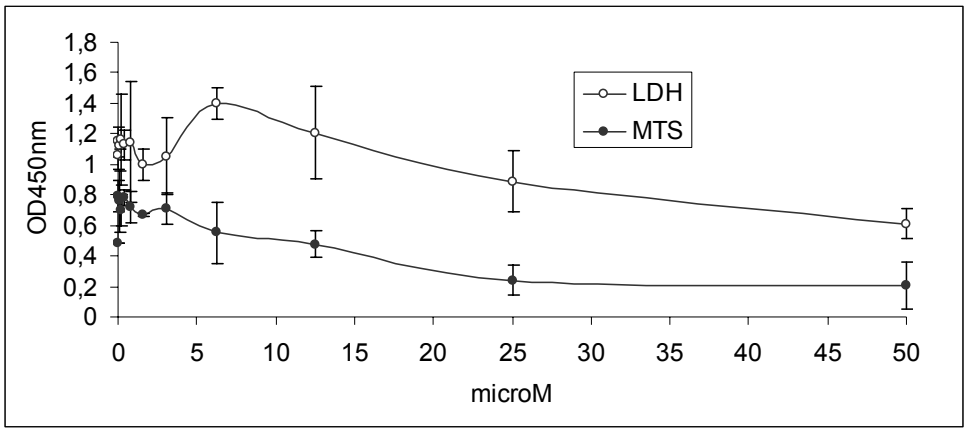

Figure 6. *LDH release from metabolically active K562 cell line (MTS) in presence TPP-C60 in non-irradiated protocols represented as OD 450 nm (mean ± SD of 6 individual experiments with triplicates/sample) [3]*

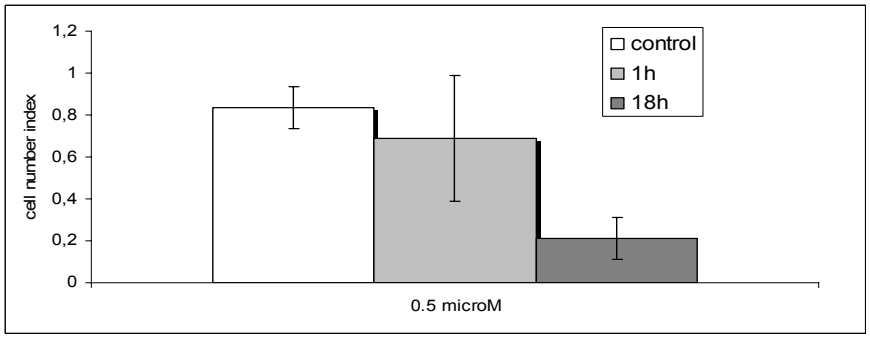

Figure 7. *Cellular number decrease in K562 cells loaded with 0.5 μM TPP-C60 after 1 and 18 h of incubation subjected to irradiation represented as index = cell number sample/cell number control (mean ± SD of 5 individual experiments with triplicates/sample)*

LDH has a high specific activity among glycolytic enzymes in tumor cell lines, whereas proliferating or growth arrested and it is co-localized with other components in the mitochondria [7]. We have used LDH testing not only as a marker for cellular membrane damage upon irradiation but as well as an indicator for mitochondrion integrity. We have obtained that, while cells after 1 h incubation in 0.5 μM subjected to irradiation have statistically the same LDH release and intracellular content (Figure 8) after a longer incubation cells display a clear increase in the LDH release while the pool of intracellular LDH diminishes.

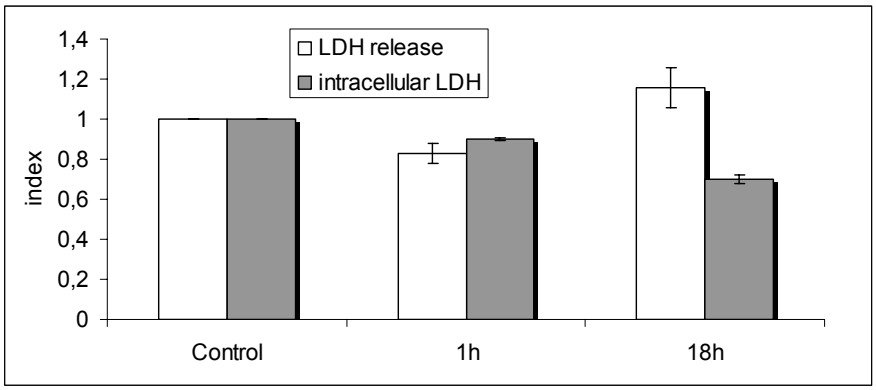

Figure 8. *LDH content and LDH release from K562 cells loaded with 0.5 μM and subjected to irradiation represented as index = OD 490 nm sample/OD 490 nm control (mean ± SD of 5 individual experiments with triplicates/sample)*

In Figure 9 are presented the viability and proliferation capacity of tumor line (K562) traced at different intervals in the presence of C60-$(TPP\text{-}Fe)_2$-O. Values are expressed as OD×1000.

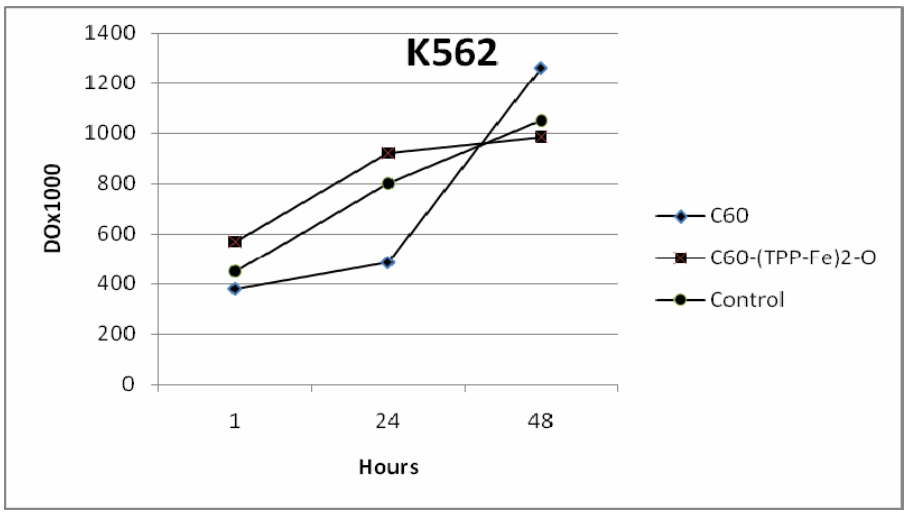

Figure 9. *Viability and proliferation capacity of C60, C60-$(TPP\text{-}Fe)_2$-O and control on K562 cell line*

CONCLUSIONS

New materials based on fullerenes (C60-$(TPP\text{-}Fe)_2$-O, C60-PVP-TPP) have been synthesized, analytically characterized and their biomedical effects studied.
The results are promising, allowing the use of compounds synthesized in the photodynamic therapy of cancer.
The presented dyad compound complexed with polyvinylpyrolidone displays on a wide range of concentration "dark" non-toxicity profile. We have shown induces apoptosis but not caspase-3 mediated. Due to its anti-tumoral activity, the TPP-C60 can be further developed as a more efficient drug delivery system in photodynamic therapy approaches.

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