

BIOMEDICAL POTENTIAL FULLERENES (C60)

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Abstract

The most abundant form of fullerenes family is buckminsterfullerene (C60) with 60 carbon atoms arranged in a spherical structure. The unique physical and chemical features of C60, have recently incited a considerable hope of its possible use in various fields of biomedicine. Many fullerene-based compounds with different biological targets have been synthesized, displaying a range of biological activities potentially useful in anticancer or antimicrobial therapy, cytoprotection, enzyme inhibition, controlled drug delivery and contrast- or radioactivity-based diagnostic imaging. The purpose of our study is the in vitro analysis of a novel compound – tetraphenyl-porphyrin-polyvinylpyrrolidone-C60 fullerene (TPP-PVP-C60) – upon K562 leukemia cell line, in order to determine the toxicological outline of this modified fullerene and to evaluate its candidature for a potential photosensitizer agent. The triple complex - PVP+C60+tetraphenylporphyrin was synthesized in this paper.

Key words: copper artefacts, archaeometallurgy

1.INTRODUCTION

From prehistory till nowadays, the modification of metal object surface is an important technological Fullerenes, the large carbon cage molecules considered to be three-dimensional analogues of benzene, represent a third carbon allotrope [1]. The most abundant form of fullerenes is buckminsterfullerene (C60) with 60 carbon atoms arranged in a spherical structure (Fig. 1). The shape of the molecule, known as truncated icosahedron, resembles that of a soccer ball, containing 12 pentagons and 20 hexagons, in which every carbon atom forms bond to three other adjacent atoms through sp² hybridization [1,2]. There are two types of bonds in the fullerene: C5–C5 single bonds in the pentagons and C5–C6 double bonds in the hexagons (Fig. 1). The unique physical and chemical features of C60, the most representative member of the fullerene family, have recently incited a considerable hope of its possible use in various fields of biomedicine.

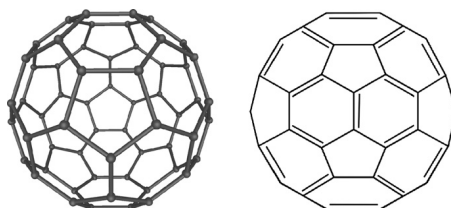


Figure. 1. The structure of C60.

The potent ability of fullerenes to photosensitize transition of molecular oxygen to highly reactive ROS makes them promising candidates for the photodynamic killing of cancer cells. The main advantage of this therapeutic approach is selectivity, achieved by tumor-specific activation of photosensitizing agent by highly focused light beam delivered to tumor region at the surface of the body or to internal tumors using optical fibers [3]. There are many studies demonstrating the efficient photodynamic action of various water-soluble C60 derivatives against different types of cultured cancer cell lines (cervical, larynx, lung and colon carcinoma) and malignant tumors in vivo [4]. A particularly promising approach involves linkage of fullerenes with other photosensitizers, such as porphyrin, exploiting the unique photophysical and redox properties that endow these C60–porphyrin dyads with extremely high capacity for ROS-mediated cytotoxicity even in the relative absence of oxygen [5]. The observed anticancer activity of fullerene derivatives was apparently dependent on generation of both singlet oxygen and superoxide anion [6, 7], and it was inversely correlated with the extent of derivatization of the fullerene cage [8,9]. The latter is consistent with the reduction of the fullerene's ROS-generating capacity that occurs upon increasing the number of covalently attached functional groups [10]. Moreover, a closer examination of the structure–activity relationship reveals that C60 derivatives containing more potent $^1\text{O}_2$ -quenching groups (e.g. –OH) display lower photodynamic activity compared to those containing the same number of groups with inferior $^1\text{O}_2$ -quenching ability (e.g. –CH) [48]. This agrees with the assumption that overall ROS production by a C60 derivative is in part determined by the ability of its functional groups to deactivate C60-generated ROS.

However, some sugar-pendant derivatives displayed different photodynamic efficiencies despite similar production of $^1\text{O}_2$ [11], while tris-malonic acid C60 was more photocytotoxic than monoadduct in spite of the higher $^1\text{O}_2$ quantum yield for the latter [12]. These data suggest that, in addition to $^1\text{O}_2$ -producing capacity, other factors, such as degree of cell membrane incorporation and cellular uptake, might profoundly influence the phototoxicity of C60-based agents.

The photodynamic antitumor action of water-soluble C60 derivatives apparently involves induction of the “programmed” cell death, known as apoptosis [9]. This type of cell demise is characterized by activation of the caspase enzyme family and fragmentation of DNA, which occurs without plasma membrane breakdown and is followed by recognition and removal of apoptotic cell by phagocytes in the absence of inflammation [13].

This is consistent with the preferential mitochondrial localization of water-soluble C60 derivatives [14], having in mind that ROS induced mitochondrial dysfunction is a key initial step in the “mitochondrial” pathway of apoptosis [15]. Interestingly, polyhydroxylated C60 was able to suppress proliferation and induce apoptosis of tumor cells in the absence of photosensitization and ROS production [16,17]. In view of the involvement of redox sensitive transcription factors, such as NF- κ B, in regulation of cell growth and apoptosis [18], these results indicate an interesting possibility that C60 could exert its antiproliferative/pro-apoptotic action not only by producing cell-damaging ROS, but also through antioxidant effects.

Unlike water-soluble C60 derivatives, C60 nanoparticles prepared by addition of conventional surfactants (e.g. SDS, Tween) or polymers (e.g. PEG, PVP) have been only sporadically tested for their photodynamic activity against cancer. This might seem somewhat surprising, as non-derivatized C60 displays higher $^1\text{O}_2$ quantum yield in comparison with functionalized water-soluble derivatives [19], so it should be a more efficient photosensitizer. Moreover, the relatively large size of these C60 nanoparticles (up to several hundreds of nm) should presumably provide high intratumor concentration through “enhanced permeability and retention” effect [20], due to abnormally large vascular pores and impaired lymphatic drainage in tumors. Indeed, PEG/C60 conjugate exhibited higher

accumulation and more prolonged retention in the tumor tissue than in normal tissues, showing a stronger tumorsuppressive photodynamic effect than conventional photosensitizer [21].

In addition to killing tumor cells, C60 could be also used for the photodynamic inactivation of bacteria, as convincingly demonstrated in studies examining the effects of water-soluble and nanoparticulate C60 on various bacterial strains [22]. The effects were significantly more pronounced in Gram positive (*Staphylococcus* spp., *Streptococcus* spp.) than in Gram negative bacteria (*Klebsiella* *Pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Streptococcus pyogenes*) [23], indicating that the bactericidal action was dependent on the fullerene insertion into the microbial cellwall, the structure of which differs between Gram positive and Gram negative bacteria. Accordingly, the antibacterial action of C60 nanoparticles was associated with alterations in membrane lipid composition and membrane fluidity in response to oxidative stress [24], and cationic C60 derivatives were more effective than neutrally or negatively charged ones, presumably as a result of the stronger interaction with bacterial cell wall [25]. Moreover, some bis-cationic and tris-cationic fullerenes performed significantly better than classical antimicrobial photosensitizer toluidine blue, under conditions in which mammalian cells were comparatively unharmed [26]. Fullerenes can also serve as building blocks in the solid phase peptide synthesis preparation of antibacterial peptides, resulting in production of fulleropeptides with higher antimicrobial activity than parental compounds [27]. In addition to antibacterial effects, both pure sonicated C60 and its derivatives have been reported to inactivate viruses belonging to *Togaviridae*, *Rhabdoviridae* and *Flaviviridae* families, as well as some bacteriophages, through either photodynamic ROS-dependent or ROS-independent actions [28-30]. One of the potentially useful ROS-independent actions is the inhibition of HIV replication through incorporation of specifically tailored C60 derivatives into the active site of HIV protease [31].

Photodynamic therapy (PDT) of malignant tumours is a new technique for treating cancers. Photodynamic therapy (PDT) is a cancer treatment based on the accumulation in malignant tissue of a photosensitiser with low systemic toxicity. Subsequent illumination induces a type II photochemical reaction with singlet oxygen production that results in destruction of biomolecules and subcellular organelles. After systemic administration porphyrins bind to albumin and lipoproteins. Accumulation occurs mainly in tumours and organs of the reticuloendothelial system. Light distribution can be assessed by using a radiation transport model and tissue optical properties, or direct measurement with light detectors. The effects of PDT depend in a complex way on: characteristics, tissue concentration and localisation of the photosensitiser; the target tissue optical properties and oxygenation; activation wavelength, power density and treatment method [31].

The lethal, acute, direct effects of PDT on tumoral cells and even normal cells depend on: type, dose and location of the photosensitizer on/or in the cell, the photodynamic efficiency of the substance in the environment, parameters of irradiation and oxygen transport. Different photosensitizers have been tested in human tumor tissues.

Currently, an extensive array of nanostructures based on allotropic forms of carbon, such as nanotubes, nanohorns and nanodiamonds, are now under investigation due to their promising application in the biomedical field [32]. Among these compounds, fullerenes represent a model of carbon-based nanoparticles (C60...C200) who have attracted considerable interest in the last years due to their distinctive properties [33]. Researches in this topic have shown that fullerenes can exhibit antioxidant and antimicrobial activities [34] and development of fullerenes derivatives is a current strategy designated to increase their hydrosolubility, biocompatibility, and their *in vivo* metabolic stability [35]. Some functionalized fullerenes with appropriate surface modifications and sizes, could contribute to antineoplastic chemotherapy efficacy displaying a low toxicity upon administration [36]. Once entered into the cells, it seems that mitochondria are a favored intracellular target of fullerenes

structures. Recently some water-soluble fullerene C60 derivatives have been proposed as a new type of efficient photosensitizers [37]. Dyads like porphyrin-C60 can efficiently generate $^1\text{O}_2$ under certain conditions and give a prolonged activated radical ion pair on photoexcitation. These particular photophysical properties of such dyads could open new opportunities in tumor treatment with promising application in photodynamic therapy of cancer. PVP is a dispersant used in cosmetics and medicines. From this point of view, the purpose of our study is the *in vitro* analysis of a novel compound – *tetraphenyl-porphyrin-polyvinylpyrrolidone-C60 fullerene* (TPP-PVP-C60) – upon K562 leukemia cell line, in order to determine the toxicological outline of this modified fullerene and to evaluate its candidature for a potential photosensitizer agent. The triple complex - PVP+C60+tetraphenylporphyrin was synthesized in this paper.

2. EXPERIMENTAL

2.1. Fullerenes compounds

Poly(vinylpyrrolidone)(PVP)-tetraphenylporphyrin(TPP)-C₆₀-complexes in DMSO:water have been studied at 293 K.

C60-toluene mixed with PVP-chloroform, solvents evaporated, and residue dissolved in water [C60] obtained was 400 µg/mL, using 100:0.8 PVP:C60 w/w. At this compound has been added TPP in toluene, mixed for 45 minutes at 45° C by ultrasonication, filtered, washed and dried.

For the analysis the method of reversed - phase high-performance liquid chromatography (HPLC) was used. Conditions of the analysis: Column: 4 mm x 25 cm, Hypersil C18.5 mem; Eluent: 44% acetonitrile in toluene, mode - isocratic; Sample: 50 µl (for a sample 1 - saturated solution in toluene); Flow: 0.6 ml/min; Detector: photometry on 350 nm. Poly(vinylpyrrolidone)(PVP) tetraphenylporphyrin (TPP)-C60-complexes in DMSO:H₂O has been studied at 293 K.

2.2. Cell culture

K562 lymphoblastic human cell line (supplier ATCC Nr.CCL-243) derived from chronic leukemia [15] was used. Cells were maintained in culture or cryopreserved as stated by the supplier. Briefly, the cells were cultivated in RPMI1640 with phenol red supplemented with 4 mM L-glutamine, 1.5 g/L NaHCO₃ and 10% fetal calf serum (FCS) at 37°C in 5%CO₂ atmosphere at a cell concentration of 1-2x10⁵ cells/mL. In 48-72h cells quadruple their number and the culture medium is changed, cells being reseeded at 1-2x10⁵ cells/mL [38]. All the tests were performed in the exponential growth phase of the cell line. Cryopreservation was performed as stated by the supplier and before utilization cells were maintained in culture at least 3 consecutive passages. 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.

2.3. Photosensitizers loading

The loading was performed at various incubation time, cell concentration and photosensitizer concentration. The following experimental conditions have been established: incubation time 1-48h, the photosensitizers loaded in the 5-250 µg/mL range and the cell suspension in the 0.05 – 1.5 x 10⁶ cells /mL concentration range. The loading degree was tested by flow-cytometry registering the cell suspension's mean fluorescence on FL3 and results are presented as indexes compared to unloaded cells.

2.4. Cell viability

Cellular viability was assessed as cell membrane integrity with Cytotox96 Non-Radioactive Cytotoxicity Assay kit (Promega) [39], test that quantifies the lactate dehydrogenase (LDH) release from intact cells. Trypan Blue exclusion test was also used in order to establish the non-toxic concentration of the photosensitizer. Cell viability tested with Trypan Blue exclusion test matched perfectly with the pattern of cell membrane integrity (LDH) dependency on concentration. Results are expressed as indexes compared to control (LDH index).

2.5. Cell proliferation

The capacity of tumoral cell line to proliferate was assessed as number of metabolically active cells with CellTiter 96Aqueous One Solution Cell Proliferation kit (Promega) [40]. The test measures the activity of intracellular dehydrogenases based on tetrazolium salt reduction to a spectrophotometrically quantifiable formazan compound. Results are expressed as indexes compared to control (MTS index).

2.6. Activation

Cell suspensions incubated for 24h in TSPP compounds were subjected to irradiation after washing them 3 times in culture medium for photosensitizers excess removal and resuspended at a 2.0×10^5 cells/mL in RPMI1640 culture medium without phenol red or FCS. The cell suspensions were irradiated at different laser wavelengths, irradiation time, and, after optimization, the parameters were $\lambda = 632.8$ nm, He-Ne laser (30 mW, 25°C, total irradiation time = 30 min, in O₂ saturated solution). 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.

2.7. Absorption and Fluorescence Spectrophotometry

A Carl Zeiss Jena M400 spectrophotometer was used for absorption spectra registration. Fluorescence spectra were obtained using a Perkin-Elmer LS 50B luminescence spectrometer using excitation wavelengths near the maximum absorbance for each photosensitizer and solvent. All solutions were measured at room temperature in 1 cm or 1 mm pathlength quartz cuvettes. Absorption spectra, fluorescence spectra and fluorescence lifetimes were taken for all solutions prepared in Water and diluted in Dulbecco's modified Eagle's medium (DMEM) with 1% FBS just before use.

Fluorescence microscopy was performed on an optical scatter imaging microscope platform, which was also fitted with an epifluorescence modality, using an inverted microscope (Eclipse T200, Nikon) with epifluorescence. A 10-nm band-pass interference filter placed in the condenser housing yielded an incident red beam centered at $\lambda = 630$ nm (V2A filter).

2.8. Fluorescence microscopy

Labeled cells were observed in situ either on the optical scatter imaging microscope platform, which was also fitted with an epifluorescence modality, using an inverted microscope (Eclipse T300, Nikon) with epifluorescence, or on a Nikon T600 confocal laser scanning microscope to monitor fluorescence emission. A 10-nm band-pass interference filter placed in the condenser housing yielded an incident red beam centered at $\lambda = 630$ nm. The images were collected with an immersion objective and displayed on a charge-coupled device camera. Data acquisition and image analysis for each specimen studied, collected 20–30 s apart, and were acquired manually switching the diameter of the variable iris.

2.9 Transmission electron microscopy (TEM)

For conventional TEM, control and subjected to PDT, K562 cells were sedimented and immediately fixed with 2.5% buffered glutaraldehyde. They were then postfixed with 1% OsO₄ in 0.1M cacodylate

buffer, included in agar, ethanol dehydrated and then processed for Epon 812 embedding at 60°C for 48 hours.

The ultra-thin sections were cut using a LKB ultramicrotome with a diamond knife and double stained with uranyl acetate and lead citrate. Formvar coated grids were used as thin section supports. Electron microscopy examination was performed with both a Philips 301 transmission electron microscope at 60 kV.

2.10. Data analysis

All data and images processing was done using Image J software (free software). All images of single cells were extracted from larger images. Each single cell image was processed to visualize and quantify the photosensitizer molecules. For rectangular selections, the x-axis represents the horizontal distance through the selection [41].

2.11. Cellular photodegradation kinetics

It were registered during irradiation with an UV-VIS spectrophotometer (Carl Zeiss Jena) with double beam, and connected to an external computer for data processing. The cells number were evaluated by means of maximum absorption at 275 nm, characteristic for DNA and cellular proteins [19] and fitted with a mathematical model [42]. A time dependency of $\log(\text{No}/\text{N})$ was plotted, representing the decrease in the actual number of intact cells during the irradiation period. As cellular controls, depending on the experimental approach, we have used cell suspensions loaded with the photosensitizers and not subjected to PDT or unloaded cells subjected to irradiation.

2.12 Irradiation protocol

Cell suspensions, pre-incubated for 1h-18h with TPP-C60, were subjected to irradiation after washing them 3x in culture medium and resuspended at 3×10^5 cells/mL. The cell suspension irradiation was performed with a polychromatic lamp in a quartz cuvette. Exposure time varied to 30 minutes. Cellular photodegradation kinetics were recorded during irradiation with an UV-VIS double-beam spectrophotometer (Carl Zeiss Jena), connected to an external computer for data processing. Cell numbers were evaluated by means of maximum absorption at the band located at 275 nm, and fitted with a mathematical model. The absorption of UV in this region in a fixed volume of solubilized cells is proportional to the cell number, and therefore can be used as a simple means of obtaining a cell count. Cell counts obtained in this way can be combined with measurements of the inhibition of DNA synthesis ([³H]-thymidine incorporation) by test compounds, to produce an index of cytotoxicity. A time dependency of $\log(\text{No}/\text{N})$ was plotted, representing the decrease in the actual number of intact cells during the irradiation period, where No = initial number of cells and N = number of cells at a given time point. As cellular controls, we have used unloaded cells suspensions subjected to irradiation.

After irradiation, cell suspensions were washed twice for removal of cell debris generated during irradiation. After PDT, cell suspension was recorded in the Counting Chamber (Roth) with 0.4% Trypan Blue Stain and the actual decrease in the number of cells subjected to PDT was presented.

2.13 Lactate dehydrogenase

LDH quantification was used for assessing both cell membrane integrity and cellular metabolism during experimental procedures. Cytotox96 Non-Radioactive Cytotoxicity Assay kit (Promega) was used for assessing released LDH from intact cells as a viability test and from cell lysates as indicator of mitochondrial functionality.

Trypan Blue exclusion test was also used in order to establish the non-toxic concentration of the photosensitizer. Results are expressed as indexes compared to control (LDH index).

2.14 Statistics

Cell functionality assessment was presented as absolute values or specific indexes. All results were expressed as mean value $\pm$ standard deviation (SD). All cellular controls consisted of cell suspensions loaded with the compounds and not subjected to PDT or unloaded cells subjected to irradiation, depending on the experimental approach.

3. RESULTS AND DISSCUSION

The unique shape of the fullerenes C60 and C70 combined with their distinct physical properties make them good candidates for preparation of large, supramolecular aggregates [43]. On the other hand, porphyrins with their electronic absorption properties have been widely used as electron-donating species in photoactive molecular dyads [44]. The porphyrins and fullerenes molecules are spontaneously attracted in solution to each other. Such supramolecular aggregates can be exploited to produce ordered arrays of interleaved porphyrins and fullerenes.

The Fullerenes (C60) was solubilized with poly(vinylpyrrolidone) (PVP) in DMSO:water, was applied to K562 cells system. On incubation of C60 with various concentrations of PVP, cell differentiation and proliferation were potently inhibited, although weaker than the vehicle controls.

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 μ M TPP-C60 concentration range, the LDH release from metabolically active K562 cells incubated for 1-6-18h displayed the same pattern (Figure 2). We have obtained at higher than 5 μ M an increase of the LDH release compared to controls while concentrations above 25 μ M kill over 50% of the cells. For studying the loading efficiency we have detailed the lower than 5 microM concentration namely 0.25-1 μ M range using 1-18h incubation time.

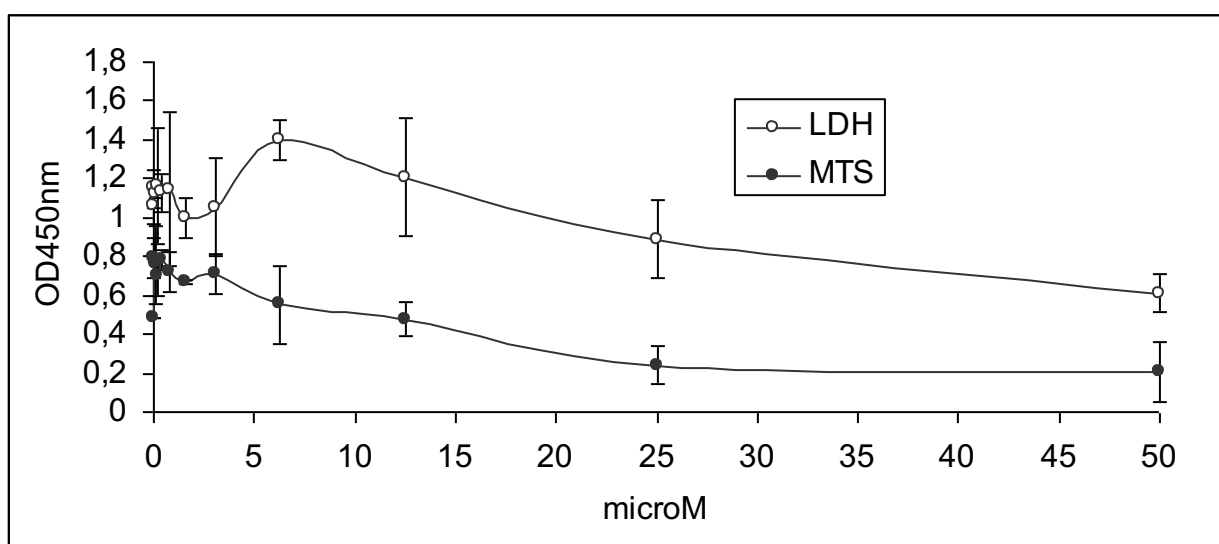


Fig.1.LDH release (LDH) from metabolically active K562 cell line (MTS) in presence TPP-PVP-C60 in non-irradiated protocols represented as OD450nm (mean $\pm$ SD of 6 individual experiments with triplicates/sample).

Cellular loading efficiency was studied indirectly by registering the supernatants and cell lysates spectra. Cells were efficiently loaded with the 0.5 μM concentration, thus for irradiation we have chosen the mentioned concentration.

During irradiation of K562 cells loaded with 0.5 μM cells after time incubation are actively destroyed (Figure 3). The actual cell counting showed that after irradiation we recover a mean of 20% when cells were loaded for 18h compared to an 80% in unloaded and irradiated controls.

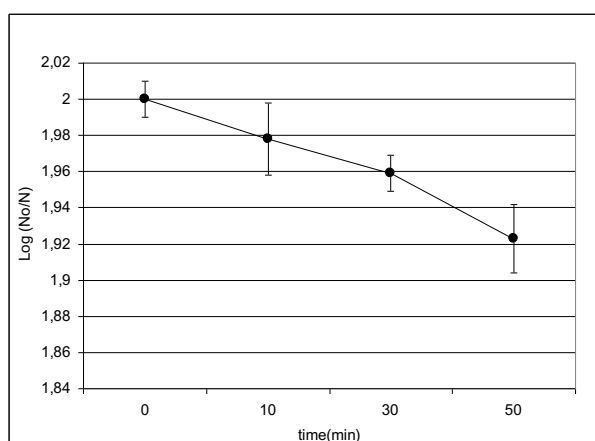


Figure 3. Photodegradation kinetics of K562 cellular suspension during the lamp irradiation

Investigated in TEM, irradiated cells both, control and TPP-C60 loaded K562 cells, showed an increased number of modified mitochondria. Mitochondria were slender and longer than in control cells and showed an electron-dense appearance, whereas they were poorly defined and less dense in the K562 cells (Figure 4). TPP-C60 loaded cells showed an increased number of mitochondria and large liposomes (Figure 4) independent of the compound concentration. TPP-C60 seems to be located free in cytoplasm, in the nuclear matrix and in mitochondria. Electron-dense 10-20 nm round isolated particles could be seen in treated K562 cells with an uneven distribution.

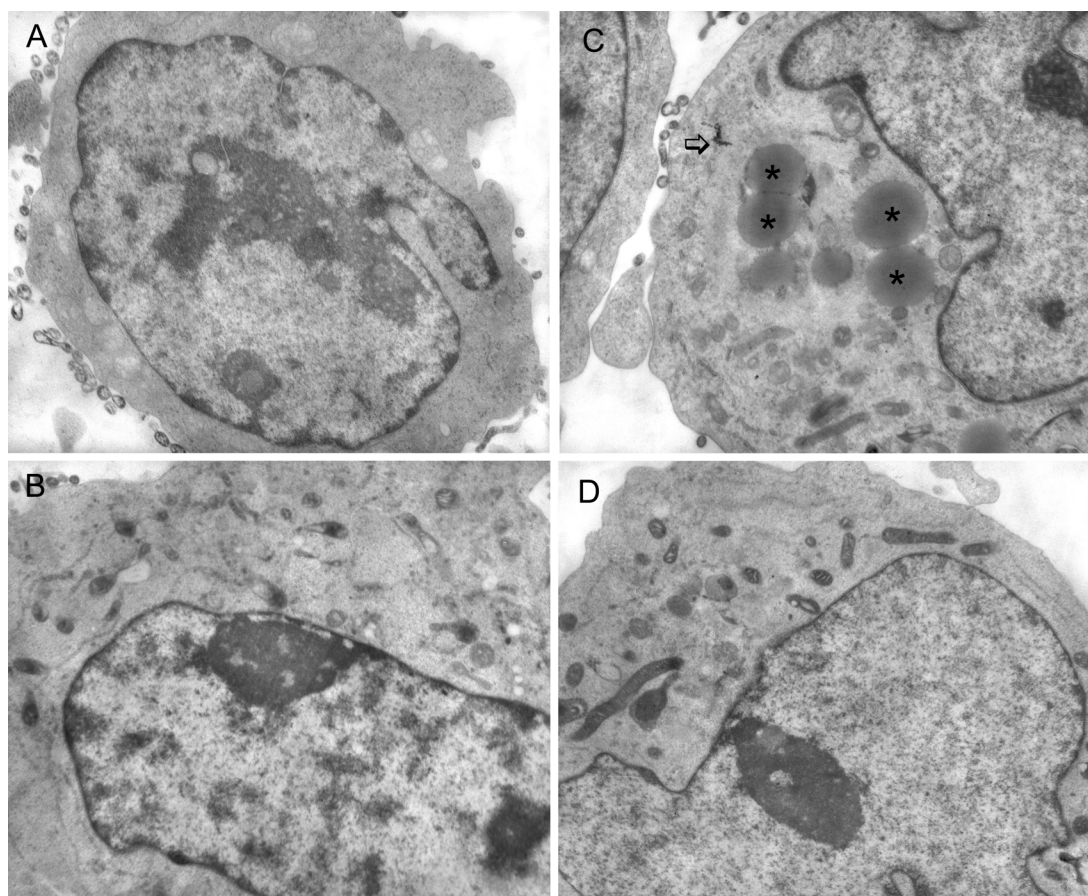


Figure 4. Irradiated cells investigated in TEM

As the intracellular localization is, among other organelle, mitochondria we have investigated the mitochondrial membrane potential. We found that the higher rate of depolarization was found when cells were irradiated after 18h of incubation with the compound and that irradiation of unloaded cells induce a good mitochondrial depolarization. The lack of a caspase-3 activation correlated with the mitochondrial depolarisation could be explained by the fact that active caspase-3 localizes in the mitochondria after translocation from the cytosol [45] and PDT protocol hindered the translocation. Moreover, we cannot exclude the findings that outer mitochondrial membrane permeabilization during apoptosis triggers caspase-independent mitochondrial potential depolarization [46].

4. CONCLUSIONS

The presented dyad compound complexed with *polyvinylpyrrolidone* displays on a wide range of concentration “dark” non-toxicity profile. The compound has a high singlet oxygen quantum yield (0.76).

We have shown that on TEM level it enters the leukaemia type of cells in organelles and that when localizing in the mitochondria induces a membrane potential depolarization that 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.

The combined use of OM, XRD and EDRXF analytical techniques have provided good insight into the bulk nature and surface corrosion products grown on the artefacts analysed during archaeological burial. OM, XRD and EDRXF results indicate on the surface damaged areas the presence of chlorine as an aggressive agent as well as giving evidence of the chemical reaction between some soil constituents (Cl, Mg, Al, Si, Fe, Ca) and degradation products. In addition, SEM-EDS, OM and XRD results show the occurrence of selective localised or general chlorine corrosion phenomena induced also by the separation of the alloying elements, which creates reactive electrochemical areas.

Finally, the chemical composition, the micro-chemical structures and the metallurgical features of the artefacts have been determined and can be used to identify some technological aspects of the ancient manufacturing processes. This information could be also used for producing Agbase reference alloys whose micro-chemical structure and chemical composition are similar to those of the ancient ones. These reference materials could be employed for carrying out accelerated degradation tests by simulating as best as possible, the specific corrosion conditions and the aggressive agents previously identified and then, to validate correctly the restoration and conservation procedures.

REFERENCES

1. Bianco A, Kostarelos K, Prato M. *Opportunities and challenges of carbon-based nanomaterials for cancer therapy*. Expert Opin Drug Deliv. 2008 Mar;5(3):331-42. Review.
2. Scott E. McNeil, *Nanotechnology for the biologist*, Journal of Leukocyte Biology 2005, Volume 78: 585-594
3. Fumelli C, Marconi A, Salvioli S, Straface E, Malorni W, Offidani AM, et al. *Carboxyfullerenes protect human keratinocytes from ultraviolet-B-induced apoptosis*. J Invest Dermatol 2000, 115: 835 - 841.
4. Tsao N, Luh T-Y, Chou C-K, Chang T-Y, Wu J-J, Liu C-C, et al. *In vitro action of carboxyfullerene*. J Antimicrob Chemother 2002; 49: 641 – 649
5. Kerns EH, Di L. *Pharmaceutical profiling in drug discovery*. Drug Discov. Today 2003, 8: 316–323.
6. Satoh M, Takayanagi I. *Pharmacological Studies on Fullerene (C60), a Novel Carbon Allotrope and Its Derivatives*, J Pharmacol Sci 2006, 100: 513 – 518
7. Hwang KC, Mauzerall D. *Photoinduced electron transport across a lipid bilayer mediated by C70*. Nature. 1993, 361: 138– 140.
8. Tokuyama H, Yamago S, Nakamura E. *Photoinduced biochemical activity of fullerene carboxylic acid*. J Am Chem Soc. 1993, 115: 7918–7919.
9. Chen B-X, Wilson SR, Das M, Coughlin DJ, Erlanger BF. *Antigenicity of fullerenes: antibodies specific for fullerenes and their characteristics*. Proc Natl Acad Sci U S A. 1998, 95: 10809–10813.
10. Shi Kam NW, Jessop TC, Wender PA, Dai H. *Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into mammalian cells*. J Am Chem Soc 2004, 126: 6850 - 6851.
11. Quintana, A., Raczka, E., Piehler, L., Lee, I., Myc, A., Majoros, I., Patri, A. K., Thomas, T.,

- Mule, J., Baker Jr., J. R. *Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor*. Pharm. Res. 2002, 19, 1310–1316.
12. Choi, H., Choi, S. R., Zhou, R., Kung, H. F., Chen, I. W. *Iron oxide nanoparticles as magnetic resonance contrast agent for tumor imaging via folate receptor-targeted delivery*. Acad. Radiol. 2004, 11, 996–1004.
13. Foley S, Crowley C, Smaih M, Bonfils C, Erlanger BF, Seta P, et al. *Cellular localization of a water-soluble fullerene derivative*. Biochem Biophys Res Commun 2002, 294:116- 119.
14. Milanesio ME, Alvarez MG, Rivarola V, Silber JJ, Durantini EN. *Porphyrin-fullerene C60 dyads with high ability to form photoinduced charge-separated state as novel sensitizers for photodynamic therapy*. Photochem Photobiol. 2005, 81(4): 891-897.
15. Schuster DI, Li K, Guldi DM, Palkar A, Echegoyen L, Stanisky C, Cross RJ, Niemi M, Tkachenko NV, Lemmetyinen H. *Azobenzene-linked porphyrin-fullerene dyads*. J Am Chem Soc. 2007, 129(51): 15973-15982.
16. D'Souza F, Maligaspe E, Karr PA, Schumacher AL, El Ojaimi M, Gros CP, Barbe JM, Ohkubo K, Fukuzumi S. *Face-to-face pacman-type porphyrin-fullerene dyads: design, synthesis, charge-transfer interactions, and photophysical studies*. Chemistry. 2008, 14(2):674-681.
17. Alvarez MG, Príncipe F, Milanesio ME, Durantini EN, Rivarola V. *Photodynamic damages induced by a monocationic porphyrin derivative in a human carcinoma cell line*. Int J Biochem Cell Biol. 2005, 37(12):2504-2512.
18. Rancan F, Rosan S, Boehm F, Cantrell A, Brellreich M, Schoenberger H, Hirsch A, Moussa F. *Cytotoxicity and photocytotoxicity of a dendritic C(60) mono-adduct and a malonic acid C(60) tris-adduct on Jurkat cells*. J Photochem Photobiol B. 2002, 67(3): 157-162.
19. Tabata Y, Ikada Y. *Biological functions of fullerene*. Pure Appl. Chem., 1999, 71: 2047-2053.
20. Gust D, Moore TA, Moore AL. *Mimicking photosynthetic solar energy transduction*. Acc Chem Res. 2001, 34(1): 40-48.
21. Alvarez MG, Prucca C, Milanesio ME, Durantini EN, Rivarola V. *Photodynamic activity of a new sensitizer derived from porphyrin-C60 dyad and its biological consequences in a human carcinoma cell line*. Int J Biochem Cell Biol. 2006, 38(12): 2092-2101.
22. Luzzio CB, Luzzio BB. *Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome*. Blood 1975, 45:321-334.
23. Olariu L, Dumitriu B, Neagu M, Manda G. *Flow cytometry measurements of intracellular Ca²⁺ in K 562 cells*. Rom J Intern Med 1999, 37:81-89.
24. Neagu M, Manda G, Constantin C, Radu E, Ion R-M. *Synthetic porphyrins in experimental photodynamic therapy induce different antitumoral effect*. J Porph Phtal 2007, 11 (1): 58-65.
25. Korzeniewski C, Callewaert DM. *An enzyme-release assay for natural cytotoxicity*. J Immunol Meth 1983, 64:313–320.
26. Barltrop JA.. *Bioorg. & Med. Chem. Lett*. 1991;1:611-614.
27. Craig C. Henderson, Paul A. Cahill, C₆₀H₂: *Synthesis of the Simplest C₆₀ Hydrocarbon Derivative*, *Science* 26 March 1993: Vol. 259. no. 5103, pp. 1885 – 1887
28. V. Lebedev, G. Török, L. Cser, A. Len, D. Orlova, V. Zgonnik, E. Melenevskaya, L.

- Vinogradova and W. Treimer, *Fullerene - polymer complexes: fractal crossover in solutions*, *J. Appl. Cryst.* (2003). 36, 646-648
29. Yamakoshi, Y.N. et al. *Chem. Comm.* 1994, 517-518; Sera, N. et al. *Carcinogenesis* 1996, 17, 2163-2169;
30. Ungurenasu, C.; Airinei, A. *J. Med. Chem.* 2000, 43, 3186-3188.
31. Prat F., Marti C., Nonell S., Xiaojun Z., Foote C.S., Gonzalez Moreno R., Bourlande J.L., Font J., *C₆₀ Fullerene-based materials as singlet oxygen O₂(¹Δ_g) photosensitizers : a time-resolved near-IR luminescence and optoacoustic study*, ***Phys. Chem. Chem. Phys.***, 2001, 3, 1638 – 1643
32. Xiao L, Takada H, Gan X, Miwa N. *The water-soluble fullerene derivative ‘‘radical sponge’’ exerts cytoprotective action against UVA irradiation but not visible-light-catalyzed cytotoxicity in human skin keratinocytes*. *Bioorg Med Chem Lett* 2006;16:1590–5.
33. Yudoh K, Shishido K, Murayama H, Yano M, Matsubayashi K, Takada H, et al. *Water-soluble C60 fullerene prevents degeneration of articular cartilage in osteoarthritis via down-regulation of chondrocyte catabolic activity and inhibition of cartilage degeneration during disease development*. *Arthritis Rheum* 2007;56:3307–18.
34. Chen C, Xing G, Wang J, Zhao Y, Li B, Tang J, et al. *Multihydroxylated [Gd@C82(OH)22]n nanoparticles: antineoplastic activity of high efficiency and low toxicity*. *Nano Lett* 2005;5:2050–7.
35. Tsao N, Luh TY, Chou CK, Wu JJ, Lin YS, Lei HY. *Inhibition of group A streptococcus infection by carboxyfullerene*. *Antimicrob Agents Chemother* 2001; 45:1788–93.
36. Rouse JG, Yang J, Barron AR, Monteiro-Riviere NA. *Fullerene-based amino acid nanoparticle interactions with human epidermal keratinocytes*. *Toxicol In Vitro* 2006;20:1313–20.
37. Tsao N, Kanakamma PP, Luh TY, Chou CK, Lei HY. *Inhibition of Escherichia coli-induced meningitis by carboxyfullerene*. *Antimicrob Agents Chemother* 1999;43:2273–7.
38. Ryan JJ, Bateman HR, Stover A, Gomez G, Norton SK, Zhao W, et al. *Fullerene nanomaterials inhibit the allergic response*. *J Immunol* 2007;179: 665–72.
39. Lu LH, Lee YT, Chen HW, Chiang LY, Huang HC. *The possible mechanisms of the antiproliferative effect of fullerenol, polyhydroxylated C60, on vascular smooth muscle cells*. *Br J Pharmacol* 1998a;123:1097–102.
40. Zakharian TY, Seryshev A, Sitharaman B, Gilbert BE, Knight V, Wilson LJ. *A fullerene-paclitaxel chemotherapeutic: synthesis, characterization, and study of biological activity in tissue culture*. *J Am Chem Soc* 2005;127: 12508–9.
41. Isobe H, Nakanishi W, Tomita N, Jinno S, Okayama H, Nakamura E. *Nonviral gene delivery by tetraamino fullerene*. *Mol Pharm* 2006;3: 124–34.
42. Bolskar RD, Benedetto AF, Husebo LO, Price RE, Jackson EF, Wallace S, et al. *First soluble M@C60 derivatives provide enhanced access to metallofullerenes and permit in vivo evaluation of Gd@C60[C(COOH)2]10 as a MRI contrast agent*. *J Am Chem Soc* 2003;125:5471–8.
43. Lin JC, Wu CH. *Surface characterization and platelet adhesion studies on polyurethane surface immobilized with C60*. *Biomaterials* 1999;20:44, 613-620

44. Pacher P, Beckman JS, Liaudet L. *Nitric oxide and peroxynitrite in health and disease*. *Physiol Rev* 2007;87:315–424.
45. Wolff DJ, Papoiu AD, Mialkowski K, Richardson CF, Schuster DI, Wilson SR. *Inhibition of nitric oxide synthase isoforms by tris-malonyl-C60-fullerene adducts*. *Arch Biochem Biophys* 2000;378:216–23.
46. Fumelli C, Marconi A, Salvioli S, Straface E, Malorni W, Offidani AM, et al. *Carboxyfullerenes protect human keratinocytes from ultraviolet-B-induced apoptosis*. *J Invest Dermatol* 2000;115:835–41.