Journal of Industrial and Engineering Chemistry 57 (2018) 171–180

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

Preparation of blue-color-emitting graphene quantum dots and their *in vitro* and *in vivo* toxicity evaluation

Md Nafiujjaman[a,1], Jeongho Kim[b,1], Hun-Kuk Park[b,*], Yong-Kyu Lee[a,c,**]

[a] Department of Green Bioengineering, Korea National University of Transportation, Chunbuk, Republic of Korea
[b] Department of Biomedical Engineering, College of Medicine, Kyung Hee University, Seoul, Republic of Korea
[c] Department of Chemical & Biological Engineering, Korea National University of Transportation, Chunbuk, Republic of Korea

ARTICLE INFO

Article history:
Received 29 May 2017
Received in revised form 8 August 2017
Accepted 11 August 2017
Available online 19 August 2017

Keywords:
Graphene quantum dot
Quantum yield
Cytotoxicity
Erythrocyte
Hemorheology

ABSTRACT

We synthesized and characterized blue-color-emitting graphene quantum dots (BGQDs) which is better than that of other GQDs synthesized from organic molecules. We investigated the biocompatibility of BGQDs with cells and erythrocytes. Evaluation of epithelial carcinoma cancer cells indicated that most cells successfully took up BGQDs and toxicity testing showed no effect on cell viability. We also examined alterations in deformability and aggregation of human erythrocytes exposed to BGQDs at different concentrations and incubation times. These results suggest that BGQDs are biocompatible and do not cause cell membrane damage, and thus, are promising nanomaterials for various medical diagnoses and treatments.

Introduction

Graphene nanomaterials and its derivatives have attracted tremendous attention in various research areas because of their optical, electrochemical, and biocompatible properties [1]. In addition, they have unique properties such as blue to green luminescence, excellent photostability, intrinsic physical and chemical characteristics, and biocompatibility [2]. There are few bioimaging and optoelectronic applications for graphene materials because of their zero bandgap properties [3]. As a result, photoluminescent graphene, which generates a bandgap, has been developed.

There are various approaches for synthesizing graphene quantum dots (GQDs), which can typically be divided into two groups: top–down and bottom up methods [4]. Top–down methods are based on exfoliation of carbon materials to form small sizes [5]. Photoluminescent GQDs can be obtained from carbon sources by a top–down method, where reaction conditions and purification processes are complicated and time-consuming. This top down method is used several steps, making it very difficult purify additional products. This method also uses acid solutions. Additionally, the top–down method produced graphene with low product yield and low quantum yield [6]. In contrast to top–down approaches, bottom-up methods fabricate GQDs from small carbon precursors [7] in a one-step process [8]. However, bottom-up methods have some limitations such as complex purification processes and their time-consuming nature.

Because GQDs are chemically inert compared to conventional quantum dots, they are not toxic to cells [9]. However, the toxicity mechanism of GQDs in a wide variety of biomedical applications requires further investigation. Yuan et al. [10] reported that the cytotoxicity of different functionalized groups on modified GQDs in human lung carcinoma (A549) and human C6 glioma cells at a concentration of 200 μg/mL. Nurunnabi et al. [11] found no *in vitro* and *in vivo* toxicity of carboxylated GQDs at concentrations of 500 μg/mL and 10 mg/kg, respectively. Jiang et al. [12] demonstrated that low-dose GQDs had little effect on zebrafish embryos and larvae.

Most *in vivo* and *in vitro* studies investigated the hemolysis and morphology of red blood cells (RBCs) to evaluate nanoparticle toxicity [13–15]. A few papers have reported the effects of nanomaterial size and aspect ratio on hemorheological changes in human RBCs such as deformability and aggregation [16,17]. Some studies of graphene toxicity have investigated the hemolysis and alteration of RBC morphology, but the effects on deformability

* Corresponding author at: Department of Biomedical Engineering, College of Medicine, Kyung Hee University, 26 Kyungheedae-ro, Seoul 02447, Republic of Korea.
** Corresponding author at: Department of Green Bioengineering, Korea National University of Transportation, Daehak-ro, Chungju-si, Chunbuk 27469, Republic of Korea.
E-mail addresses: sigmoidus@khu.ac.kr (H.-K. Park), leeyk@ut.ac.kr (Y.-K. Lee).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.jiec.2017.08.019
1226-086X/

and aggregation of RBCs remain unknown. GQDs are less hemolytic than other nanomaterials are at higher concentrations [13,18–20]. Their results demonstrated that GQD-treated RBCs showed significant morphological alterations such as echinocytic or acanthocytic shapes.

The purpose of this study was to synthesize blue-color emitting GQDs (BGQDs) with high quantum yield using a simple and rapid bottom-up method by carbonizing L-glutamic acid, based on a previous method with minor modifications [6,21]. We evaluated *in vitro* toxicity on normal cells (MDCK) and cancer cells (KB) as well as *in vivo* in mice. We also investigated the hemolysis, deformability, and aggregation of RBCs exposed to BGQDs and observed RBC shapes by scanning electron microscopy (SEM).

Materials and methods

Synthesis of BGQDs

BGQDs were synthesized by pyrolyzing L-glutamic acid as previously reported with minor modifications [6,21]. Briefly, 2 g L-glutamic acid was added to a glass bottle and heated to 220 °C using a heating mantle. The solid L-glutamic acid changed to liquid, and the boiling colorless liquid turned brown, indicating the formation of BGQDs. Next, 10 mL water was added to the solution followed by stirring for 30 min. The solution was cooled to room temperature, centrifuged at 10,000 × *g* for 30 min, and the supernatant was collected.

Characterization

The synthesized BGQDs were characterized by ultraviolet (UV) spectroscopy (Optizen 2120UV; Mecasys Co., Ltd., Daejeon, Korea) to analyze the absorbance of BGQDs. Photoluminescence (PL) spectroscopy (FS-2; SCINCO, Seoul, Korea) was performed to evaluate the emission profiles of BGQDs. The morphology and height of BGQDs were characterized using an atomic force microscope (AFM) (Nanoscope IV; Veeco Instruments Inc., Plainview, NY, USA) operating in tapping mode. BGQD morphology was observed using a JSM-7610F field emission scanning electron microscope (FE-SEM; JEOL, Tokyo, Japan). The average diameter of BGQDs in distilled water was measured by Dynamic light scattering (DLS) at a fixed scatting angle of 90° at 25 °C using a Zetasizer Nano S90 (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633 nm He–Ne gas laser. Thermogravimetric analysis (TGA) was performed using a SDT 2960 (TA Instruments, Inc., New Castle, DE, USA). The morphology of BGQDs was observed using a JEM-2100F field-emission transmission electron microscopy (TEM) (JEOL USA, Inc., Peabody, MA, USA). An energy-dispersive X-ray spectroscope (EDS 51-XM X1034; Oxford Instruments, Inc., Abingdon, UK) attached to the FE-SEM with an operating voltage of 5 kV, was used to analyze the relative carbon, nitrogen, and oxygen content of the BGQDs. The quantum yield of BGQDs was calculated by measuring the integrated PL intensity in aqueous solution against Rhodamine B with a quantum yield 31% [22].

In vitro *cell uptake assay*

The cellular uptake of BGQDs was evaluated in epithelial carcinoma (KB) cells and Madin Darby Canine Kidney (MDCK) cells using a confocal laser scanning microscope (LSMS 10; Zeiss, Oberkochen, Germany). Lyophilized BGQDs were dispersed in fresh Roswell Park Memorial Institute-1640 medium at a concentration of 250 μg/mL. The cells were incubated with the BGQD solution for 4 h, washed three times with phosphate-buffered saline (PBS), and then fixed with 4% formaldehyde in PBS. All procedures were performed in the dark. Cell images were collected by confocal laser scanning microscopy paired with a long pass emission filter (488–543 nm) and the BGQDs inside the cells were visualized [23].

In vitro *cellular toxicity*

KB and MDCK cells were seeded onto a 96-well plate (5×10^4 cells/well), incubated for 24 h, and then treated with different concentrations of BGQDs using PBS as the control. The cells were incubated for another 24 h in the dark at 37 °C, and then 50 μL of aqueous MTT solution (Sigma–Aldrich, St. Louis, MO, USA) was added to each well of the 96-well plate 4 h before terminating the 24-h incubation. The cells were incubated for another 4 h, the upper layer of the solution was discarded, and then MTT solubilization solution (100 μL) was added to each well to dissolve the formazan crystals by stirring with a pipette. Next, optical absorbance of the resulting solution was measured using a Varioskan Flash (Thermo Scientific, Waltham, MA, USA) at a wavelength of 570 nm, and converted to cell viability by constructing a standard curve (absorbance vs. cell numbers) [24]. Cell viability was then calculated using the following equation:

$$\text{Cell viability } (\%) = \frac{\text{ABS}_{\text{sam}}}{\text{ABS}_{\text{cont}}} \times 100 \quad (1)$$

where ABS_{sam} and ABS_{cont} are the absorbance values of the test and control samples, respectively.

Live/dead viability/cytotoxicity assay

The live/dead viability/cytotoxicity assay kit containing calcein acetoxymethyl ester (AM) (Thermo Fisher Scientific) and propidium iodide (Sigma–Aldrich) induced the live and dead cells to produce green and red emission, respectively; this was observed using fluorescence microscopy. Calcein AM shows green fluorescence after AM is hydrolyzed by intracellular esterases in live cells. Propidium iodide penetrates the nuclei of dead cells once the integrity of their cell membranes is compromised. KB cells (1×10^4 cells/well) were grown on 8-well plates for 24 h, the medium was replaced with fresh cell culture medium, and the cells were treated with BGQDs and PBS. Cells were further incubated for 24 h and then treated with the live/dead viability/cytotoxicity kit reagents before acquiring fluorescent microscopic images using an oil immersion 20× objective lens in two channels (488 and 543 nm excitation for calcein AM and propidium iodide, respectively). This processed allowed for concurrent comparison of cells exposed to light and those without light exposure [25].

In vivo *fluorescence imaging*

The 7–8-week-old nude mouse model was purchased from Orient Bio, Inc. (Gyeonggi, Korea) and were maintained under specific pathogen-free conditions. All experiments were approved by the Institutional Animal Care and Use Committee of the Korea National University of Transportation in accordance with NIH guidelines [26]. Subcutaneous tumors were implanted by inoculating epithelial carcinoma (KB) cells into one side of the mouse body. When the tumor volume was approximately 100 mm^3, 100 μL of 5 mg/kg of BGQDs were injected intravenously via the tail vein into mice. Mice were then anesthetized with ketamine via intraperitoneal injection and images of BGQD-injected mice were taken by a time domain diffuse optical tomography system. All images were acquired using a 4000 MM Pro digital imaging system (Kodak, Rochester, NY, USA). Exposure time was 30 s, and