Published in final edited form as:

React Oxyg Species (Apex). 2018 September ; 6(17): 338–348. doi:10.20455/ros.2018.855.

Graphene Quantum Dots Protect against Copper Redox-Mediated Free Radical Generation and Cardiac Cell Injury

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Abstract

In this work, we investigated the effects of graphene quantum dots (GQDs) on copper redox-mediated free radical generation and cell injury. Using electron paramagnetic resonance (EPR) spectrometry in conjunction with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trap, we found that GQDs at a concentration as low as 1 µg/ml significantly inhibited Cu(II)/H₂O₂-mediated hydroxyl radical formation. GQDs also blocked Cu(II)-catalyzed nucleophilic addition of H₂O to DMPO to form a DMPO-OH adduct in the absence of H₂O₂, suggesting a potential for GQDs to inhibit copper redox activity. Indeed, we observed that the presence of GQDs prevented H₂O₂-mediated reduction of Cu(II) to Cu(I) though GQDs themselves also caused the reduction of Cu(II) to Cu(I). To further investigate the effects of GQDs on copper redox activity, we employed the Cu(II)/hydroquinone system in which copper redox activity plays an essential role in the oxidation of hydroquinone to semiquinone radicals with consequent oxygen consumption. Using oxygen polarography as well as EPR spectrometry, we demonstrated that the presence of GQDs drastically blocked the oxygen consumption and semiquinone radical formation resulting from the reaction of Cu(II) and hydroquinone. These results suggested that GQDs suppressed free radical formation via inhibiting copper redox activity. Lastly, using cultured human cardiomyocytes, we

demonstrated that the presence of GQDs also protected against Cu(II)/H₂O₂-mediated cardiac cell injury as indicated by morphological changes (e.g., cell shrinkage and degeneration). In conclusion, our work shows, for the first time, the potential for using GQDs to counteract copper redox-mediated biological damage.

Keywords

Copper redox; Electron paramagnetic resonance; Graphene quantum dots; Human cardiomyocytes; Hydrogen peroxide; Hydroxyl radical; Hydroquinone; Nanotechnology; Oxygen polarography; Spin-trapping

1. INTRODUCTION

Graphene quantum dots (GQDs) are defined as single to several layers of graphene of a size typically 10 nm. GQDs have been emerging as a nanotech modality of great diagnostic and therapeutic potential in biomedicine due to their favorable biocompatibility and unique electro-optical properties resulting from quantum confinement and edge effects [1, 2]. The size-dependent electro-optical properties have led to the widespread use of GQDs in molecular imaging of biological processes, including drug delivery and targeting [1, 2]. On the other hand, the electrochemical and redox properties of GQDs might make them useful in protecting against oxidative stress, a pathophysiological process widely implicated in human diseases [3, 4]. Indeed, early work reported potential free radical-scavenging activities of GQDs in cell-free systems [5]. In the present study, we report for the first time that GQDs potentially protected against copper redox-mediated free radical generation and cardiac cell injury. In this context, copper mobilization and the resulting redox reactions have been suggested to play an important role in oxidative tissue injury, including myocardial ischemia-reperfusion injury [6, 7].

2. MATERIALS AND METHODS

2.1. Materials

GQDs (a product of Dotz Nano, Tel-Aviv, Israel) were obtained from Sigma–Aldrich (Cat. No. 900708, St. Louis, MO, USA). The GQDs with a diameter < 5 nm, emit blue fluorescence upon excitation and have a topographic height of 1–2 nm, indicative of one to a few layers of graphene. The GQDs were dispersed in distilled water and stored at 4°C. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Enzo Life Sciences (Ann Arbor, MI, USA). Dulbecco's phosphate-buffered saline (DPBS) was from GIBCO/Thermo Fisher (Cat. No. 14190-144, Waltham, MA, USA). All other chemicals of analytical grade were from Sigma–Aldrich. The distilled water (Cat. No. 15230-147) obtained from GIBCO/Thermo Fisher was used to prepare the solutions when applicable.

2.2. Characterization of GQDs

The GQDs at 1 mg/ml were exposed to ultraviolet light and the blue fluorescence was captured using an iPhone camera. The excitation and emission wavelength of GQDs at 10

ng/ml were determined using a fluorescence spectrometer (LS 55, PerkinElmer, Waltham, MA, USA).

2.3. Electron Paramagnetic Resonance (EPR) Spectrometry

EPR spectra were obtained using an X-band EPR spectrometry system from Bruker (Billerica, MA, USA) under the following conditions [8]: microwave frequency, 9.78 GHz; microwave power, 30 mW; modulation frequency, 86 kHz; modulation amplitude, 1 G; and time constant, 5.12 ms. The EPR sample reactions were carried out in 0.1 ml DPBS unless otherwise indicated, and the samples were loaded into 50- μ l capillary tubes (Drummond Scientific, Broomall, PA, USA) before subjecting to the ERP measurement.

2.4. Assay for Measuring Cu(I) Formation

The reduction of Cu(II) to Cu(I) by H₂O₂ was determined by using the Cu(I)-specific reagent bathocuproinedisulfonic acid (BCS) [9]. In brief, Cu(II) (CuSO₄) was incubated with H₂O₂ in the presence or absence of GQDs in 1 ml DPBS containing 0.3 mM BCS at 37°C. The time-dependent formation BCS-Cu(I) complex was monitored by measuring its absorbance at 480 nm using a Beckman DU-8 spectrophotometer (Brea, CA, USA).

2.5. Oxygen Polarography

Oxygen consumption caused by the hydroquinone/Cu(II) system was monitored with a Clark-type biological oxygen detection system (YSI 5300, Yellow Springs, OH, USA) upon mixing hydroquinone with Cu(II) in the presence or absence of GQDs in 2.5 ml air-saturated DPBS at 37°C. Oxygen consumption was expressed as percentage of saturation oxygen [9].

2.6. Cell Culture

Primary human cardiomyocytes were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO₂ [10]. For cytotoxicity studies, the cells were seeded into 48-well plates at a density of 2×10^4 /well and 24 h later, the cells were exposed to H₂O₂ (100 μ M) and Cu(II) (10 μ M) in complete PBS (DPBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% D-glucose) in the presence or absence of 10 μ g/ml of GQDs at 37°C for 3 h. Immediately after this incubation, the morphological changes of the cells were examined under a phase-contrast light microscope (Nikon Elipse PSE TS 100, Tokyo, Japan) and photomicrographs were taken using a Nikon DS-Fi1 camera with an NIS-Elements D3.0 software (Nikon).

2.7. Statistical Analysis

Graphical data are expressed as means $\pm$ standard derivation (SD) from at least three separate experiments unless otherwise indicated. Differences between mean values of multiple groups were analyzed by one-way analysis of variance followed by Student–Newman–Keuls test. Differences between two groups were analyzed by Student's t test. Statistical significance was considered at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Inhibition by GQDs of Cu(II)/H₂O₂-Mediated Hydroxyl Radical Formation

The GQDs from Dotz Nano are among the graphene-based nano-products of the highest quality. The blue fluorescence and the characteristic excitation and emission wavelengths revealed in Figure 1 are in line with the characteristics described by the manufacture. As noted earlier, the Dotz Nano GQDs with a diameter < 5 nm possess a topographic of 1–2 nm, corresponding to one to a few layers of graphene.

Reaction of H₂O₂ with transition metal ions, including copper ions plays a major role in oxidative tissue injury via the generation of the highly reactive hydroxyl radicals [11]. Hence, we used the Cu(II)/H₂O₂ as a model system along with DMPO-spin trapping technique to determine the effects of GQDs on hydroxyl radical generation. As presented in Figure 2, incubation of DMPO with Cu(II)/H₂O₂ led to the formation of the typical DMPO-hydroxyl radical (DMPO-OH) spin adduct. This is in line with the formation of hydroxyl radicals from the reaction of H₂O₂ with Cu(II). In this context, Cu(II) is first reduced to Cu(I) by H₂O₂ (i.e., $\text{H}_2\text{O}_2 + \text{Cu(II)} \rightarrow \text{Cu(I)} + \text{HO}_2 + \text{H}^+$), and the Cu(I) formed in turn reacts with additional H₂O₂ to form hydroxyl radical via the so-called Fenton-type reaction: $\text{Cu(I)} + \text{H}_2\text{O}_2 \rightarrow \text{Cu(II)} + \text{OH}^\bullet + \text{OH}^-$ [12, 13]. Incubation of DMPO with Cu(II) alone (in the absence of H₂O₂) also resulted in the formation of the DMPO-OH adduct. Previous studies suggested that copper ions could catalyze the nucleophilic addition of water to DMPO forming the DMPO-OH adduct via a hydroxyl radical-independent, copper redox-mediated mechanism [14]. Nevertheless, the presence of GQDs nearly completely blocked the formation of the DMPO-OH adduct from either Cu(II)/H₂O₂ or Cu(II) alone (Figure 1A, spectra d and e). Notably, GQDs inhibited Cu(II)/H₂O₂-mediated DMPO-OH adduct formation in a concentration-dependent manner; an 80% inhibition was achieved with GQDs at 1 µg/ml (Figure 2B). We did not study the effects of GQDs at concentrations lower than 1 µg/ml. Based on the data, we speculated an inhibitory effect by GQDs at ng/ml concentrations. Such lower levels of GQDs in combination with the favorable biocompatibility of GQDs could be readily achieved in vivo without causing unwanted effects.

3.2. Inhibition by GQDs of H₂O₂-Mediated Reduction of Cu(II) to Cu(I)

Blockage of DMPO-OH adduct formation from the DMPO/Cu(II) reaction (Figure 2A, spectrum d) suggested a possible inhibition of copper redox activity by GQDs. Toward this end, we determined the effects of GQDs on H₂O₂-mediated reduction of Cu(II) to Cu(I) [the first step of the reactions leading to the formation of hydroxyl radicals from the reaction of Cu(II) and H₂O₂]. As shown in Figure 3, incubation of H₂O₂ with Cu(II) led to a time-dependent formation of Cu(I). This is in agreement with previous findings that H₂O₂ is able to reduce Cu(II) to Cu(I) [12, 13]. Incubation of Cu(II) with GQDs also led to time-dependent formation of Cu(I) though the rate was much lower than that of H₂O₂-mediated reduction. The ability of GQDs to reduce Cu(II) to Cu(I) suggested a potential electron transfer from GQDs to Cu(II). Indeed, an early study indicated that the electron transfer from GQDs to a copper complex enhanced the nuclease activity of the copper complex [15]. In addition, as noted earlier, GQDs are redox active, possessing potential antioxidant

activities [5]. Hence, it is not surprising that GQDs could reduce Cu(II) to Cu(I). Although GQDs were capable of reducing Cu(II) to Cu(I), their presence nearly completely blocked H₂O₂-mediated reduction of Cu(II) to Cu(I) (Figure 3, second curve from top). The data suggested that GQDs might sequester Cu(II), preventing H₂O₂-mediated reduction of Cu(II) to Cu(I).

3.3. Inhibition by GQDs of Copper Redox-Mediated Oxidation of Hydroquinone

The reduction of Cu(II) to Cu(I) by GQDs (Figure 3) along with the nearly complete inhibition of Cu(II)/H₂O₂-mediated hydroxyl radical formation (Figure 2A) suggested that the Cu(I) formed from the GQDs/Cu(II) was not redox active, and thus, was incapable of reacting with H₂O₂ to form hydroxyl radicals. In other words, GQDs might bind to Cu(I), preventing its oxidation to Cu(II) by oxidants, such as H₂O₂. To further investigate this potential mechanism, we next used a hydroquinone/Cu(II) system, where a Cu(II)/Cu(I) redox cycle determines the oxidation of hydroquinone with consequent oxygen consumption and semiquinone radical formation [16, 17]. As presented in Figure 4, addition of Cu(II) to hydroquinone caused a drastic increase in the oxygen consumption rate, which was in agreement with our early findings that copper mediates oxidation of hydroquinone [16, 17]. Notably, the presence of GQDs (100 µg/ml) completely blocked the hydroquinone/Cu(II)-induced oxygen consumption. As a Cu(II)/Cu(I) redox cycle is essential for Cu(II)-catalyzed oxidation of hydroquinone, leading to oxygen utilization, the complete blockage of hydroquinone/Cu(II)-induced oxygen consumption by GQDs was consistent with our hypothesis that GQDs inhibited copper redox-mediated reactions. Our previous work has shown that oxidation of hydroquinone by Cu(II) via a Cu(II)/Cu(I) redox cycle mechanism leads to the formation of semiquinone radicals [16]. We thus determined if GQDs could also inhibit the semiquinone radical formation from the hydroquinone/Cu(II) system. As demonstrated in Figure 5, the formation of semiquinone radicals from the hydroquinone/Cu(II) system was dramatically reduced in the presence of GQDs (100 µg/ml). The Cu(I)-selective chelator BCS as well as the reduced form of glutathione (GSH), a biological copper chelator also blocked the formation of the semiquinone radical from the hydroquinone/Cu(II) system. Collectively, these observations pointed to an inhibitory effect of GQDs on copper redox-mediated chemical reactions and free radical generation.

3.4. Inhibition by GQDs of Cu(II)/H₂O₂-Mediated Cardiac Cell Injury

As mentioned above, redox-active copper ions play a critical role in various oxidative stress-associated pathophysiological conditions, such as myocardial ischemia-reperfusion injury [6, 7]. Accordingly, we used cultured human primary cardiomyocytes to determine if GQDs could also protect against Cu(II)/H₂O₂-mediated cardiac cell injury. In line with the inhibitory effects on Cu(II)/H₂O₂-mediated free radical generation, the presence of GQDs drastically protected the cardiac cells from Cu(II)/H₂O₂-induced damage, as revealed by the prevention of the remarkable cell shrinkage and presence of cell debris caused by Cu(II)/H₂O₂ (Figure 6). It is of note that GQDs alone at the concentration used exerted no significant effects on cell morphology, which is consistent with the notion that GQDs, as compared with other nanomaterials, possess favorable biocompatibility and low toxicity in biological systems [1, 2].

4. CONCLUSION

In conclusion, the GQDs studied in this report appear to be able to potentially block copper redox-mediated free radical generation and cardiac cell injury at biologically relevant concentrations. Studies are currently underway to investigate the biological effects of GQDs in other oxidative stress-related disease conditions. Such studies would advance our understanding of the biological basis of using graphene-based nanomaterials for potential intervention of human diseases involving an oxidative stress mechanism.

Acknowledgments

Y.R.L., H.Z., Z.J. were supported in part by research grants from the National Institutes of Health (CA192936, HL129212, and GM124652), and an Institutional Development Grant from the North Carolina Biotechnology Center (2015-IDG-1018) for establishing the EMX^{plus} EPR Free Radical Research Facility at Campbell Medical School. The authors declare no conflicts of interest.

ABBREVIATIONS

BCS	bathocuproinedisulfonic acid
DMEM	Dulbecco's modified Eagle medium
DMPO	5,5-dimethyl-1-pyrroline <i>N</i> -oxide
DMPO-OH	DMPO-hydroxyl radical spin adduct
DPBS	Dulbecco's phosphate-buffered saline
EPR	electron paramagnetic resonance
GQDs	graphene quantum dots
GSH	reduced form of glutathione
HQ	hydroquinone

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FIGURE 1. Characteristics of GQDs

Panel A shows blue fluorescence of GQDs in a microfuge tube at 1 mg/ml in distilled water upon exposure to ultraviolet light. Panel B shows the excitation and emission wavelengths of GQDs at 10 ng/ml.

FIGURE 2. Effects of GQDs on DMPO-hydroxyl radical adduct (DMPO-OH) formation from the Cu(II)/H₂O₂ system

In panel A, DMPO (80 mM) was incubated, in 0.1 ml DPBS, with 10 μM CuSO₄, or 10 μM CuSO₄ + 100 μM H₂O₂ in the presence or absence of 100 μg/ml of GQDs at 37°C for 5 min. Immediately after this 5-min incubation, the samples were subjected to the EPR measurement, as described under the Materials and Methods section. In panel B, DMPO (80 mM) was incubated with 10 μM CuSO₄ + 100 μM H₂O₂ in the presence or absence of the indicated concentrations of GQDs at 37°C for 5 min. Immediately after this 5-min incubation, the samples were subjected to the EPR measurement, as described under the Materials and Methods section. Data represent means ± SD (n = 3). *, p < 0.05 compared with control.

FIGURE 3. Effects of GQDs on H_2O_2 -mediated reduction of Cu(II) to Cu(I)

The formation of Cu(I) was continuously monitored spectrophotometrically at 480 nm following incubation of BCS with Cu(II)/ H_2O_2 in the presence or absence of GQDs in DPBS at 37°C for 30 min. The effect of GQDs on reduction of Cu(II) to Cu(I) in the absence of H_2O_2 was also monitored. Concentrations of the reactants: H_2O_2 , 100 μM ; CuSO_4 , 10 μM ; GQDs, 100 $\mu\text{g/ml}$; and BCS, 0.3 mM. The curves are representative of 3 experiments.

FIGURE 4. Effects of GQDs on hydroquinone/Cu(II)-mediated oxygen consumption

In panel A, oxygen consumption was continuously monitored using a Clark-type oxygen electrode in a reaction volume of 2.5 ml DPBS at 37°C for the indicated time. GQDs (100 µg/ml), hydroquinone (1 mM), and CuSO₄ (10 µM) were added at the indicated time points. In panel B, the oxygen level was determined 5 min after the addition of the Cu(II), and the data represent means ± SD (n = 3). *, p < 0.05.

FIGURE 5. Effects of GQDs on semiquinone radical formation from the hydroquinone/Cu(II) system

In panel A, the formation of semiquinone radicals was determined using EPR spectrometry. Briefly, 1 mM hydroquinone (HQ) was incubated with 10 μ M CuSO₄ in the presence or absence of GQDs (100 μ g/ml), BCS (1 mM), or the reduced form of glutathione (GSH) (1 mM) in DPBS at 37°C for 5 min followed by subjecting to the EPR measurement as described under the Materials and Methods section. In panel B, the semiquinone radical EPR signal intensity resulting from the reaction of HQ (1 mM) and Cu(II) (10 μ M) in the presence or absence of GQDs (100 μ g/ml) was determined under the same experimental condition described above in panel A, and the data represent means $\pm$ SD (n = 3–4). *, p < 0.05 compared with HQ; #, p < 0.05 compared with “HQ+Cu(II)”.

FIGURE 6. Effects of GQDs on Cu(II)/H₂O₂-induced cell injury in primary human cardiomyocytes

Human cardiomyocytes were seeded into 48-well plate and the completely confluent cells were incubated with 100 μ M H₂O₂ plus 10 μ M CuSO₄ in the presence or absence of GQDs (10 μ g/ml) in complete PBS at 37°C for 3 h followed by the examination of cell morphological changes under a phase-contrast light microscope as described under the Materials and Methods section.