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Selective Toxicity of Hydroxyl-Rich Carbon Nanodots for Cancer Research

Tak H. Kim¹, Joseph P. Sirdaarta², Qian Zhang³, Ehsan Eftekhari¹, James St. John^{3,4}, Derek Kennedy³, Ian E. Cock², and Qin Li¹

¹ Queensland Micro and Nano Technology Center & School of Engineering
Griffith University, 170 Kessels Rd, Nathan QLD 4111, Australia

² Australian Rivers Institute & School of Natural Sciences
Griffith University, 170 Kessels Rd, Nathan QLD 4111, Australia

³ Griffith Institute for Drug Discovery
Griffith University, 46 Don Young Rd, Nathan QLD 4111, Australia

⁴ Menzies Health Institute Queensland
Griffith University, Parkland Drive, Southport QLD 4222, Australia

Highly biocompatible carbon nanodots were synthesized, which are abundant in hydroxyl functional groups on particle surface. Interestingly, the nanoparticles display divergent antiproliferative activities against two ovarian choriocarcinoma cell lines, which may be further explored for cancer drug discovery.

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¹ Queensland Micro and Nano Technology Center & School of Engineering, Griffith University, 170 Kessels Rd, Nathan QLD 4111, Australia

² Australian Rivers Institute & School of Natural Sciences, Griffith University, 170 Kessels Rd, Nathan QLD 4111, Australia

³ Griffith Institute for Drug Discovery, Griffith University, 46 Don Young Rd, Nathan QLD 4111, Australia

⁴ Menzies Health Institute Queensland, Griffith University, Parkland Drive, Southport QLD 4222, Australia

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ABSTRACT

The toxicity of nanoparticles in a biological system is an integration of effects arising from surface functionality, particle size and ionic dissolution etc. Its complexity suggests that generalization on a material's toxicity may be inappropriate. Moreover, from medicinal point of view, toxicity can be used for treatment of malignant cells, such as cancer. In this study, highly biocompatible carbon nanodots (named gCDs) were synthesized by reacting citric acid and urea in glycerol, which resulted in abundant hydroxyl functional groups on particle surface. gCDs show excitation-dependent photoluminescence, but with brighter green to yellow emission. Importantly, a series of toxicity assessments showed that as-synthesized gCDs possess exceptional biocompatibilities to various biological entities including 18 bacteria species, *Petunia axillaris* seedlings and *Artemia franciscana* nauplii. Furthermore, the particles were shown to have low- to non-toxic effects on human embryonic kidney (HEK-293) cell lines, breast (MCF-7), oral squamous (CAL-27) carcinoma cell lines. Of particular interest, the gCDs display antiproliferative activities against ovarian choriocarcinoma cells (JAR / Jeg-3 cell lines), which may be further explored for cancer drug discovery.

KEYWORDS

carbon nanodots, fluorescence, nanotoxicity, choriocarcinoma cells, thioredoxin reductase

1. Introduction

Nanoparticles have been envisaged to provide localized cure for challenging diseases, such as cancer, by facilitating targeted delivery and

Address correspondence to Qin Li, qin.li@griffith.edu.au

improved bioavailability. The functionalized nanoparticles can either act as the vehicle for potent drugs or they themselves can act as the therapeutic agents[1]. Carbon dots (CDs) have attracted significant research interest in the past decade owing to their excellent optical performance, low toxicity, chemical stability and flexibility and versatility in synthesis[2]. Their bright and tunable fluorescence has prompted development of CDs for a wide range of applications including biological imaging[3,4], sensing[5], solid state lighting[6,7], and drug delivery[8,9]. At its inception, CDs were generally deemed as biocompatible and of low-toxicity to nontoxic[10]. However, caution should be exercised in generalizing this conclusion. Numerous new methods with novel recipes have been developed for synthesizing CDs with improved optical properties, diverse functionalities and easier methods[11,12]. Since the interactions of nanoparticles with biological systems are an integration of effects arising from surface functionality, particle size and ionic dissolution, the toxicity of CDs needs to be evaluated case by case, instead of being generalized. On the other hand, effects on the inhibition of cellular proliferation may provide leads for drug discovery. Overall, a comprehensive understanding of the toxicity of CDs in various biological systems are required for validating the use of CDs' for further in vivo and in vitro studies.

Significant research efforts have also been made on tuning the emission colors[5,13], and understanding the fluorescence mechanisms[14] of CDs. Most of the reported CDs emit blue[15] or green fluorescence[10], which limits their broad applications, particularly in bioimaging. Although exact answers are not yet available, a consensus has been reached that in the case of CDs the surface states (both density and moiety) have a stronger influence on the emissive properties than particle

size does[13,14]. To establish a better understanding of the relationship between surface states and emission properties, comparative studies with a variety of ligands on the surface of similar CDs are

required.

Herein, we report a new type of CD that is synthesized by a hydrothermal reaction with citric acid and urea in glycerol, named gCDs. The resultant gCDs exhibit red-shifted PL emission, in comparison to CDs synthesized in water using the same synthetic method and precursors (wCDs)[16]. Interestingly, the gCDs were completely non-toxic to plants (*Petunia axillaris* seedlings), brine shrimps (*Artemia franciscana* nauplii) and several bacterial species, which is opposite to the outcome of wCDs. The gCDs exhibited nontoxic to low toxicity towards HEK-293 cells, MCF-7 and CAL-27 carcinoma cells. In contrast, the gCDs exhibited antiproliferative activities towards the ovarian choriocarcinoma cells, JAr/Jeg-3, which over-produce thioredoxin[17,18]. The study offers new insight into the in vivo and in vitro biological activities of gCDs. The selective antiproliferative activities displayed on the aggressive ovarian choriocarcinoma cells, JAR, is deserves attention and may offer a new drug lead or scaffold for the development of cancer chemotherapeutics.

2. Results and Discussion

2.1 Synthesis

The CD samples were prepared by a one-step hydrothermal method. Urea and citric acid were mixed in a 10 : 1 molar ratio (0.5 g urea and 0.16 g citric acid) in 10 mL of glycerol. The mixture was placed in a hydrothermal reactor, followed by a 24-hour reaction at 180°C (CDs samples synthesized in water, wCDs, were prepared for control experiments and results for wCDs are available in Supplementary Material). The formation of CDs in a hydrothermal system has been recognized as a

carbonization of the precursors which is considered a complex route, involving dehydration and polymerization stages[19,20]. Urea decomposes into ammonia and carbon dioxide gas under the reaction conditions, whilst citric acid forms the backbone of the CDs' framework. It has been established that pressurized ammonia gas can achieve both N doping **Figure 1** (a) Atomic force microscopy images (Inset: DLS), (b) HRTEM image and (c) Raman spectrum of 1k-gCDs; (d) IR comparison between as-synthesized and 1k-gCDs, high resolution (e) C1s, (f) N1s XPS spectrum of the 1k-gCDs, (g) UV-Vis absorption and PL emission of 1k-gCDs, (h) pictures of 1k-gCDs PL emission under room light and various excitations. The corresponding data of the 3.5k-gCDs are available in Supplementary Material.

CDs were further processed and purified via dialysis at 3.5 kD (3.5k-gCDs) and 1 kD (1k-gCDs), respectively (The sample preparation processes are illustrated in Scheme S1).

2.1 Physiochemical properties

The CDs' particle sizes were characterized by atomic force microscopy (AFM) and dynamic light scattering (DLS). As presented in Figure 1 (a), the majority of particles collected from 1kD membrane (1k-gCDs) were measured between 1 and 4 nm by AFM. The size distribution from the DLS measurement was in agreement (Figure 1 (a) inset). However, the distribution from DLS was slightly larger with particles reaching up to 20 nm. The relative increase in particle distribution in the DLS is

in the conjugated carbon structure and amine surface functionalization[21]. Additionally, adding hydroxyl rich glycerol into the system results in hydroxylamine formation, followed by reduction of the imine[22,23]. During the reaction, polymerization proceeds, resulting in nucleation and nanoparticles growth by aggregation[24]. The crude as-synthesized

likely attributable to the hydration layer from the peripheral functionality and the agglomeration of the particles. The surface charge of the gCDs determined by the DLS measurement is -15 mV, whilst the wCDs exhibited almost neutral charge (-1 mV).

TEM characterization in Figure 1 (b) indicates that the particles are spherical with multiple carbon layers. Successive planes of carbon lattices can be observed and the distance between successive planes is ~0.36 nm. The Raman spectrum of the 1k-gCDs (Figure 1 (c)) displayed two notable peaks at 1368 and 1595 cm^{-1} corresponding to the D and G band, respectively. The broad 2D band between 2700 and 2800 cm^{-1} , with a strong G band intensity, further indicates that the particles contain multiple graphitic layers[25]. The weak shoulder at 1549 cm^{-1} from the asymmetric G band may indicate the presence of

structural defects and/or oxidized products on the surface of the particles[26].

The Infrared (IR) spectra in Figure 1 (d) indicate that all of the gCDs have strong hydroxyl ($3600\text{--}3200\text{cm}^{-1}$ stretch) bands. The C=O and C-N stretches between 1750 and 1550 cm^{-1} from the as-synthesized gCDs confirms the formation and functionalization of the carbon nanoparticles. Both the as-synthesized and 1k-gCDs show relatively strong stretch O-H ($3600\text{--}3200\text{ cm}^{-1}$) and C-H stretch ($3000\text{--}2750\text{ cm}^{-1}$) due to the hydroxyl rich environment (glycerol as a solvent) for the synthesis. These strong C-H and O-H bands indicate the incorporation of glycerol on CDs surface via esterification or hydroxylamine formation[27], affording multiple hydroxyls on the particle surface. Furthermore, the 1k-gCDs display relatively stronger C=O stretch (1780 and 1655 cm^{-1}) and N-H bend (1565 cm^{-1}) than the as-synthesized CDs, indicating the existence of other functional groups such as carboxyl and amine. The C-N stretch at 1400 cm^{-1} further supports the functionalization of amine on the surface of the carbon cores[28]. Furthermore, the N-O bend with the overlapping N-O stretch at 1300 and 1510 cm^{-1} respectively further confirms the formation of hydroxylamine in glycerol, similar to other systems reported[22,23].

The samples were further characterized by x-ray photoelectron spectroscopy (XPS) to probe the chemical structures of the core and the surface functionalities. The XPS survey spectrum of the 1k-gCDs indicates that the 1k-gCDs primarily consist of carbon ($\sim 67\%$). Oxygen and nitrogen contents were 27 and 6% respectively (Figure S4 and Table S1). The high resolution C1s spectra (Figure 1 (e)) could be deconvoluted into four peaks corresponding to C-C/C=C (284.6 eV), C-O/C-N (286.0 eV), C=O (287.6 eV) and O-C=O (288.6 eV)[29]. The distinctively large amount of C-O/C-N indicates rich surface functionalities due to the participation of urea and glycerol in reaction. The high resolution spectra of N1s in Figure 1 (f) revealed four peaks at 398.4 , 399.6 , 401.1 and 402.3 eV ascribed to pyridinic N, amine, quaternary N and nitro from hydroxylamine respectively[30,31]. The presence of nitro functionality from XPS additionally supports the

formation of hydroxylamine. Furthermore, the pyridinic N in the gCDs demonstrates N-doping in the carbon ring structure during hydrothermal reaction.

The oxygen containing functional groups present in glycerol (hydroxyl) and citric acid (carboxyl) may interact with the amines in urea, contributing to the incorporation of N into the carbon nanoparticles through the hydroxylamine and imine formation followed by transformation of imine[22,23,32] to pyridinic N during carbonization on the gCDs[33,34]. In comparison, the reaction in the absence of glycerol but water may have resulted in the decomposition of urea and the formation of carbon nitride and ammonia, which would act as an intermediate for the formation of a N dopant in the carbon core[35]. This would results in rich amines on the surface of wCDs (Figure S5)[16]. The results from XPS correlate well with the data from IR characterizations.

UV-Vis characterizations in Figure 1 (g) reveal the characteristic properties of gCDs. The absorption edge indicate that the samples have absorption maxima shorter than 250 nm , which can be ascribed to $\pi\text{--}\pi^*$ transition. Shoulder peaks at $\sim 340\text{ nm}$, adjacent to its absorption edge due to $n\text{--}\pi^*$ transition, are also noted.⁴⁵ PL spectra of the 1k-gCDs in Figure 1 (g) display excitation dependent PL emission spectra similar to previous reports. Yellow PL emission was also noted when the 1k-gCDs are excited over the blue spectral range (e.g. 460 nm , Figure 1 (h) and Figure S6). Notable red-shifts were also observed from 3.5k-gCDs ($\lambda_{\text{Em}} = 520\text{ nm}$ for 1k-gCDs; cf. $\lambda_{\text{Em}} = 550\text{ nm}$ for 3.5k-gCDs at $\lambda_{\text{Ex}} = 460\text{ nm}$, Figure S6). These red shifts of PL emission are highly likely caused by the larger particle size ($1\text{--}10\text{ nm}$ 1k-gCDs; cf. $4\text{--}20\text{ nm}$ 3.5k-gCDs, Figure S1)[36]. Quantum yields of the 1k-gCDs were measured at 10% ($\lambda_{\text{Ex}} = 360\text{ nm}$).

2.2 Toxicity assessment

The toxicity of the gCDs were assessed against various biological entities including bacteria, brine shrimps (*Artemia franciscana* nauplii), *Petunia axillaris* seedlings, human embryonic kidney (HEK-293) cell

lines, breast (MCF-7), oral squamous (CAL-27) carcinoma cell lines and ovarian choriocarcinoma (JAr / Jeg-3) cell lines. 1k- and 3.5k-wCDs were also tested under identical conditions for comparison.

The CDs' antibacterial activities were assessed by Kirby-Bauer antibiotic test, which showed no antibacterial activities for neither gCDs nor wCDs (Table S2). However, when assessed against *Artemia franciscana* nauplii (brine shrimps) lethality bioassay at a concentration of $1.5 \text{ mg}\cdot\text{mL}^{-1}$ [37]. None of the gCDs displays noticeable toxic effect whilst the wCDs show mortalities comparable with the reference toxin (potassium dichromate, $1 \text{ mg}\cdot\text{mL}^{-1}$), as shown in Figure 2 (a). Fluorescence microscopy

Figure 2 (a) Mortality of the CDs tested against brine shrimps, (b) fluorescence microscopy images of brine shrimps ((1) negative control, (2) 1k-gCDs and (3) 3.5k-gCDs, top: $\lambda_{\text{Ex}} = 405$ and bottom: $\lambda_{\text{Ex}} = 488 \text{ nm}$), (c) 1k-gCDs infiltrated into *Petunia axillaris* seedling leaves ((1) after 10 m and (2) after 2 h, top: room light and bottom: 365 nm UV light) and (d) cell viability assessed at $450 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CD concentration after 24 h incubation.

images of the brine shrimps in Figure 2 (b) clearly show uptake and distribution of the sample within the brine shrimps whilst the images from the control group show no obvious visuals. The brine shrimps incubated in synthetic seawater doped with 1k-gCD and 3.5k-gCDs for 48 hrs, respectively, were clearly photoluminescent, confirming the uptake of the gCDs by the brine shrimps.

The toxicity of 1k-gCD and 1k-wCD samples were further tested by infiltrating the 1k-CDs on *Petunia axillaris* seedlings[38]. The CD samples in the leaf show visible emission under a 365nm UV lamp (Figure 2 (c), S7 and S8). The *petunia axillaris* seedling injected with 1k-gCDs did not show any noticeable

toxic effects. The leaf infiltrated by the 1k-gCDs was devoid of signs of dehydration or abnormality and the leaf returned to normal condition within 1 hour. The emission from the leaf could not be observed after one day. The seedling was observed for four weeks and showed no obvious toxic effects. This indicates that the infiltrated 1k-gCDs were metabolized by the *Petunia axillaris* seedling. However, the leaf infiltrated by the 1k-wCDs showed signs of dehydration after 2 hours, resulting in complete dehydration after 24 hours (Figure S8). Another set of assessments were performed by feeding the 1k-gCDs and 1k-wCDs directly to the *Petunia axillaris* seedlings for four weeks. No visible signs of toxicity were observed. No further effects on the growth were observed compared with the control sample (Figure S9, S10 and S11). However, the seedling grown in the presence of the CDs showed higher luminescent under UV.

The gCDs are rich in hydroxyl groups whilst the wCDs have relatively higher amounts of amine and carboxyl. Therefore, the mortality caused by the wCDs is likely originated from the amine/carboxyl on the surface, which can have strong interactions with biological entities[39]. In contrast, the benignity of gCDs could be attributed to the rich hydroxyls on the particle surface and the overall negative charge (-15mV).

In vitro toxicity was assessed on HEK293, CAL27 and MCF7 using an alamarBlue cell viability assay and JAr and Jeg-3 via an MTS assay system at an initial CD concentration of $450 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$. The results in Figure 2 (d) indicate that none of the CD samples displayed notable toxic effects (dose responses Figure S12). Interestingly, introducing the 1k-gCDs prior to the cell adhesion (shorter incubation periods) resulted in inhibition of cellular proliferation, evidenced by the results from both the MTT and alamarBlue assays (Figure S12 (e) and Figure 3 (a) respectively). The results from the JAr and Jeg-3 cell assays were particularly interesting, displaying substantial inhibition of cellular proliferation. Indeed, when dosed with $450 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ 1k-gCDs prior to cell adhesion, the JAr and Jeg-3 cell proliferation was reduced 35% and 5%, respectively, as shown in

Figure 3(a). It is noteworthy that under the incubation times/conditions utilized in these assays the cells had not adhered to the base of the well prior to addition of the test CDs. It is possible that the 1k-gCDs may block processes associated with cell adherence and thus affect cellular proliferation. Several anticancer drugs are known to function by

Figure 3 (a) JAr and Jeg-3 proliferation assessed with 1k-gCDs introduced before cell adhesion ($\mu\text{g}\cdot\text{mL}^{-1}$), confocal images of a (b) JAr and (c) Jeg-3 cell incubated with 1k-gCDs (from left: image captured at $\lambda_{\text{Ex}} = 405, 473$ nm and differential interference contrast image at $\lambda_{\text{Ex}} = 473$ nm). No noticeable fluorescence was detected from the control JAr and Jeg-3 cells (Figure S13). (d) schematic illustration of the Trx system (upper) and the Trx system with 1k-gCDs (lower).

demonstrated similar antiproliferative properties.

To further evaluate the antiproliferative efficacy of the 1k-gCDs, a dose response relationship was assessed across a range from 55 to 450 $\mu\text{g}\cdot\text{mL}^{-1}$ (Figure 3 (a)). The IC_{50} values were determined to be 215.3 and 37.7 $\mu\text{g}\cdot\text{mL}^{-1}$ for JAr and Jeg-3 cells respectively. Interestingly, under the incubation conditions resulting in non-adhered cells, 1k-gCDs inhibited both JAr and Jeg-3 proliferation. Jeg-3 cells were more susceptible with subsequently lower IC_{50} values.

Jeg-3 cells produce higher levels of thioredoxin

mechanisms which give similar effects[40]. These drugs are able to bind to tubulin monomers, blocking their polymerization[41]. This not only affects microtubule formation and thus cell division, but also affects cell adherence. Furthermore, nanoparticles including nanospheres, nanocapsules[42], and Au nanoparticles[43] have

(Trx)[17] and thus would be expected to be more resistant to oxidative toxins. However, cellular redox defences not only require Trx but also need a system of other proteins/enzymes to ensure that the Trx is in a reduced form, allowing it to overcome oxidative stress[18]. As presented in Figure 3 (d), the Trx system consists of a redox active protein (Trx, with two active cysteines), a homodimeric seleno-protein (thioredoxin reductase, TrxR) and a nicotinamide adenine dinucleotide phosphate (NADPH). The Trx undergoes reversible oxidation forming a disulfide linkage. If one of the proteins (e.g. TrxR) is not present in high enough levels or Trx is not in a

reduced state (e.g. Trx-(SH)₂), there may be an accumulation of the oxidised form[44]. Consequently, despite having higher Trx levels, the redox state of the Jeg-3 cells may result in the Trx being present in an oxidized state (e.g. Trx-S₂). If so, this would push the cells towards oxidative stress and thus higher antiproliferative effects. The 1k-gCDs in the cells (Figure 3 (b) and (c)) presumably may interact with the reduced Trx or/and TrxR, similar to other systems reported previously[45–47]. Thus, the Trx system inhibition by the 1k-gCDs may contribute to the greater antiproliferation affects, evident in the Jeg-3 cells. Further studies are ongoing to confirm if this is the case.

Figure 4 (a) TEM of 1k-gCDs^{3rd}, (b) IR spectra of the fractionated 1k-gCDs by column chromatography, PL emission intensity responses of the 1k-CDs against (c) cysteine, (d) sodium selenate, PL emission intensity responses of the 3rd fraction of 1k-gCDs against (d) cysteine, (e) sodium selenite (PL responses of the other fractions are presented in Supplementary Material).

2.3 Further sample fractionation and surface functionality

In order to evaluate the plausible interactions of the

1k-gCDs with Trx and/or TrxR, sulfur and selenium containing chemicals (cysteine and sodium selenate) were assessed by PL intensity responses of the 1k-gCDs. It is well recognized that PL emission properties of CDs are highly dependent on their surface states[5,16]. Thus, if these analytes are subject to binding onto the surface of the 1k-gCDs, changes in the PL emission would be expected. Preliminary results from the PL intensity response assessment of the 1k-gCDs against cysteine and sodium selenate are presented in in Figure 4 (c) and (d). The PL intensity of the 1k-gCDs show consecutive decreases by adding increasing amounts of the analytes (cysteine and sodium selenate, any changes of PL intensities from sodium chloride was not observed (Figure S14)). This is highly likely due to the binding of the analytes onto the surface of the 1k-gCDs. However, no changes of PL emission intensities were observed from the 1k-wCDs (figure S14) which correlates well with no cell proliferative responses from the 1k-wCDs.

In an attempt to describe the inhibition of Trx system by the 1k-gCDs' specific surface functionalities, we extended this PL assessment to further fractionated 1k-gCDs. The 1k-gCDs were fractionated into 5 different fractions by column chromatograph. Each fraction displayed different optical properties including notable changes in UV-Vis absorption peak at ~340 nm and PL emission intensities (Figure S15).

Further TEM characterization reveal that the particles in the first (1k-gCDs^{1st}) and the fifth fraction (1k-gCDs^{5th}) of the 1k-gCDs are amorphous or oligomer/polymer based carbons since no particles could be detected via the TEM characterization[14,48]. However, the TEM from the third fraction (1k-gCDs^{3rd}, Figure 7 (a)) shows that the particles are spherical with multiple carbon layers. Carbon lattices can also be observed which are separated at a distance of ~0.36 nm. These observations are identical to the TEM data from the unfractionated 1k-gCDs (Figure 1 (c)).

IR spectra of the fractionated 1k-gCDs in Figure 4 (b) further indicate that each fraction can be differentiated from each other. In contrast with the IR spectrum of the 1k-gCDs, the IR from the 1k-gCDs^{1st}

shows a notably increased amount of amine (N-H bend, 1565 cm^{-1}) and reduced hydroxyl (O-H bend, 1035 cm^{-1}). The second fraction (1k-gCDs^{2nd}) shows a relatively higher amount of both amine and hydroxyl. Hydroxyls are dominant functional groups in the third and fourth fractions (1k-gCDs^{3rd} and 1k-gCDs^{4th}). Subsequently, the majority of the functional groups on the graphite/graphene-like carbon particles are hydroxyls with other groups including carboxyls and amines. It is also notable that the 1k-gCDs^{5th} contains a significantly reduced amount of functional groups. This may indicate that the particles in 1k-gCDs^{5th} are not well functionalized. Accordingly, PL emission intensity of 1k-gCDs^{5th} is considerably decreased, in contrast with the other fractions.

The fractionated 1k-gCDs' PL responses against the analytes (cysteine, sodium selenite and sodium chloride) were further tested. As shown in Figure 7 and S16, the hydroxyl dominant 1k-gCDs^{3rd} and 1k-gCD^{4th} display comparable PL intensity responses with the 1k-gCDs (Figure 7 (c) and (d) vs. Figure 7 (e) and (f) / S16 (d)). The amine dominant 1k-gCDs^{1st} and 1kg-CDs^{2nd} show no notable PL intensity changes, indicating no interaction between the analytes and the amine functionality/carbon core. Furthermore, no changes were also observed from the 1k-gCDs^{5th} which is likely due to the lack of the surface functional groups. These findings suggest that hydroxyls on the surface or the carbon core of the 1k-gCDs may interact with sulfide and/or selenide in the Trx and/or TrxR, inhibiting the Trx system. The PL intensity responses may demonstrate a conceivable pathway of Trx system inhibition, although the analytes tested are not exactly identical to cysteine and selenocysteine in the Trx system. Further studies will be required in order to evaluate the precise mechanism of the antiproliferative activities from the 1k-gCDs.

3. Conclusions

In summary, we have synthesized a multivalent and hydroxyl-rich carbon dots, namely gCDs, by reacting citric acid and urea in glycerol under hydrothermal

condition at 180°C for 24 hours. Glycerol is likely to be tethered on the CDs surface through esterification or hydrolamine formation. The enriched surface functionalities have an impact not only on the optical properties of the gCDs, namely exhibiting bright yellow emission, but also on the dots' interactions with biological entities.

Our finding suggest that the CD samples displayed different toxic responses depending on the biological entities tested. In our comparative study using the pair of g-CDs and wCDs, since the particle sizes are comparable between the samples, the surface functional groups of the CDs are likely to be responsible for the various toxicity results. The 1k-wCDs' toxic effects against *Petunia axillaris* seedlings and *A. franciscana* nauplii may originate from carboxyl and amine surface functionalities. In contrast, the gCDs, which are predominantly covered with hydroxyls derived from glycerol, displayed very good biocompatibilities throughout the biological entities tested in this study.

Moreover, the variations in the application of gCDs in cell culture demonstrated that 1k-gCDs possess antiproliferative properties, potentially affecting cell adhesion. The antiproliferation activities were higher toward Jeg-3 choriocarcinoma cells in comparison to JAr, whereas Jeg-3 produces much higher amount of Trx. We further demonstrated that the combined effect of hydroxyl functional groups and the particle size (1 – 2 nm) of the 1k-gCDs may be responsible for the substantial antiproliferative activities of Jeg-3 via Trx system inhibition. Such an effect may be further explored for cancer drug discovery.

4. Experimental Section

Sample synthesis and fractionation: All the chemicals used for the sample preparation were purchased from Chem-Supply Pty Ltd, Australia and used as received. Synthesis of the samples was carried out in a 30mL PTFE-lined autoclave. Masses of 0.5 g of urea and 0.16 g of citric acid were mixed (10:1 molar ration) in either glycerol or water (10 mL) and placed in the autoclave. The autoclave was sealed and maintained at 180°C for 24 hours and then the autoclave was cooled to room temperature

and samples were collected for further processing. Dialysis membranes were acquired from Spectrum Laboratories, Inc. Dialysis was performed twice at different molecular weight cut-offs (MWCOs) for obtaining pure and mono-dispersed CD samples. Prior to dialysis, all the samples were filtered through 0.22 μ m syringe filters in order to remove large aggregates. The filtrate was subsequently placed in a 3.5kDa membrane bag (the volume of each dialysis bag was prepared at 80mL) and dialyzed against large amount of water (20mL of sample solution/6L of water for 36 hours, 500mL of water was added every 2 hours and left for 12 hours further). The sample collected in the 3.5KDa membrane bag was named 3.5kCDs. The sample out of the 3.5 kDa bag was condensed in a rotary evaporator and the condensed sample was placed in a 1 kDa membrane bag for further dialysis. The sample collected in the 1kDa bag was named 1k-CDs. 1k-gCDs were further fractionated by a silica gel filled column and methanol was used as an eluent. The methanol in the fractionated 1k-gCDs was evaporated by a rotavap.

Artemia franciscana nauplii toxicity screening: The *Artemia franciscana* nauplii (brine shrimp larvae) lethality bioassay was employed to examine the toxicity of CDs. *Artemia franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). The cysts were hatched in synthetic seawater and the nauplii were separated from the shells and used for the test. A volume of 400 μ L of seawater containing approximately 50 *A. franciscana* nauplii were added to wells of a 48-well plate and immediately used for bioassay. A volume of 400 μ L of samples or a reference toxin (1 mg·mL⁻¹, potassium dichromate, Chem-Supply, Australia) were transferred to the wells and incubated at 25°C under artificial light (1000 Lux). A negative control (400 μ L seawater) was run in triplicate for each plate. All treatments were performed in triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 48 hours all nauplii were sacrificed by adding 100 μ L of acetic acid (concentrated, Chem-Supply, Australia) to each well and counted to facilitate the

total % mortality per well.

Plant toxicity: The tests were performed using commercially available *Petunia axillaris* seedlings from a local nursery which were grown hydroponically. In order to feed CD samples directly to the seedlings, the soils on their root were washed and placed in flasks with cotton wool. A 3mL volume of the CD samples (concentration 200 μ g·mL⁻¹) were fed every 2 days for 4 weeks in a controlled environment. The sample infiltration tests were performed by infiltrating 0.5 mL of the CD samples at a concentration of 200 μ g·mL⁻¹ by 1 mL syringes without needles on the back of the target leaves.

Evaluation of antimicrobial activity: Antimicrobial activity of all plant extracts was determined using a disc diffusion assay. Briefly, 100 μ L of the test bacteria were grown in 10 mL of fresh nutrient broth media until they reached a count of approximately 10⁸ cells·mL⁻¹. An amount of 100 μ L of bacterial suspension was spread onto nutrient agar plates. The CDs were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were impregnated with 10 μ L of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2 hours before incubation with the test CDs. Plates were incubated at 30°C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Standard discs of ampicillin (2 μ g) and chloramphenicol (10 μ g) were obtained from Oxoid Ltd. and served as positive controls for antibacterial activity. Filter discs impregnated with 10 μ L of distilled water were used as a negative control.

Cell lines: Tongue Squamous (CAL27) carcinoma cell line was obtained from School of Life Science, Sun Yat-sen University (Guangzhou, China). Human breast (MCF7) carcinoma cell line and Human Embryonic Kidney (HEK293) cell line were donated by QIMR Berghofer Medical Research Institute (Brisbane, QLD). All the adherent cell lines were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 medium, Life technologies) supplemented with 10%

foetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO₂ in air. JAr and Jeg-3 choriocarcinoma cell lines used in this study were obtained from American Type Culture Collection (Rockville, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), supplemented with 20 mM HEPES, 10 mM sodium bicarbonate, 50 µg·mL⁻¹ streptomycin, 50 IU·mL⁻¹ penicillin, 2 mM glutamine and 10 % fetal calf serum (Life Technologies). The cells were maintained as monolayers in 75 ml flasks at 37 °C, 5 % CO₂ in a humidified atmosphere until approximately 80 % confluent.

HEK293, CAL27 and MCF7 Cell viability/inhibition assay: Cells at 70-80% confluency were harvested and plated with optimal cell density in the 384-well microplate and incubated at 37°C, 5% CO₂ overnight. The cells were treated with the sample CDs and were incubated for 24 h at 37°C in 5% CO₂. A 5 µL volume of alamarBlue (Thermo Scientific) were added in the wells after the incubation. The plate was read using a Biotek Synergy 2 multi-mode microplate reader with excitation of 560 nm and emission of 590 nm after 24 h. A medium without cells was set as the blank control whilst 0.5% DMSO served as the vehicle control. The cell inhibition was expressed as fluorescence intensity in the presence of the test CDs as a percentage of that in the vehicle control using the following formula (Equation 1):

$$\text{Cell inhibition (\%)} = 100 - \frac{F_{Exp} - F_{BC}}{F_{VC} - F_{BC}} \times 100 \quad \text{Equation 1}$$

F_{Exp} is the fluorescence intensity of the test CDs, F_{BC} is the fluorescence intensity of the blank control F_{VC} is the fluorescence intensity of the vehicle control. All the experiments were performed in biological triplicates.

Jeg-3 and JAr Cell proliferation test: A 1 ml volume of trypsin (Sigma) was added to the culture flasks and incubated at 37°C, 5 % CO₂ for 15 min to dislodge the cells. The cell suspensions were then transferred to a 10 mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 mL of

fresh media. Aliquots of the resuspended cells (70 µL, containing approximately 5000 cells) were added to the wells of a 96 well plate. A volume of 30 µL of the test CDs or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO₂ for 24 hours in a humidified atmosphere. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for further 24 hours. Absorbances were recorded at 490 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in at least triplicate and triplicate controls were included on each plate. The antiproliferative activity of each test was calculated as a percentage of the negative control using the following formula (Equation 2):

$$\text{Cell Proliferation (\%)} = \frac{A_{ct}}{A_{cc}} \times 100 \quad \text{Equation 2}$$

A_{ct} is the corrected absorbance for the test CD solution (calculated by subtracting the absorbance of the test CD solution in media without cells from the extract cell test combination) and A_{cc} is the corrected untreated control (calculated by subtracting the absorbance of the untreated control in media without cells from the untreated cell media combination). IC₅₀ values were calculated using OriginPro 9.0.0 software packages.

1k-gCDs photoluminescence quenching test: 1mL of 1k-gCDs or fractionated 1k-gCDs were mixed with 1mL of analyte solutions (cysteine, sodium selenate and sodium chloride) prepared at 5 to 50 µM. PL intensity responses were measured by fluorescent spectrometer. Cysteine and sodium chloride were purchased from Chem-Supply, Australia and sodium selenate was acquired from Sigma-Aldrich. The chemicals were used without further purification.

Sample characterization: Atomic force microscopy (AFM) images were captured on a NT-MDT NTEGRA Spectra AFM by semi-contact mode. Hydrodynamic particle size and zeta potential were measured on a Malvern Zetasizer Nano ZS using dynamic light scattering (DLS) technique. High

resolution transmission electron microscopy images were acquired from a FEI Titan G2 80-200 at 100kV. Fourier transform infrared (FTIR) spectra were recorded on a Perkin Elmer Spectrum Two FTIR spectrometer using an attenuated total reflection (ATR) attachment and a baseline correction was applied after the measurement. X-ray photoelectron spectroscopy (XPS) spectra were acquired using a Kratos Axis ULTRA X-ray photoelectron spectrometer incorporating a 165mm hemispherical electron energy analyser. Atomic concentration and peak fittings were carried out using a CasaXPS version 2.2.73 software packages. Raman spectra were recorded on a Reinshaw InVia using 514nm excitation and a baseline correction was applied. Fluorescence emission spectra were recorded on a Thermo Scientific Lumina fluorescence spectrometer and UV visible (UV-vis) absorption spectra were measured in an Agilent 8453 UV-vis spectrometer. Quantum yields (QY) were calculated by comparing the integrated photoluminescence intensity values and the absorption values with a reference sample. Fluorescence microscopy images of *A. franciscana nauplii* were captured on an Olympus IX70 epifluorescence microscope at the excitation of 405 and 488 nm and the exposure time for each excitation was 450 and 1500 ms respectively. Laser scanning confocal microscope fluorescent and differential contrast images were taken using an Olympus FV1000 microscope and processed on FV10-ASW 3.01.01.09 Viewer software packages. In order to enhance presentation, brightness and contrast were adjusted uniformly across the field of view using the FV10-ASW 3.1 Viewer software and ImageJ 1.47t software packages.

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Electronic Supplementary Material: Physiochemical and Optical properties of wCDs, Toxicity results of gCDs and wCDs, Confocal images of reference JAR and Jeg-3 cell, optical properties and PL responses of the column fractionated 1k-gCDs are available in the online version of this article at http://dx.doi.org/10.1007/s12274-***-****-

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Electronic Supplementary Material

Selective Toxicity of Hydroxyl-Rich Carbon Nanodots for Cancer Research

Tak H. Kim¹, Joseph P. Sirdaarta², Qian Zhang³, Ehsan Eftekhari¹, James St. John^{3,4}, Derek Kennedy³, Ian E. Cock², and Qin Li¹(✉)

Type author addresses here. The font is Times New Roman 9. The affiliation should be the institution where the work was conducted. If the present address of an author differs from that at which the work was carried out, please give the present address as well. For example,

¹ Queensland Micro and Nano Technology Center & School of Engineering, Griffith University, 170 Kessels Rd, Nathan QLD 4111, Australia

² Australian Rivers Institute & School of Natural Sciences, Griffith University, 170 Kessels Rd, Nathan QLD 4111, Australia

³ Griffith Institute for Drug Discovery, Griffith University, 46 Don Young Rd, Nathan QLD 4111, Australia

⁴ Menzies Health Institute Queensland, Griffith University, Parkland Drive, Southport QLD 4222, Australia

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Scheme S1 Sample synthesis and the fractionation process.

Figure S1 (a) AFM, (b) DLS and (c) Raman spectrum of 3.5k-gCDs.

Figure S2 (a) AFM, (b) DLS of 1k-wCDs, (c) AFM, (d) DLS of 3.5k-wCDs and (e) HRTEM of 1k-wCDs.

Figure S3 IR spectra of (a) starting materials (b) gCDs and (c) wCDs.

Figure S4 XPS (a) survey spectra, high resolution (b) C1s, (c) N1s and (d) O1s spectra of fractionated gCDs.

Figure S5 XPS (a) survey spectra, high resolution (b) C1s, (c) N1s and (d) O1s spectra of fractionated wCDs.

Table S1 Carbon, oxygen and nitrogen contents.

Samples	C (At. %)	N (At. %)	O (At. %)
1k-gCDs	66.8	6.0	27.2
3.5k-gCDs	65.3	7.8	26.9
1k-wCDs	58.4	6.1	35.5
3.5k-wCDs	57.6	5.8	36.6

Figure S6 PL emission map of PL map of (a) 1k-gCDs, (b) 3.5k-gCDs, (c) 1k-wCDs and (d) 3.5k-wCDs.

Figure S7 A leaf of a *Petunia axillaris* seedling infiltrated by 1k-gCDs after 10 min under (a) room light, (b) UV light, 2 hr under (c) room light, (d) UV light, 24 hr under (e) room light and (f) UV light.

Figure S8 A leaf of a *Petunia axillaris* seedling infiltrated by 1k-wCDs after 10 min under (a) room light, (b) UV light, 2 hr under (c) room light, (d) UV light, 24 hr under (e) room light and (f) UV light.

Figure S9 A *Petunia axillaris* seedling hydroponically grown by water for reference at (a) 0 week, (b) 4 weeks (c) 1 week under room light and (d) 1 week under UV light.

Figure S10 A *Petunia axillaris* seedling hydroponically grown by 1k-gCD sample at (a) 0 week, (b) 4 weeks (c) 1 week under room light and (d) 1 week under UV light.

Figure S11 A *Petunia axillaris* seedling hydroponically grown by 1k-wCD sample at (a) 0 week, (b) 4 weeks (c) 1 week under room light and (d) 1 week under UV light.

The *A Petunia axillaris* seedlings grown in the presence of the CDs showed higher luminescent under UV. It was noticeable that the emission from the plants were close to that of the UV lamp. The emission of the plants was likely reflection of the UV light by trichomes on the seedlings. The seedlings grown by the CD samples might provide extra nutrients, resulting in a higher number of trichome growth on them compared to the control seedling.

Table S2 Antibacterial test on 18 different bacterium species.

Bacteria Species	Zone of inhibition (mm)						
	1k-gC Ds	3.5k-g CDs	1k-wCD s	3.5k-w CDs	Ampicillin (+ve control)	Chloramphenicol (+ve control)	Negative control
Gram-negative rods							
<i>A. faecalis</i>	-	-	-	-	8	-	-
<i>A. hydrophilie</i>	-	-	-	-	6	21	-
<i>C. fruendi</i>	-	-	-	-	-	19	-
<i>E. coli 0157</i>	-	-	-	-	6	19.5 ± 0.71	-
<i>K.pneumoniae</i>	-	-	-	-	-	-	-
<i>P. mirabilis</i>	-	-	-	-	15	18	-
<i>P. fluorescens</i>	-	-	-	-	-	22	-
<i>S. newport</i>	-	-	-	-	9	7	-
<i>S. marescens</i>	-	-	-	-	-	19	-
<i>S. sonei</i>	-	-	-	-	8	6	-
<i>E. aerogenes</i>	-	-	-	-	8	21	-
Gram-negative bacilli							
<i>P. aeruginosa</i>	-	-	-	-	-	-	-
<i>A. baylyi</i>	-	-	-	-	-	13	-
Gram-positive rod							
<i>B. cereus</i>	-	-	-	-	-	13	-
Gram-positive cocci							
<i>S. aureus</i>	-	-	-	-	-	-	-
<i>S. epidermidis</i>	-	-	-	-	-	-	-
<i>S. pyogenes</i>	-	-	-	-	8.5 ± 0.71	-	-
<i>E. faecalis</i>	-	-	-	-	-	-	-

Figure S12 Dose response of human embryonic kidney cell line (HEK293), tongue squamous cell carcinoma cell line (CAL27) and human breast cancer cell line (MCF7) of (a) 1k-gCDs, (b) 3.5k-gCDs, (c) 1k-wCDs, (d) 3.5k-wCDs and (e) dose response of 1k-gCDs assessed under shorter incubation periods (1k-gCDs introduced prior to the cell adhesion).

Figure S13 Confocal images of a control (a) JAr cell and (d) Jeg-3 cell incubated *without* 1k-gCDs (from left: image captured at 405, 473 nm and differential interference contrast image).

Figure S14 PL intensity responses of the 1k-CDs against (a), (b) sodium chloride, (c), (d) cysteine, (e) and (f) sodium selenate. (Left column: 1k-gCDs (a), (c), (e), right column: 1k-wCDs (b), (d), (f)).

Figure S15 Optical properties of the column fractionated 1k-gCDs.

Figure S16 PL intensity responses of the fractionated 1k-gCDs against the analytes assessed. (a) 1k-gCDs^{1st}, (b) 1k-gCDs^{2nd}, (c) 1k-gCDs^{3rd}, (d) 1k-gCDs^{4th} and (e) 1k-gCDs^{5th} against 1st column: cysteine, 2nd column: sodium selenate and 3rd column: sodium chloride.