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# AQUEOUS C60 FULLERENE SOLUTION EFFECTS ON CELL VIABILITY

Lība Sokolovska[#], Maksims Čistjakovs, Alīna Sultanova, and Modra Murovska

Institute of Microbiology and Virology, Rīga Stradiņš University, 5 Rātsupītes Str., Rīga, LV-1067, LATVIA

[#] Coresponding author, liba.sokolovska@rsu.lv

Contributed by Modra Murovska

*Fullerenes are carbon nanoparticles with the ability to quench reactive oxygen species. The biomedical potential of fullerenes is diminished by their low solubility in water, but many approaches have been developed to bypass this problem, like chemical modification of the carbon cage and the use of the solvent exchange method to transfer fullerenes from one solvent to the other. These two approaches were used in this study. Carboxylated fullerene aqueous solution was acquired using solvent exchange method transferring fullerene nanoparticles (C60) from toluene to water. Effects of varying concentration (0.5, 1, 1.5, 2, 2.5, 3, 5, 10 μM) of aqueous fullerene solution on cell viability and their antioxidative capabilities were evaluated on PC-3 and on monocytes isolated from a blood donor using Resazurin Cell Viability Assay. PC-3 cell viability was drastically affected by the 10 μM fullerene solution but remained relatively stable when treated with other concentrations even after longer periods of incubation with resazurin dye. Elevated cell viability was observed in monocytes treated with various fullerene concentrations, possibly indicative of fullerene protective activity against oxidative stress.*

**Key words:** *carboxyfullerene, antioxidant, oxidative stress, monocytes, PC-3.*

## INTRODUCTION

Buckminsterfullerene (shortened to fullerene) was discovered in 1985 and is the third carbon allotrope, following diamond and graphite. Fullerenes are highly symmetric with different cage sizes — consisting of different numbers of carbon atoms. The most abundant fullerene is C60, which consists of 60 carbon atoms forming 12 pentagons and 20 hexagons (Goodarzi *et al*., 2017; Rašović, 2017). Shortly after their discovery, researchers created a way of producing them. We know that fullerenes exist in nature and in interstellar space, and they have even been implicated in the creation of life (Goodman *et al*., 2012).

Fullerene nanoparticles have high potential to be used in biomedical applications due to their antioxidative capabilities. Their ability to quench reactive oxygen species (ROS) has earned them the name "radical sponges". ROS like singlet oxygen are highly reactive and can react with various biologically crucial macromolecules, such as DNA, lipids, proteins etc. (Markovic and Trajkovic, 2008). Additionally to ROS quenching, fullerenes possess a polarly opposite property — upon photoexcitation they can generate ROS (Trpkovic *et al*., 2012). Most studies concerning these fullerene capabilities have been done using C60, so not only is this fullerene the most abundant, it is also the most studied.

Both of the described C60 properties can be facilitated in developing new therapeutics. The ability to generate ROS can be used both as an antimicrobial agent and in cancer treatment. Many *in vitro* and *in vivo* studies have demonstrated the photodynamic action of C60 against different cancer cell types and tumours *in vivo* (Chen *et al*., 2012; Grebinyk *et al*., 2018). The antioxidant nature of fullerenes and their ability to localise in mitochondria (Foley *et al*., 2002; Chirico *et al*., 2007) can be facilitated in developing cytoprotective antioxidant treatments. A lot of focus has been given to fullerene use in neurodegeneration treatment, and results acquired from mice models are promising, since treatment with fullerenes demonstrated reduced age-associated oxidative stress in the brain, significantly extended lifespan and prevented age-related cognitive impairment in mice (Quick *et al*., 2008). C60 fullerenes have also been studied as tools for diagnostic procedures, as they can potentially be used in X-ray and magnetic resonance imaging (Rašović, 2017).

The low solubility of fullerenes poses a great obstacle for their use in biomedical applications. Fullerenes have been dissolved in organic solvents like toluene, xylene, tetrahydrofuran, but these solvents are not applicable for fullerene solution use in biological systems. Multiple approaches to increase fullerene solubility in water have been devised, like chemical modification of the carbon cage, fullerene incorporation in water soluble supramolecular structures, the use of solvent exchange to transfer fullerenes from an organic solvents to water using ultrasonification, and the prolonged stirring of fullerenes in pure water (Markovic and Trajkovic, 2008).

Knowing that C60 fullerenes possess antioxidative properties and that their aqueous solutions have biomedical applications, the aim of this study was to evaluate how various C60 fullerene aqueous solution concentrations affect cell viability and whether they exert protective effects on cells via oxidative stress reduction.

## MATERIALS AND METHODS

**Fullerene.** (1,2-Methanofullerene C60)-61-carboxylic acid powder was acquired from Sigma-Aldrich. Purity of the acquired powder was 97% (determined by high performance liquid chromatography, HPLC). The C60 fullerene cage was functionalised with a carboxyl group (Fig. 1). The empirical formula was $C_{62}H_2O_2$ and molecular weight was 778.68 g/mol.

**Cell cultures.** Two cell culture types were used in this study — immortalised and primary. Human prostate adenocarcinoma cell line PC-3 (ATCC® CRL1435 ™) was used in this study. PC-3 cells were cryopreserved prior to the experiments. Human monocytes were isolated by sedimentation from peripheral blood mononuclear cells (PBMCs). The PBMCs were isolated from donors' whole blood using a Ficoll gradient. Isolated PBMCs were washed using Phosphate-buffered saline (PBS), counted and immediately used for further experiments

**Aqueous fullerene solution preparation.** (1,2-Methanofullerene C60)-61-carboxylic acid powder (Sigma-Aldrich) was dissolved in toluene at a concentration of 200 μM. As fullerenes dissolved in toluene, the solution acquired a pink hue. Fullerene powder was allowed to fully dissolve in toluene overnight at 4 $^{o}$C, with periodic ultrasonification.

Aqueous fullerene solution was obtained by the solvent exchange method (Mikheev *et al*., 2018). Aqueous solution acquisition was done in 4 $^{o}$C. 2 ml distilled water added to 1 ml of fullerene solution in toluene and mixed thoroughly followed by periodical ultrasonification. Mixing and ultrasonification was continued until toluene had evaporated and the aqueous phase had acquired a brownish hue. To ascertain that the fullerenes had dispersed in the water phase, the absorbance of the brownish aqueous solution was measured using the Nanodrop in UV-vis mode. Peaks in absorbance were measured as reported elsewhere (Belousova *et al*., 2015). The obtained 100 μM aqueous fullerene solution was stored at 4 $^{o}$C and later used to treat cells.

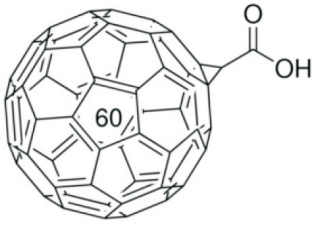


*Fig. 1.* (1,2-Methanofullerene C60)-61-carboxylic acid. C60 fullerene carbon cage functionalised with a carboxyl group (Sigma Aldrich, Catalog Nr. 658847).

**Cell culturing and treatment with fullerene aqueous solution.** Eight fullerene concentrations were tested – 0.5, 1, 1.5, 2, 2.5, 3, 5, 10 μM. Eight wells were assigned to each of the concentrations and eight were untreated wells — cells in growth medium without fullerenes.

Frozen PC-3 cells were thawed in 37 $^{o}$C water bath and cultured in prewarmed RPMI-1640 (10% foetal bovine serum) medium. The cells were incubated at 37 °C in a 5% $CO_2$ in air atmosphere until they were ready to be subcultured. Subculturing was performed when cells reached confluency of about 90%. To subculture cells, media was removed, the cells were washed with PBS, and 0.25% trypsin 0.53 mM Ethylenediamine tetraacetic acid (EDTA) solution was added and incubated for 5–10 min until cells detached. Enzymatic action of trypsin-EDTA was stopped by adding growth medium. PC-3 cells were seeded in 96 well plates containing 200 μl growth medium. Each well contained approximately 28 000 cells. Cells were allowed to attach overnight in 37 °C in a 5% $CO_2$ in air atmosphere, after which the growth medium was removed and a new growth medium containing different concentrations of fullerene was added.

Immediately after isolation from a donor blood, PBMCs were seeded on 96 well plate and were allowed to attach overnight in 37 °C in a 5% $CO_2$ in air atmosphere. Each well contained approximately 40 000 cells. Next morning, the medium containing unattached cells was removed, after which a new growth medium containing different concentrations of fullerene was added.

Cells were incubated with fullerenes for 24 h, after which growth medium containing fullerenes was removed. Medium was also removed from untreated wells.

**Fullerene effects on cell viability.** Resazurin Cell Viability Assay (Abcam. UK, ab129732) was used to evaluate the effects of fullerene nanoparticles on cell viability and their antioxidative activity.

After the growth medium containing fullerenes was removed, 100 μl fresh growth medium was added to every well. 20× Cell Viability Stain (Abcam, UK, ab129732) was diluted 10× in growth medium. 100 μl of the diluted stain (2× Cell Viability Stain) was added to each well. Plates were incubated in 37 °C in a 5% $CO_2$ in air atmosphere. After 4, 5, 6, and 24 hours of incubation, absorbance at 540 nm was measured. Although the manufacturer indicated that

four hours of incubation was the most optimal, we measured absorbance repeatedly to evaluate whether fullerenes exert protective effects, knowing that the dye after longer periods of incubation can induce oxidative stress (Erikstein *et al*., 2010).

**Statistical analysis.** The experiment was repeated three times for both PC-3 cells and monocytes — in each repetition, each concentration was tested in eight wells. Acquired data were analysed with appropriate analysis methods (nonparametric t tests) and graphed using GraphPad Prism 6 software (GraphPad Software, USA).

## RESULTS

**Fullerene effects on PC-3 cell viability.** Cell viability tests after 4-hour incubation indicated that the most significant drop in cell viability compared to untreated cells was observed in cells treated with 10 μM fullerene solution (median optical density (OD) 0.720 [interquartile range (IQR): 0.710–0.727] vs 0.670 [IQR: 0.669–0.679]; $p = 0.0002$). Viability increased as the fullerene concentration decreased, reaching viability similar to untreated cells at around 2 μM. Cells treated with 2 μM fullerene solution showed marginally higher viability than the untreated cells, but not statistically significantly (median OD 0.720[IQR: 0.710–0.727] vs 0.728 [IQR: 0.722–0.739]; $p = 0.2909$). Similar cell viability patterns among untreated and treated cells were observed at all time points (5, 6, and 24 hours) (Fig. 2). After 24-hour incubation with the resazurin dye, cell viability was overall higher compared to previous time points. Untreated cell viability in the first three time points was 0.745 (IQR: 0.726–0.753) and after the 24-hour incubation — 0.936 (IQR: 0.931–0.951) ($p < 0.0001$). This difference could be explained by the fact that the cells might have gone through another division, as the cell culture supernatant measurements after 24-hour incubation showed similar values — median OD was 0.739 (IQR: 0.731–0.744) (Fig. 3).

**Fullerene effects on monocyte viability.** Fullerenes seemed to affect monocytes differently than PC-3, as stark drops in cell viability after 4-hour incubation were not ob-

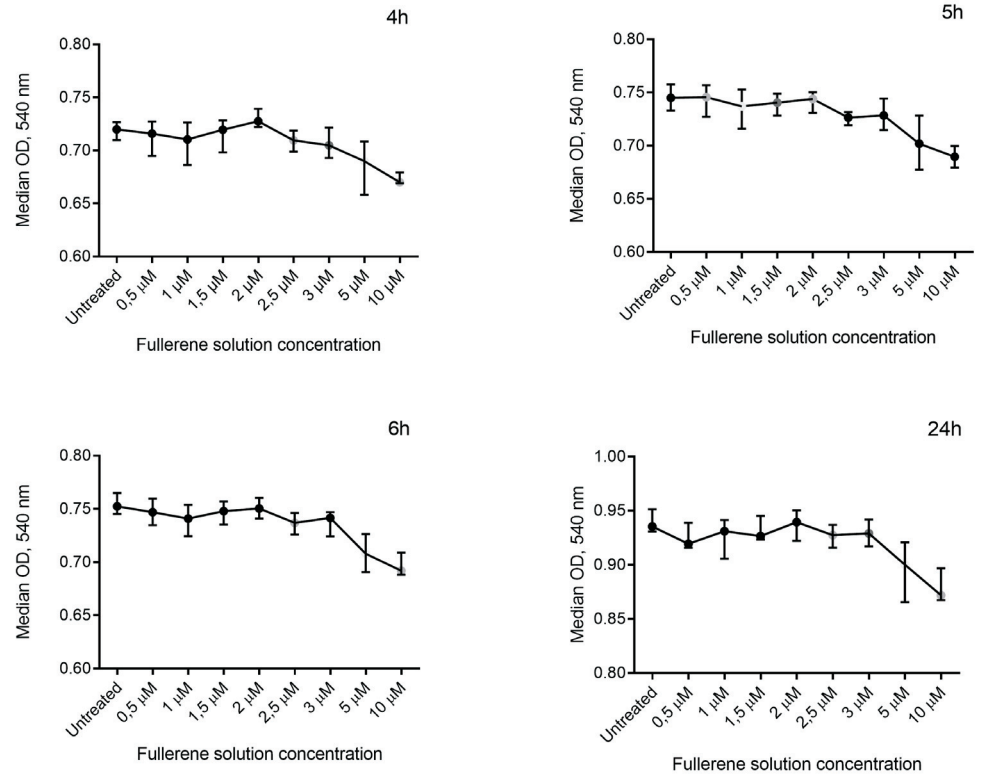


*Fig. 2.* Effects of various fullerene solution concentrations on PC-3 cell viability as determined by using Resazurin Cell Viability Assay (ab129732). Cell viability results after 4 hours, 5 hours, 6 hours, and 24 hours after incubation with resazurin dye.

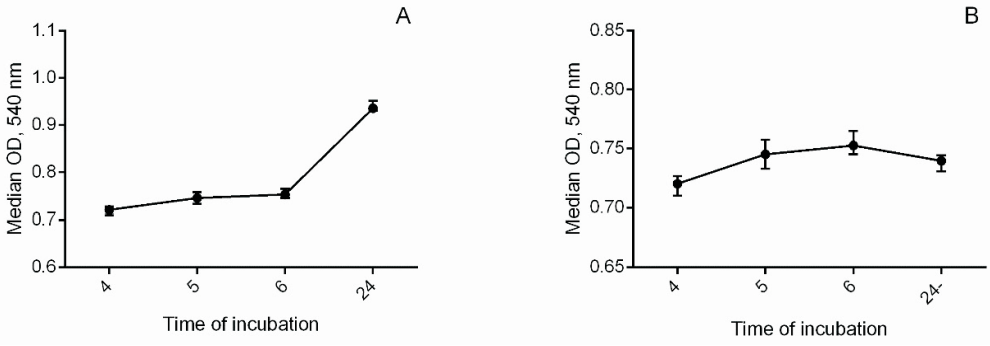


*Fig. 3.* Viability of untreated PC-3 cells after 4-, 5-, 6-, and 24-hour incubation with resazurin dye as determined by using Resazurin Cell Viability Assay (ab129732) (A). Viability measurements of PC-3 cell culture supernatant cells after 4-, 5-, 6- and 24-hour incubation with resazurin dye as determined by using Resazurin Cell Viability Assay (ab129732) (B).

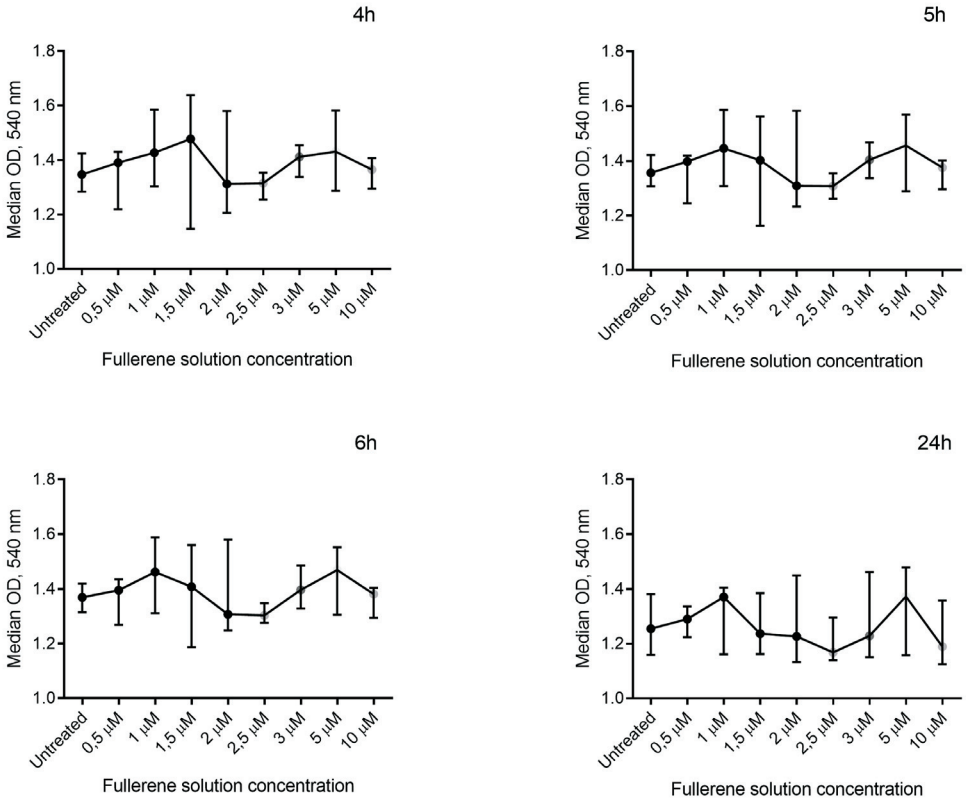


*Fig. 4.* Effects of various fullerene solution concentrations on monocyte viability as determined by using Resazurin Cell Viability Assay (ab129732). Cell viability results after 4 hours, 5 hours, 6 hours and 24 hours after incubation with resazurin dye.

served, even in cells treated with 10 µM fullerene solution. At all time points, the highest cell viability was observed in cells treated with 5, 1.5, and 1 µM fullerene solution. After 4 hours, highest cell viability compared to untreated cells was observed in cells treated with 1.5 µM fullerene solution, although without statistical significance (median OD 1.347 [IQR: 1.284–1.424] vs 1.478 [IQR: 1.148–1.639]; $p$ = 0.476). Similar cell viability patterns among untreated and treated cells were observed at all time points —5, 6, and 24 hours (Fig. 4).

Untreated cell viability remained relatively stable during the first three time points (median OD 1.347 [IQR: 1.284–1.424] vs 1.356 [IQR: 1.307–1.422] vs 1.369 [IQR: 1.314–1.419], $p$ = 0.845), but decreased after 24-hour incubation with the cell viability dye (median OD 1.255 [IQR me points ($p$ = 0.474, $p$ = 0.179, $p$ = 0.180) (Fig. 5).

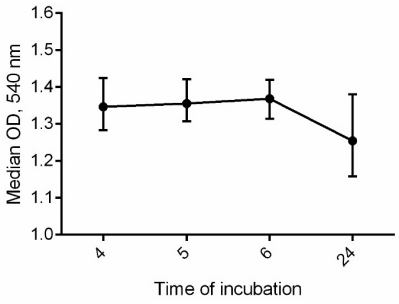


*Fig. 5.* Viability of untreated monocytes after 4-, 5-, 6-, and 24-hour incubation with resazurin dye as determined by using Resazurin Cell Viability Assay (ab129732)

## DISCUSSION

Accumulation of ROS and the oxidative stress caused by them are important factors in both human aging and the development of various diseases like autoimmunity, atherosclerosis, diabetes, neurodegenerative disorders and others. Oxidative stress theory of aging states that age-associated functional losses are due to the accumulation of oxidative damage to macromolecules like DNA, lipids and proteins by ROS, which means that the identification of new antioxidative agents is an important field of study (Liguori *et al*., 2018).

Functionalised fullerenes, such as the ones used in this study, additionally to their increased solubility in water may possess greater ability in scavenging ROS like singlet oxygen, superoxide anion and hydroxyl radicals, compared to pristine fullerene, but the differences in scavenging ability have been scarcely assessed, in part due to the vast diversity on fullerene functionalisation possibilities (Markovic and Trajkovic, 2008).

Resazurin dye is a redox reactive compound widely used in cell biology assays, such as of cell viability and metabolic activity. Resazurin acts as an electron acceptor in the electron transport chain within the inner mitochondrial membrane and is known to accept electrons from free radicals in a way that promotes ROS generation, which means that prolonged exposure of cells with resazurin can lead to oxidative stress (Erikstein *et al*., 2010). Since fullerenes have been shown to localise in cell mitochondria ( Foley *et al*., 2002; Chirico *et al*., 2007), we evaluated whether fullerene solution could alleviate the effects of resazurin dye via their antioxidative properties.

Although cell viability variations were observed in this study, in only one instance a statistically significant drop in cell viability was observed — in the case of PC-3 treated with 10 μM. This may be partially explained by the fact that functionalised fullerenes, like the carboxylated C60 fullerenes used in this study, in addition to their increased solubility, seem to be less cytotoxic and at the same time might have anticancer properties (Trpkovic *et al*., 2012).

Elevated cell viability in monocytes treated with some of the fullerene concentrations, compared to that of untreated cells, may indicate some level of fullerene protective ability against oxidative stress. Similar results were reported in a study on C60 carboxyfullerene protective activity against oxidative stress-induced apoptosis in PBMCs. These nanoparticles exerted protection against apoptosis via their ROS scavenging capabilities. Carboxyfullerenes protected PBMCs from induced oxidative stress-related apoptosis at both 2 μM and 10 μM concentrations (Monti *et al*., 2000). We did not see a protective effect at 10 μM like the study by Monti *et al*. reported, but this discrepancy can be explained by the fact that Monti *et al*. used a slightly differently chemically modified C60 fullerene.

While the results of this study showed minor anti-oxidative properties of aqueous fullerene solution in monocytes primary culture, it did not show severe signs of cytotoxicity at various concentrations. That means that there is a possibility to use carboxylated fullerenes for other research applications, for example, to further investigate their antioxidative activity and to investigate their use as carriers by conjugation with other molecules. Due to their hydrophobic core, these particles can easily cross the cell membrane. The carboxyl group on these fullerenes also provides various types of couplings. Every molecule that has a free amino group (peptides, proteins, DNA, etc.) could be coupled via carbodiimide reaction. Also, the fullerene's property of making clusters was shown to be effective for forming stronger immunogenic response when it carries an antigen, i.e. great application for vaccine development (Alarcón *et al*., 2011). Some studies have found that fullerenes seemingly do not influence T-cell reactivity, but could potentially activate cells of the innate immune system (Klein *et al*., 2012).

Another interesting observation was that a high concentration of fullerene aqueous solution (10 μM) showed strong cytotoxic properties on prostate cancer cells, but not on primary monocytes. This indicates possible anti-cancer properties; however, this should be investigated in greater detail using various cancer cell cultures and applying methods of induced fullerene ROS generation like fullerene photoexcitation. This approach has demonstrated a decrease in cell viability in some cancer cell cultures (Markovic and Trajkovic, 2008; Franskevych *et al*., 2017).

For more precise evaluation of effects of fullerene solution on cell viability and their protective abilities, some procedures like the total organic carbon technique to confirm the concentrations of the analysed fullerene solution should be applied in future studies. Also some procedures should be applied to guarantee that all of the toluene has been evaporated and that there is no residual toluene in the aqueous fullerene solution, to exclude negative effects of the organic solvents on the analysed cells (Mikheev *et al*., 2016). The measurement of ROS could also add to further elucidation of fullerene effects on cell viability and their protective ability, as this would help both to assess their ROS scavenging ability and exclude the chance of ROS production.

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## C60 FULERĒNU ŪDENS ŠĶĪDUMA IETEKMES UZ ŠŪNU DZĪVOTSPĒJU IZPĒTE

Fulerēni ir oglekļa nanodaļinas, kurām piemīt spēja mazināt skābekļa reaktīvo formu daudzumu. Fulerēnu pielietojumu biomedicīnā mazina to zemā šķīdība ūdenī, taču izveidoti vairāki paņēmieni, kā šo problēmu apiet, piemēram, modificējot nanodaļiņas vai izmantojot šķīdinātāju apmaiņas metodi, kurā fulerēni no viena šķīdinātāja tiek pārcelti otrā. Iepriekš minētie paņēmieni tika pielietoti arī šī pētījuma ietvaros, jo karboksilēto fulerēnu ūdens šķīdums tika iegūts, izmantojot šķīdinātāju apmaiņu — no toluola uz ūdeni. Dažādu fulerēnu ūdens šķīduma koncentrāciju (0,5, 1, 1,5, 2, 2,5, 3, 5, 10 μM) ietekme uz šūnu dzīvotspēju un to antioksidatīvās īpašības tika pētītas, izmantojot PC-3 šūnas un no asins donora perifēro asiņu mononukleārajām šūnām izdalītus monocītus, pielietojot resazurīna šūnu dzīvotspējas testu. PC-3 šūnu dzīvotspēju izteikti ietekmēja 10 μM fulerēnu šķīduma klātbūtne, taču dzīvotspēja citu koncentrāciju klātbūtnē saglabājās vienmērīga, pat pēc ilgāka inkubācijas laika ar resazurīna krāsvielu. Paaugstinātu monocītu dzīvotspēju novēroja pie vairākām fulerēnu šķīduma koncentrācijām, norādot uz fulerēnu iespējamo spēju aizsargāt šūnas pret oksidatīvo stresu.