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# $C_{60}$ fullerene accumulation in human leukemic cells and perspectives of LED-mediated photodynamic therapy

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ABSTRACT

Recent progress in nanobiotechnology has attracted interest to a biomedical application of the carbon nanostructure $C_{60}$ fullerene since it possesses a unique structure and versatile biological activity. $C_{60}$ fullerene potential application in the frame of cancer photodynamic therapy (PDT) relies on rapid development of new light sources as well as on better understanding of the fullerene interaction with cells.

The aim of this study was to analyze $C_{60}$ fullerene effects on human leukemic cells (CCRF-CEM) in combination with high power single chip light-emitting diodes (LEDs) light irradiation of different wavelengths: ultraviolet (UV, 365 nm), violet (405 nm), green (515 nm) and red (632 nm). The time-dependent accumulation of fullerene $C_{60}$ in CCRF-CEM cells up to 250 ng/$10^6$ cells at 24 h with predominant localization within mitochondria was demonstrated with immunocytochemical staining and liquid chromatography mass spectrometry. In a cell viability assay we studied photoexcitation of the accumulated $C_{60}$ nanostructures with ultraviolet or violet LEDs and could prove that significant phototoxic effects did arise. A less pronounced $C_{60}$ fullerene phototoxic effect was observed after irradiation with green, and no effect was detected with red light. A $C_{60}$ fullerene photoactivation with violet light induced substantial ROS generation and apoptotic cell death, confirmed by caspase3/7 activation and plasma membrane phosphatidylserine externalization. Our work proved $C_{60}$ fullerene ability to induce apoptosis of leukemic cells after photoexcitation with high power single chip 405 nm LED as a light source. This underlined the potential for application of $C_{60}$ nanostructure as a photosensitizer for anticancer therapy.

## 1. Introduction

Photodynamic therapy is a non-surgical approach aimed on the selective elimination of cancer cells. The main idea of PDT is to combine two non-toxic components – photosensitizing molecule and visible light – which in the presence of oxygen gain a pronounced toxicity [1–3]. Anticancer PDT effects are realized directly through the induction of cancer cell death and/or indirectly when damage of the vascular system and activation of the immune response are provoked [4,5]. Rapid development of endoscopic fiber optic devices [6,7] allows to test PDT in treatment not only for skin malignacies, but for brain, lung, esophagus, colon, pancreas, liver, bile duct, breast, bladder, prostate and neck cancers as well [1,4,8].

Over the past decade, the application of nanoparticulate agents has been established both in pharmaceutical research and in clinical settings [4,9]. The constantly increasing interest in novel nanotechnology platforms for biomedical applications stimulated the investigation of carbon nanomaterials, including fullerenes and their most prominent representative – $C_{60}$ fullerene [10]. Pristine non-modified $C_{60}$ fullerene is a lipophilic, spheroidal shaped and symmetrical molecule with 0.72 nm in diameter [11,12]. Due to the specific packing of atoms in penta- and hexagon units the surface of $C_{60}$ is three times smaller than expected for biological molecules with the same molecular weight of 720 Da. The unusual structure of $C_{60}$ fullerene determines its unique physicochemical properties and biological activity [9,11–14].

Today considerable attention is devoted to $C_{60}$ fullerene as potential

*Abbreviation:* HPLC-ESI-MS, High Performance Liquid Chromatography-Electro Spray Ionization -Mass Spectrometry

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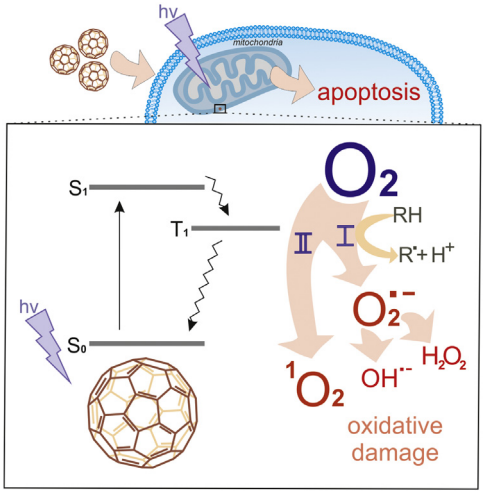


**Fig. 1.** Schematic mechanism of $C_{60}$ fullerene photodynamic cancer therapy. An absorbed photon excites $C_{60}$ fullerene to the first excited singlet state $S_1$, that relaxes to the more long lived triplet state $T_1$. The $C_{60}$ triplet interacts with oxygen either through type I or type II, resulting in the intensification of reactive oxygen species generation and induction of apoptotic cell death.

regulator of oxidative balance in biological systems. Since a $C_{60}$ molecule consists entirely of $sp^2$-hybridized carbon atoms [14], it is able to generate reactive oxygen species (ROS) after UV–vis light irradiation with a quantum yield of 1.0 [15]. $C_{60}$ fullerene advantages compared to conventional photosensitizing molecules are the higher photostability and lesser photobleaching [2]. There are two ways of ROS production by photoexcited $C_{60}$: by energy (type I) or electron (type II) transfer from photoexcited $C_{60}$ to oxygen (Fig. 1) [2,15–19]. The produced reactive oxygen species are excellent oxidizing agents that react with a wide range of biological targets. Oxidative stress, which occurs when ROS generation overwhelms the cell antioxidant defense system can lead to cell death by apoptosis [16–18]. Mitochondria have been found to be an important subcellular target for many photosensitizing drugs due to its role in apoptosis induction [3,17].

$C_{60}$ fullerene-mediated PDT efficiency in vitro and in vivo was shown to a large extent with its hydrophilic derivatives hydroxy- [14,17,20], carboxy- [16], PEG [10,12,17] -$C_{60}$ and $C_{60}$ with various organic substitutes [15,18,20–23]. Functionalization of $C_{60}$ improves its water solubility and increases its biocompatibility by decreasing the aggregate size [14], but on the other hand, inhibits its interaction with cellular lipid membranes and changes the pattern of cellular uptake [14,18,19,22,23]. $C_{60}$ diffuses through bilayered membrane from six [22] to nine [24] orders of magnitude faster as compared with its hydrophilic derivatives, which interact with polar groups on the membrane surface instead of entering the cell. Higher lipophilicity promotes diffusion of pharmaceutical agents across the plasma membrane and further relocation to other cellular membranes, thus facilitating intracellular uptake [17]. Hydrophobic drugs are shown to attack the cancer cells mainly by direct interactions, weather hydrophilic agents act indirectly by damaging blood vessels [18]. Moreover, the presence of functional groups on $C_{60}$ fullerene surface decreases the quantum yield of singlet oxygen production after molecule photoexcitation [15,16]. So, the cellular uptake and further biological effects of pristine $C_{60}$ and its derivatives could be different. Pristine $C_{60}$ fullerene may be applied in PTD in the form of liposome-based delivery systems [2,8,16,25,26] or water colloidal $C_{60}$ solutions [16,27–30]. Previously, a negligible toxicity of pristine $C_{60}$ stable colloid solution [30] against normal cells was shown [29,30]. At the same time a pronounced proapoptotic effect was detected in leukemic cells treated with pristine $C_{60}$ fullerene and irradiated with UV/Vis light in the range of 320–600 nm [11,27,31]. These data indicate the potential of $C_{60}$ as an effective photosensitizer in cancer therapy.

The use of high power single chip light-emitting diodes is expected to promote PDT application, since they have a higher portability and extremely lower cost, compromising the efficiency [32,33] of lasers as the classical PDT light sources. LED-based equipment has a high potential to simplify PDT's technical part and to reduce costs [34]. In this paper, we report first data concerning the pristine $C_{60}$ fullerene accumulation and localization inside human leukemic cells and its phototoxic effects potentially induced by UV, violet, green and red high power single chip LEDs light irradiation.

## 2. Materials and methods

### 2.1. Chemicals

RPMI 1640 liquid medium, phosphate buffered saline (PBS), Fetal Bovine Serum (FBS), Penicillin/Streptomycin and L-glutamin were obtained from Biochrom (Berlin, Germany). Poly-D-lysine hydrobromide, Triton X100, Bovine Serum Albumin (BSA), 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI), glycerol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and sucrose were obtained from Sigma-Aldrich Co. (St-Louis, USA). Dimethylsulfoxide (DMSO), trypan blue, paraformaldehyde, toluene, methanol, 2-isopropanol, acetonitrile, tris(hydroxymethyl)aminomethane (Tris) and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) were used. 3-(*N*-morpholino)propanesulphonic acid (MOPS) was purchased from ICN Biomedicals Inc. (Ohio, USA).

### 2.2. $C_{60}$ fullerene synthesis

The pristine $C_{60}$ fullerene aqueous colloid solution was prepared as described in [28] by $C_{60}$ fullerene transfer from toluene to water using continuous ultrasound sonication. Obtained $C_{60}$ colloid solution was characterized by high $C_{60}$ fullerene concentration ($2 \times 10^{-4}$ M, purity 99%), stability and homogeneity.

### 2.3. Spectrophotometric analysis

Samples (100 µl of $C_{60}$ colloid solution) were placed into a 96-well plate Sarstedt (Nümbrecht, Germany), $C_{60}$ absorbance spectrum was measured with the multimode microplate spectrometer Tecan infinite M200 PRO (Grödig, Austria) at the following parameters: wavelength range 200–900 nm, wavelength step size: 2 nm, number of flashes per well: 10.

### 2.4. Cell culture

The human cancer cell line of leucosis origin – CCRF-CEM (ACC 240) – was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Cells were maintained in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin and 2 mM Glutamine, using 25 $cm^2$ flasks at a 37 °C with 5% $CO_2$ in a humidified incubator Binder (Tuttlingen, Germany). The number of viable cells was counted using 0.1% trypan blue staining and a Roche Cedex XS Analyzer (Basel, Switzerland).

### 2.5. Immunofluorescence staining

CCRF-CEM cells ($2 \times 10^5$/ml) were seeded in 6-well plates (Sarstedt, Nümbrecht, Germany) on cover slips (Carl Roth, Karlsruhe, Germany), previously coated with poly-D-Lysine, and incubated for 24 h. Cells were treated with 20 µM $C_{60}$ colloid solution for further 24 h. Then cells were washed with PBS, stained with MitoTracker Orange FM (Invitrogen Molecular Probes, Carlsbad, USA) for 30 min at 37 °C and then fixed with 4% paraformaldehyde for 15 min at room temperature (RT) in the dark. After washing with PBS, cells were permeabilized with