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Investigation the cytotoxicity and photo-induced toxicity of carbon dot on yeast cell

Zeinab Bagheri[a,*], Hamide Ehtesabi[a], Zahra Hallaji[b], Hamid Latifi[c], Ebrahim Behroodi[c]

[a] Faculty of Life Sciences and Biotechnology, Shahid Beheshti University G.C., Velenjak, Tehran, Iran
[b] Protein Research Center, Shahid Beheshti University G.C., Velenjak, Tehran, Iran
[c] Laser & Plasma Research Institute, Shahid Beheshti University G.C., Velenjak, Tehran, Iran

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ABSTRACT

Carbon dots (CDs) as a new fluorescent material with excellent water solubility, chemical inertness, and easy surface modification are a good candidate for bioimaging and biosensing due to their low toxicity and good biocompatibility. Although carbon is not an intrinsically toxic substance, carbon nanomaterials such as CDs may cause risks to human health and the potentially hazardous effects of CDs on various living systems must be completely determined. So far, cytotoxicity studies of CDs have focused on human cells and are mainly conducted on limited cell lines. In the present study, toxicity assessment of CDs was evaluated on yeast cells *Pichia pastoris* as a unicellular eukaryotic model. Results revealed dose-dependent toxicity of CDs on yeast cells and less relative cell growth in 25 mg/ml of CDs as compared to the control group. CDs binding curve confirmed the interaction between CDs and surface of yeast cells. SEM images showed that the CDs caused cell shrinkage and hole formation on the surface of yeast cells and also induced slightly cell deformation. It was demonstrated that CDs could generate the ROS dose-dependently. Finally, results showed the growth inhibition and ROS generation effects of CDs were enhanced at light exposure, as an important environmental factor. These findings could have important implications for applications of CDs.

1. Introduction

Carbon dots a new class of graphene nanoparticle have attracted great attention of many research groups due to their unique properties, including high photoluminescence, well solubility, low toxicity and excellent biocompatibility that are widely used in bioimaging, drug delivery and theranostic (Hu et al., 2014; Ostadhossein and Pan, 2017; Zheng et al., 2015; Zhu et al., 2015). Apart from biomedical applications, CDs possess several interesting features, such as resistance to photobleaching, large surface area, wavelength-dependent emission, up-conversion capability and ease of synthesis, makes them promising materials in many industrial fields as well (Cayuela et al., 2016; Hola et al., 2014; Shen et al., 2012; Wang et al., 2016b; Wang et al., 2015a,2015b). While considering the new uses of CDs in wide range of novel applications such as LED fabrication, photocatalysis, supercapacitors and batteries, cause the entry and spread of the CDs to the environment (Wang et al., 2016c). Although carbon is not an intrinsically toxic substance, the specific material and structural configuration of carbon nanomaterials may cause risks to human health and the potentially hazardous effects on various living systems must be completely determined (Feng and Liu, 2011; Seabra et al., 2014; Zhu et al., 2007, 2015).

Cytotoxicity studies of CDs have focused on human cells and are mainly conducted on limited cell lines, such as Hela cells, which restricts the generalization of the results to other cells, this could be performed using cells and simpler organisms (Dhawan and Sharma, 2010; Li et al., 2017; Wang et al., 2016a). Unicellular eukaryotic model organisms are widely used to study the toxicity of chemicals such as heavy metals, anticancer drugs, herbicides and food preservatives because of similarities of their biochemical structure, molecular mechanisms and cellular organization to those of higher organisms. According to our knowledge, there is no report has been conducted to investigate the toxicity effect of CDs on yeast cells (Wang et al., 2016a; Willaert et al., 2016).

Additionally, the enormous variety of synthesized CDs with diverse physicochemical properties, is the next challenge to explore the toxicity of CDs (Havrdova et al., 2016; Jeon et al., 2017). One approach to overcome this challenge is trying to understand the mechanism of CDs toxicity at the cellular level (Seabra et al., 2014; Wang et al., 2015a,2015b). several studies demonstrated that various CDs cause

* Corresponding author.
E-mail address: ze_bagheri@sbu.ac.ir (Z. Bagheri).

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ROS generation on account of their surface chemistry and edge effect (Wang et al., 2016a). Wang et al. reported that the CDs-induced release of ROS caused DNA damage in fibroblast cells (NIH/3T3) (Wang et al., 2015a, 2015b). Jiang et al. also showed that the green fluorescent CD synthesized from GO through one-step hydrothermal method caused the ROS generation in HeLa cells dose-dependently (Jiang et al., 2015). Another recent study on NIH/3T3 cells, also demonstrated that the contribution of the ROS generation to the toxicity of CDs is dominantly related to the property of the nanomaterial itself, not to the properties of coating polymers (Havrdova et al., 2016). Additionally, Chong et al. demonstrated that CDs in the absence of light can efficiently scavenge a number of free radicals. However, upon exposure to blue light, exhibit significant phototoxicity through increasing intracellular ROS levels and reducing cell viability (Wu et al., 2013).

The objective of this study was to evaluate the potential hazards of CDs nanoparticle using the yeast *Pichia pastoris* as a unicellular eukaryotic model. Water-soluble fluorescent CDs were prepared from citric acid using pyrolysis method and then characterized by UV–vis spectroscopy, spectrofluorimetry, Dynamic light scattering and transmission electron microscopy. In the next step, the relative cell growth of yeast cells was assessed in the presence of different concentration of CDs. The spectrofluorometry was used to answer the question of whether the interaction between CDs and yeast cells and or CDs could adsorb on the cell surface. Additionally, the morphological changes of yeast treated with CDs were examined by scanning electron microscopy (SEM). Because the light is the important environmental factor, its impact on toxicity of CDs was also determined. Finally, the intercellular ROS generation induced CDs was examined. The finding of this study could be used for further application of CDs in biological systems.

2. Material and methods

2.1. Chemicals and reagents

Anhydrous citric acid, sodium hydroxide, yeast extract, soy peptone, D-glucose, agar, glutaraldehyde, sulfuric acid, and phosphate buffered saline (PBS) were purchased from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), osmium tetra oxide, quinine sulphite and 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA), were obtained from Sigma-Aldrich (GmbH Munich, Germany). Ethanol and butanol were purchased from Mojallali (Tehran, Iran).

2.2. Synthesis and characterization of CDs

CDs were prepared using a citric acid pyrolysis method following our previous report (Bagheri et al., 2017). Briefly, 1 gr of anhydrous citric acid was heated to 160 °C for 50 min and then neutralized to pH of 7.0 by adding sodium hydroxide (NaOH) aqueous solution (0.5 M) under continuous stirring. The stock solution of CDs was stored at 4 °C until use. Optical properties of the CDs solution were determined using ultraviolet–visible (UV–vis) spectroscopy (LAMBDA 950 UV/Vis/NIR Spectrophotometer, PerkinElmer, USA). Photoluminescence (PL) and photoluminescence excitation (PLE) spectra were obtained using a spectrophotometer (Varian Cary Eclipse Fluorescence Spectrophotometer, Agilent, USA). Quantum yield (QY) of prepared CDs was measured using a quinine sulphite standard solution (quinine sulphite dissolved in 0.05 M solution of H2SO4). The particle size distribution of CDs was determined using dynamic light scattering (DLS) (Nanophox, Sympatec GmbH, Germany). The general morphology of prepared CDs was characterized by a transmission electron microscope (TEM) (Zeiss – EM10C, Germany) operating at 80 kV. For TEM specimen preparation, the stock solution of CDs was diluted in water and sonicated for 10 min using an ultrasonic cleaner (Misonix– S3000, USA). Then a small drop of that was placed onto a copper grid supported with carbon film and dried at room temperature.

2.3. Cultivation of yeast cells and toxicity testing

To examine the toxicity effect of CDs on yeast cells, the biomass increase of treated yeast with various concentrations of CDs were compared to control. The yeast biomass was evaluated by the optical density (OD) measurement of the cell suspensions at 600 nm (Kasemets et al., 2012). *P.pastoris* wild type was grown on yeast peptone dextrose (YPD) medium (1% yeast extract/ 2% peptone / 2% dextrose). One ml aliquots of the overnight cultures of yeast cell suspension and 1 ml of different concentrations of CDs were added into the tubes, obtaining 2 ml mixtures containing yeast cells with an OD_{600} of 0.1 and CDs with the following concentrations: 0, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5 and 25 mg/ml. The mixtures were cultured at 30 °C with stirring at 200 rpm for 16 h and then the absorbance were recorded at 600 nm. All experiments were performed in triplicate; the relative cell growth is the ratio of cell number in treated sample to untreated control. Results were expressed as mean ± SD of triplicate analyses.

Pichia pastoris wild type was grown on yeast peptone dextrose (YPD) medium; 1% yeast extract, 2% peptone, 2% dextrose at 30 °C. 1 ml aliquots of the overnight cultures of yeast cell suspension and 1 ml of different concentrations of CDs were added into the tubes, obtaining 2 ml mixtures containing yeast cells with an OD_{600} of 0.1 and CDs with the following concentrations: 0, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5 and 25 mg/ml.

2.4. Mitochondrial activity assay

For better understanding of the toxicity effect of CDs on yeast cells, Cell proliferation and their mitochondrial activity were investigated by dimethylthiazol-2-y1–2,5-diphenyltetrazolium bromide (MTT) assay (Rahimifard et al., 2014). In brief, CDs-treated cells were exposed to 50 µl MTT reagent (0.5 mg/ml) for 3 h at 37 °C. Then, purple formazan crystal, which appeared by reducing MTT in living cells, was dissolved by adding 150 µl dimethyl sulfoxide (DMSO) to each samples. After 30 min shaking, the absorbance intensity was assessed at 570 nm using an ELISA reader (Biotech, Winooski, VT).

2.5. Investigation the interaction of CDs with surface of yeast cell

To investigate the possibility of CDs binding and interaction to the cell surface, yeast cells were incubated with different concentration of CDs (0, 5, 10, 15, 20, 25 mg/ml) for 3 h at 30 °C, while slowly shaking. The OD of cells in the yeast/CDs suspensions was 0.1. After incubation, the yeast cells were centrifuged for 5 min at 5000 rpm and the pellet washed twice with sterilized PBS to remove unbound CDs. In the next step, the cell pellet was collected and resuspended in 3 ml of PBS under gentle vortex. Finally, the emission intensity of cell-treated CDs was recorded using a spectrofluorometer with excitation and emission wavelengths were 360 and 460 nm, respectively (Verma and Stellacci, 2010).

2.6. Cells morphological examination by SEM

The morphological changes of *P.pastoris* treated with CDs were demonstrated using SEM (HITACHI S-4160). For this purpose, 10 ml of 25 mg/ml CDs solution was added into 10 ml of 2 × YPD medium. 500 µl of yeast suspension at OD_{600} 0.1 was then added into the tube. The yeast/CDs suspensions were kept at 30 °C for 16 h with an agitation of 200 rpm in a dark incubator chamber. In addition, a sample was cultivated with the same conditions without CDs, as a test of control. After the incubation, cells from both tubes were harvested by light centrifugation and fixed by 2% glutaraldehyde (in 0.1 M Na-cacodylate buffer) for 3 h, and then were post-fixed in the same buffer with 1% osmium tetroxide for 1 h. Next, samples were dehydrated through a graded series of ethanol (30%, 50%, 70%, 90%, and 100%) for 10 min for each ethanol concentration and followed by centrifugation. After