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Graphene Quantum Dots Potently Block Copper-Mediated Oxidative DNA Damage: Implications for Cancer Intervention

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ABSTRACT | Our early work suggested that graphene quantum dots (GQDs) block Cu(II)/Cu(I) redox cycle in biological systems. Here we report that GQDs could also potently protect against copper redox-mediated oxidative DNA damage. Using Cu(II)/hydrogen peroxide, Cu(II)/hydroquinone, and Cu(II)/ascorbic acid as three biologically relevant systems for inducing oxidative DNA damage, we demonstrated that GQDs protected against the above system-induced DNA strand breaks in ϕx-174 plasmid DNA in a concentration-dependent manner. Notably, a significant protection was observed with GQDs at 1 μg/ml, and a nearly complete protection was shown with 10 and 100 μg/ml of GQDs. Using electron paramagnetic resonance (EPR) spectrometry in conjunction with α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN)-spin trapping, we showed that the above three systems generated hydroxyl radicals, as evidenced by the formation of a POBN-CH_3 radical adduct in the presence of 0.5 M dimethyl sulfoxide (DMSO). Consistent with the protective effects of GQDs on DNA damage, the hydroxyl radical formation was markedly reduced in the presence of GQDs in a concentration dependent manner. A nearly complete blockage of the hydroxyl radical generation was seen with GQDs at 10 and 100 μg/ml. Taken together, our results showed that GQDs potently protected against oxidative DNA damage. Considering the critical role of copper in cancer development, our findings might have important implications for cancer intervention with GQD-based nanotech modality.

KEYWORDS | Ascorbic acid; Copper; DNA strand breaks; Electron paramagnetic resonance; Graphene quantum dots; Hydrogen peroxide; Hydroquinone; Hydroxyl radical; Nanotech; α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone

ABBREVIATIONS | ASC, ascorbic acid; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; GQDs, graphene quantum dots; HQ, hydroquinone; POBN, α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone; POBN-CH_3, POBN-methyl radical adduct

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CONTENTS

1. INTRODUCTION

Copper plays an important role in cancer progression likely due to its redox activity in mediating oxidative DNA damage and genomic instability as well as its stimulation of cellular processes crucial for cancer development, especially angiogenesis [1, 2]. Accordingly, copper depletion has been proposed as a novel strategy for suppressing cancer progression and metastasis [3]. Indeed, copper-chelating compounds are effective in various animal models of cancer [3, 4]. However, the adverse effects associated with conventional copper-chelating compounds limit their use in cancer intervention. Hence, developing more effective copper-targeting anticancer drugs is warranted. Over the past several years, graphene quantum dots (GQDs) have been emerging as a nanotech modality with tremendous applications in biomedicine, especially in bioimaging and cancer drug delivery [5]. Recently, we have demonstrated that GQDs may block copper redox-mediated free radical generation and cardiac cell injury [6]. Hence, we proposed that GQDs may also block copper redox-mediated oxidative DNA damage. In the present study, we demonstrated for the first time that GQDs potently blocked copper redox-mediated oxidative DNA damage by hydrogen peroxide (H_2O_2), hydroquinone (HQ), and ascorbic acid (ASC).

2. MATERIALS AND METHODS

2.1. Materials and Agents

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. ϕX-174 RF I DNA and Lambda DNA-*Hind*III digest (DNA markers) were purchased from New England Biolabs (Beverley, MA, USA). α-(4-Pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) 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 unless indicated otherwise. The distilled water (Cat. No. 15230-147) obtained from GIBCO/Thermo Fisher was used to prepare the solutions when applicable.

2.2. Assay for DNA Strand Breaks

DNA cleavage was assessed by detecting the conversion of supercoiled ϕX-174 RF I double-stranded DNA to open circular and linear forms [7]. Briefly, 0.3 μg DNA was incubated with Cu(II)/H_2O_2, Cu(II)/HQ, or Cu(II)/ASC in the presence or absence of GQDs in a final volume of 24 μl at 37°C for 30 min. Following incubation, the samples were immediately put on ice and mixed with 3 μl of DNA gel loading buffer, and then loaded into 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate, and 2 mM ethylenediaminetetraacetic acid (EDTA) and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer. After electrophoresis, the gels were stained with EZ-Vision in-gel fluorescent DNA dye (Amresco, Solon, OH, USA) for 30 min, followed by another 30 min destaining in water.

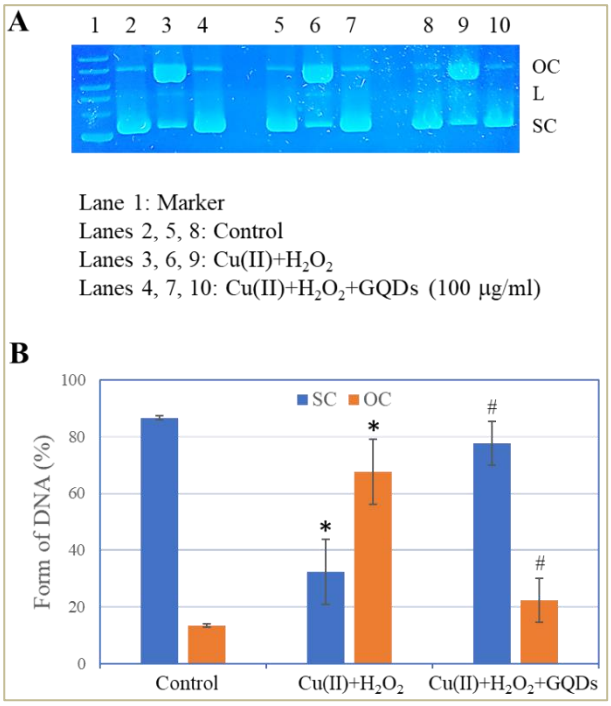

FIGURE 1. Effects of GQDs (100 µg/ml) on Cu(II)/H_2O_2-mediated DNA strand breaks. The ϕX-174 RF I DNA was incubated with Cu(II)/H_2O_2 (10 µM/25 µM) in the presence or absence of 100 µg/ml GQDs at 37ºC for 30 min and the different forms of the DNA, namely, supercoiled (SC), open circular (OC), and linear (L), were resolved upon gel electrophoresis followed by DNA staining as described under the Materials and Methods section. Data in panel B represent mean ± SD (n = 3). *, $p < 0.05$ compared to "Control"; #, $p < 0.05$ compared to "Cu(II)+H_2O_2".

The gels were then photographed under ultraviolet illumination and quantified using software associated with the Alpha Innotech Imaging system (San Leandro, CA, USA).

2.3. Electron Paramagnetic Resonance (EPR) Assay for Hydroxyl Radical Formation

EPR spectra were obtained using an X-band EPR spectrometry system from Bruker (Billerica, MA, USA) under the following conditions: 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, and the samples were loaded into 50-µl capillary tubes (Drummond Scientific, Broomall, PA, USA) before subjecting to the ERP measurement [6].

2.4. Statistical Analysis

Graphical data are expressed as means ± standard derivation (SD) from at least three separate experiments unless otherwise indicated. Differences between two groups were analyzed by Student's t test. Statistical significance was considered at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. GQDs Blocked Oxidative DNA Damage Induced by Cu(II)/H_2O_2, Cu(II)/HQ, and Cu(II)/ASC

Copper is perhaps the most redox-active transition metal ion involved in oxidative DNA damage. Indeed, Cu(II) reacts with H_2O_2 as well as other endogenous biological reductants, including ASC, forming DNA-damaging radical species [8]. In addition to the above cellular molecules, exogenous chemicals, including HQ and many polyphenolic compounds also react with Cu(II), generating free radical species that cause DNA damage [9, 10]. Hence, we chose Cu(II)/H_2O_2, Cu(II)/HQ, and Cu(II)/ASC as three separate systems to induce DNA damage in a plasmid DNA system. In this regard, induction of single-strand breaks to the supercoiled double-stranded ϕX-174 RF I DNA leads to the formation of open circular DNA, while the generation of a linear form of DNA is indicative of double-strand breaks. More extensive double-strand breaks may lead to the occurrence of a DNA smear upon gel electrophoresis. As shown in **Figure 1**, incubation of the ϕX-174 RF I DNA with Cu(II)/H_2O_2 (10 µM/25 µM) for 30 min caused a significant conversion of the supercoiled DNA to the open circular form, suggesting induction of DNA single-strand breakage by Cu(II)/H_2O_2 under the above experimental conditions. Notably, the presence of GQDs at 100 µg/ml almost completely prevented the Cu(II)/H_2O_2-induced DNA strand breaks.

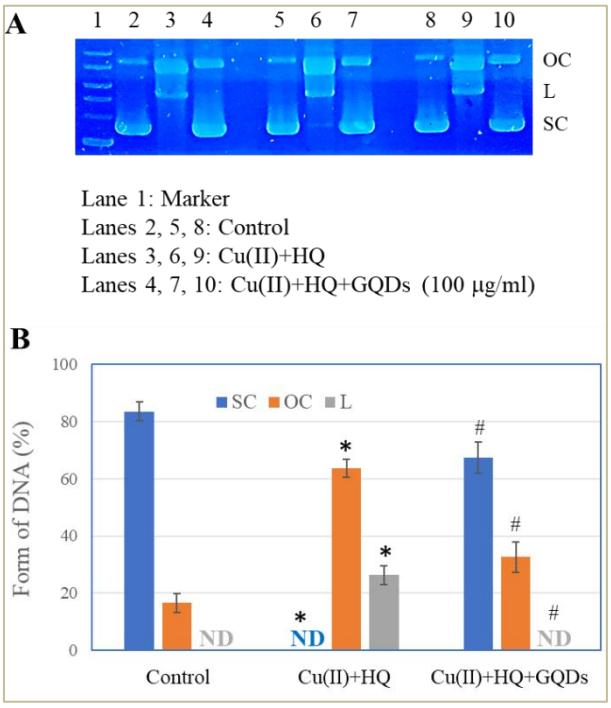

FIGURE 2. Effects of GQDs (100 µg/ml) on Cu(II)/HQ-mediated DNA strand breaks. The ϕX-174 RF I DNA was incubated with Cu(II)/HQ (10 µM/50 µM) in the presence or absence of 100 µg/ml GQDs at 37°C for 30 min and the different forms of the DNA, namely, supercoiled (SC), open circular (OC), and linear (L), were resolved upon gel electrophoresis followed by DNA staining as described under the Materials and Methods section. Data in panel B represent mean ± SD (n = 3). *, $p < 0.05$ compared to "Control"; #, $p < 0.05$ compared to "Cu(II)/HQ". ND, not detectable (i.e., the linear form in "Control", the supercoiled form in "Cu(II)+HQ", and the linear form in "Cu(II)+HQ+GQDs" groups were not detectable).

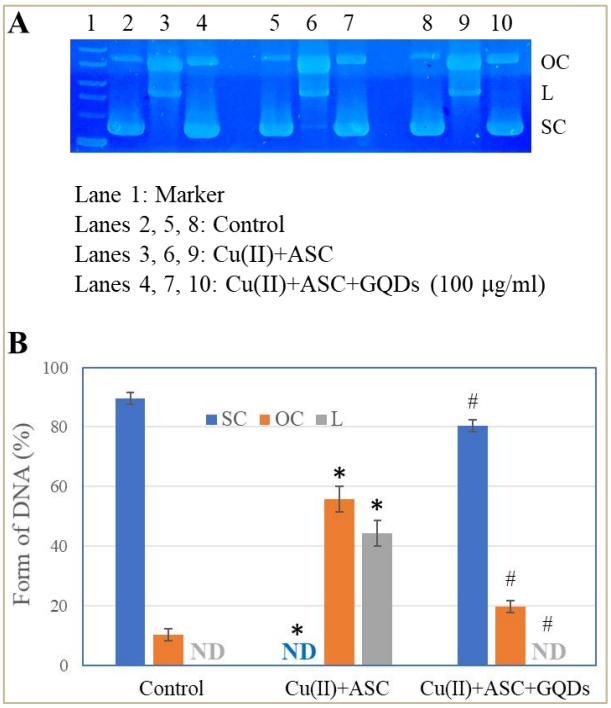

FIGURE 3. Effects of GQDs (100 µg/ml) on Cu(II)/ASC-mediated DNA strand breaks. The ϕX-174 RF I DNA was incubated with Cu(II)/ASC (10 µM/50 µM) in the presence or absence of 100 µg/ml GQDs at 37°C for 30 min and the different forms of the DNA, namely, supercoiled (SC), open circular (OC), and linear (L), were resolved upon gel electrophoresis followed by DNA staining as described under the Materials and Methods section. Data in panel B represent mean ± SD (n = 3). *, $p < 0.05$ compared to "Control"; #, $p < 0.05$ compared to "Cu(II)/ASC". ND, not detectable (i.e., the linear form in "Control", the supercoiled form in "Cu(II)+ASC", and the linear form in "Cu(II)+ASC+GQDs" groups were not detectable).

In line with findings reported in previous studies, incubation of ϕX-174 RF I DNA with either the Cu(II)/HQ (10 µM/100 µM) system or the Cu(II)/ASC (10 µM/100 µM) system for 30 min led to the complete conversion of the supercoiled DNA to the open circular and linear forms, indicative of the induction of both single- and double-strand breaks by the above two systems. It is of note that Cu(II)/HQ and Cu(II)/ASC under the above conditions also caused the formation of a DNA smear, indicating extensive DNA double-strand breaks resulting in DNA degradation (**Figures 2** and **3**). Once again, the DNA damage was nearly completely blocked by the presence of GQDs (100 µg/ml). The effects of GQDs at lower concentrations (0.1, 1, and 10 µg/ml) were also determined. As shown in **Figure 4**, GQDs at 10 µg/ml showed a protective effect against oxidative DNA damage similar to that ob-

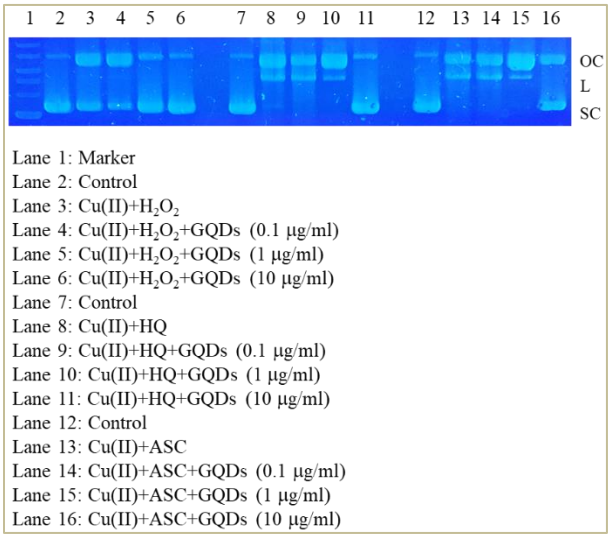

FIGURE 4. Concentration-effects of GQDs (0.1–10 µg/ml) on DNA strand breaks induced by Cu(II)/H_2O_2, Cu(II)/HQ, and Cu(II)/ASC. The ϕX-174 RF I DNA was incubated with Cu(II)/H_2O_2 (10 µM/25 µM), Cu(II)/HQ (10 µM/50 µM), or Cu(II)/ASC (10 µM/50 µM) in the presence or absence of the indicated concentrations (0.1, 1, and 10 µg/ml) of GQDs at 37ºC for 30 min and the different forms of the DNA, namely, supercoiled (SC), open circular (OC), and linear (L), were resolved upon gel electrophoresis followed by DNA staining as described under the Materials and Methods section.

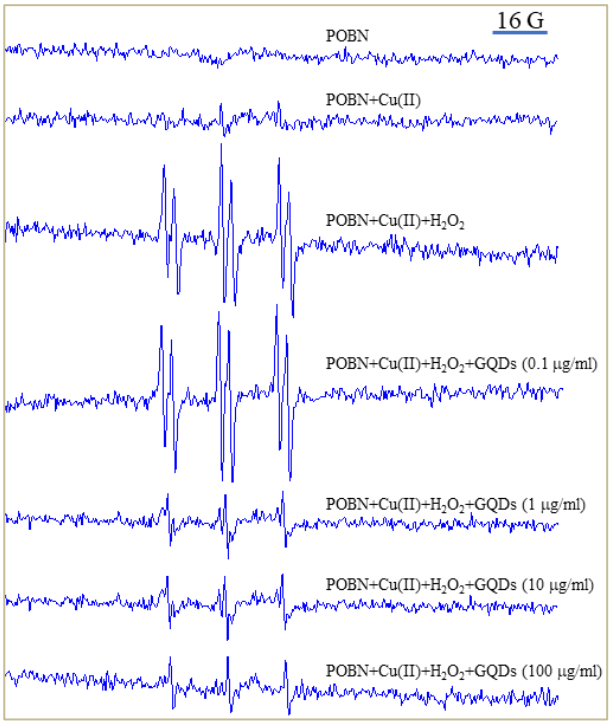

FIGURE 5. Effects of GQDs (0.1–100 µg/ml) on Cu(II)/H_2O_2-mediated hydroxyl radical formation. POBN (100 mM) was incubated with Cu(II)/H_2O_2 (10 µM/1 mM) in the presence or absence of the indicated concentrations (0.1, 1, 10, and 100 µg/ml) of GQDs in DPBS at 37ºC for 5 min followed by subjecting to EPR measurement as described under the Materials and Methods section.

served with 100 µg/ml GQDs. Although not complete, a significant protection was also seen with 1 µg/ml GQDs. Notably, the formation of a DNA smear (DNA degradation) caused by Cu(II)/HQ or Cu(II)/ASC was completely prevented with 1 µg/ml GQDs. In contrast, GQDs at 0.1 µg/ml exhibited no significant protection against DNA strand breaks induced by the above three systems.

3.2. GQDs Blocked Hydroxyl Radical Formation from Cu(II)/H_2O_2, Cu(II)/HQ, and Cu(II)/ASC

We recently showed that GQDs could potently block the Cu(II)/Cu(I) redox cycle likely via sequestering the copper ions, thereby preventing them from participating in the redox reactions [6]. Accordingly, inhibition of the Cu(II)/Cu(I) redox cycle may be primarily responsible for the protective effects of GQDs on oxidative DNA damage. As Cu(II)/Cu(I) redox cycle is involved in the formation of the DNA-damaging hydroxyl radicals from H_2O_2, HQ, and ASC, we next determined the dose-dependent effects of GQDs on hydroxyl radical formation. Towards this end, we used the POBN spin-trapping technique to specifically detect the hydroxyl radical formation [11]. As POBN has a relatively low affinity to hydroxyl radicals, dimethyl sulfoxide (DMSO, 0.5 M) was used to react with hydroxyl radicals to form methyl radicals ($CH_3^{\bullet}$), which then react readily with POBN giving rise to a POBN-CH_3 adduct with a characteristic EPR spectrum [11]. As shown in **Figure 5–7**, each of the three systems led to the formation of hydroxyl radicals. Consistent with the protective effects on DNA strand breakage, GQDs

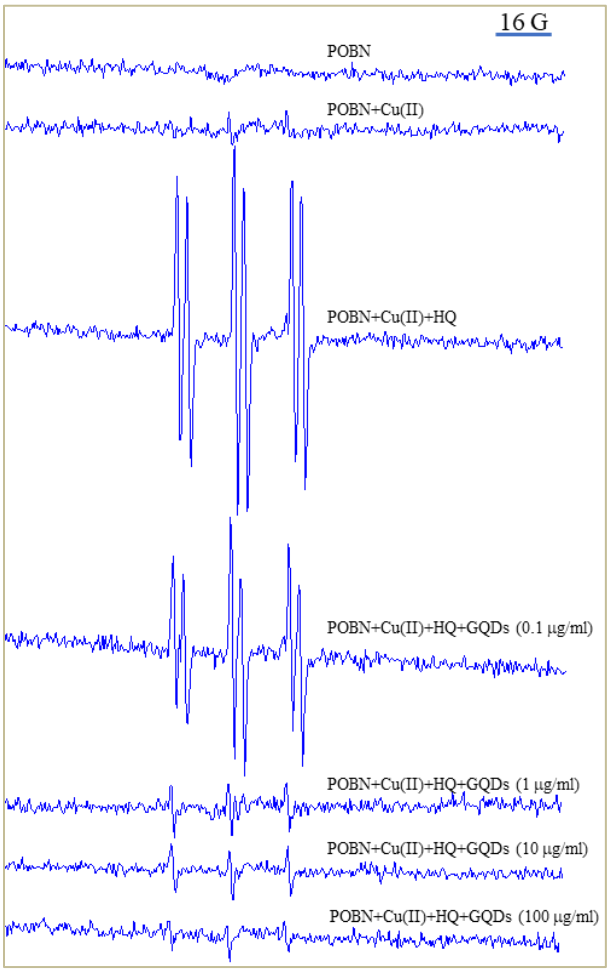

FIGURE 6. Effects of GQDs (0.1–100 µg/ml) on Cu(II)/HQ-mediated hydroxyl radical formation. POBN (100 mM) was incubated with Cu(II)/HQ (10 µM/100 µM) in the presence or absence of the indicated concentrations (0.1, 1, 10, and 100 µg/ml) of GQDs in DPBS at 37°C for 5 min followed by subjecting to EPR measurement as described under the Materials and Methods section.

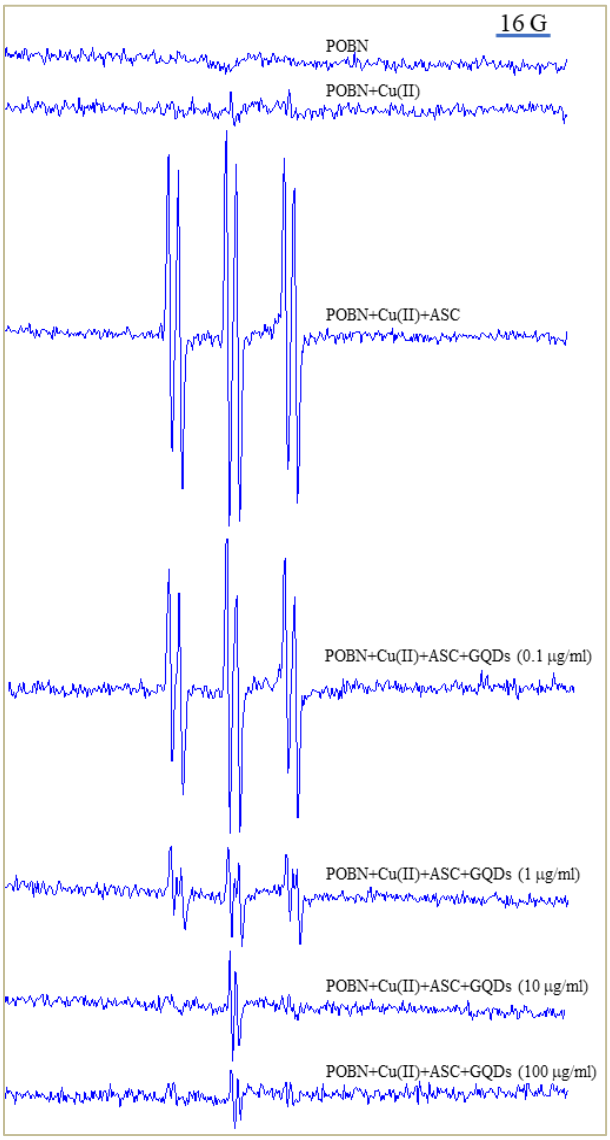

FIGURE 7. Effects of GQDs (0.1–100 µg/ml) on Cu(II)/ASC-mediated hydroxyl radical formation. POBN (100 mM) was incubated with Cu(II)/ASC (10 µM/100 µM) in the presence or absence of the indicated concentrations (0.1, 1, 10, and 100 µg/ml) of GQDs in DPBS at 37°C for 5 min followed by subjecting to EPR measurement as described under the Materials and Methods section.

(1–100 µg/ml) exerted a concentration-dependent blockage of the hydroxyl radical formation. Although GQDs at 0.1 µg/ml showed no protection against DNA damage, this concentration appeared to slightly reduce the formation of hydroxyl radicals from the Cu(II)/HQ and Cu(II)/ASC systems. The failure of translating this slight reduction in hydroxyl radical formation to into DNA protection might be due to the limited sensitivity of the gel electrophoresis assay for DNA strand breakage.

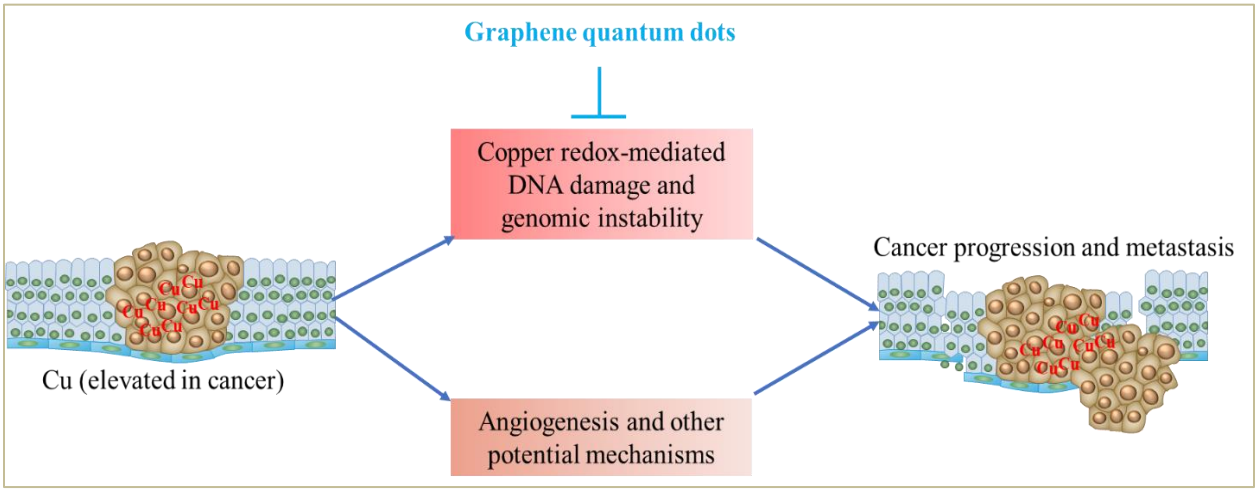

FIGURE 8. A hypothetic scheme depicting the possible use of GQDs in cancer intervention. Via blocking copper redox-mediated DNA damage and genomic instability, GQDs might be useful in retarding cancer progression and metastasis.

4. CONCLUSION

In conclusion, our results demonstrated that GQDs potently protected against oxidative DNA damage and hydroxyl radical formation induced by Cu(II)/H_2O_2, Cu(II)/HQ, and Cu(II)/ASC. Copper ions are elevated in cancer and may play an important role in cancer progression and metastasis [1, 2]. While the exact mechanisms of copper involvement in cancer development remain to be established, it has been suggested that copper ions stimulate angiogenesis, a critical event leading to cancer progression. On the other hand, copper ions react with both endogenous and exogenous compounds leading to the formation of the DNA-damaging hydroxyl radicals. Indeed, hydroxyl radicals are among the most reactive species that cause gene mutations and genomic instability, two additional critical events involved in cancer progression and metastasis. In line with the above notion, copper depletion via pharmacological agents has been shown to be effective in retarding cancer progression in animal models as well as certain pilot clinical studies [4]. Hence, due to their favorable biocompatibility, GQDs might be used as a potentially novel modality for inhibiting copper redox-mediated genomic instability in cancer progression and metastasis (**Figure 8**).

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