Electronic Supplementary Material (ESI) for Green Chemistry.

Sustainable synthesis of single crystalline sulphur-doped graphene quantum dots for bioimaging and beyond

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This file contains figures S1 - S10 and tables S1 – S5.

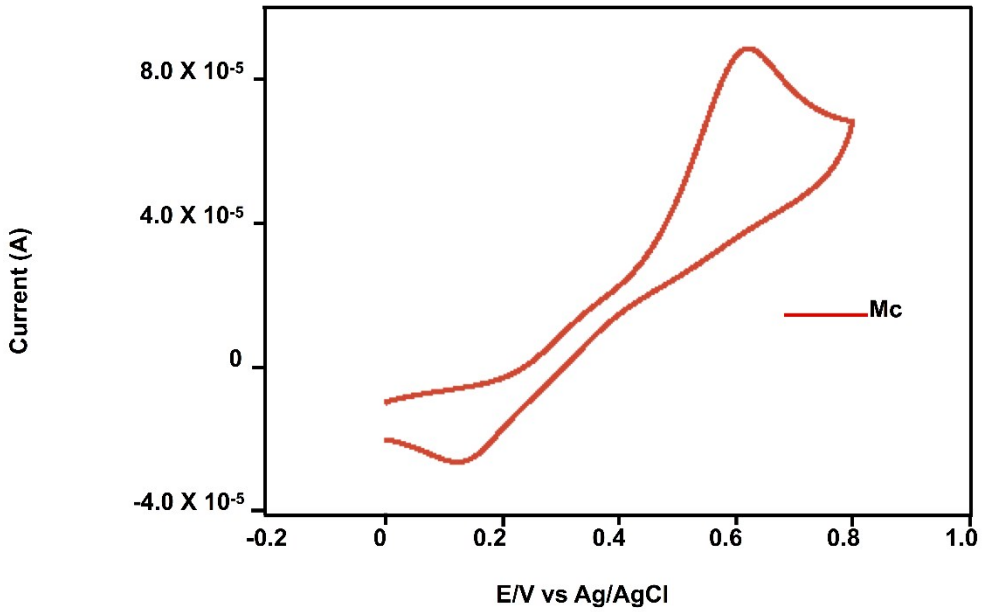

Figure S1. Conductivity study of Molasses Cake

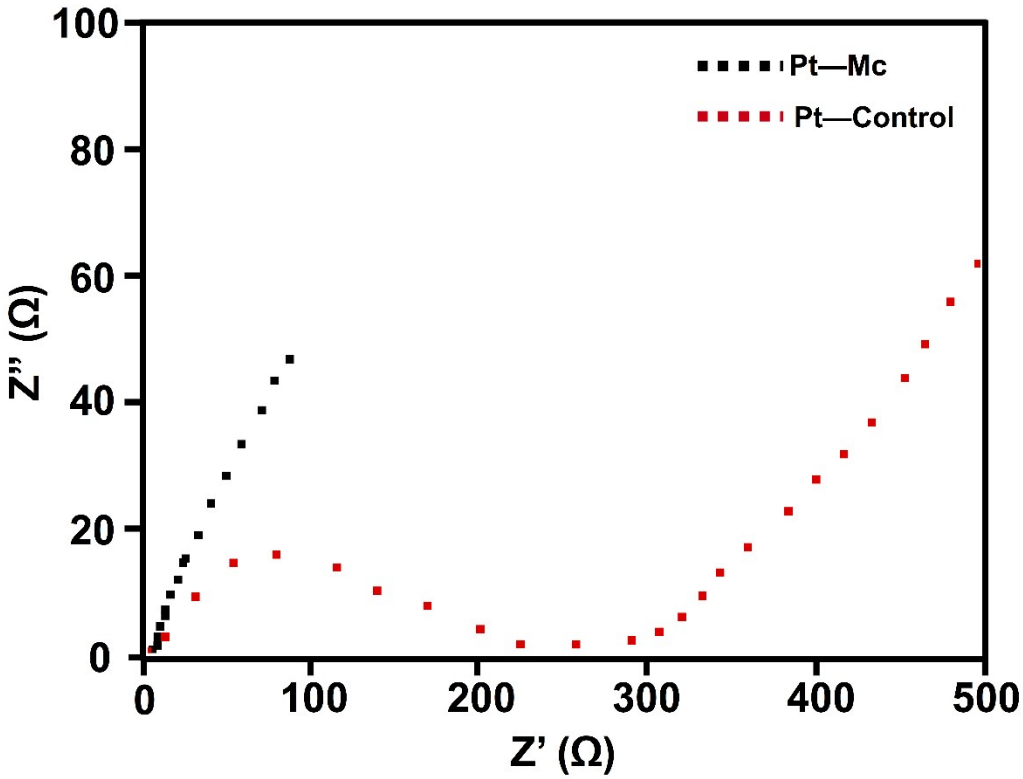

Figure S2. Electrochemical impedance spectrometry of molasses cake

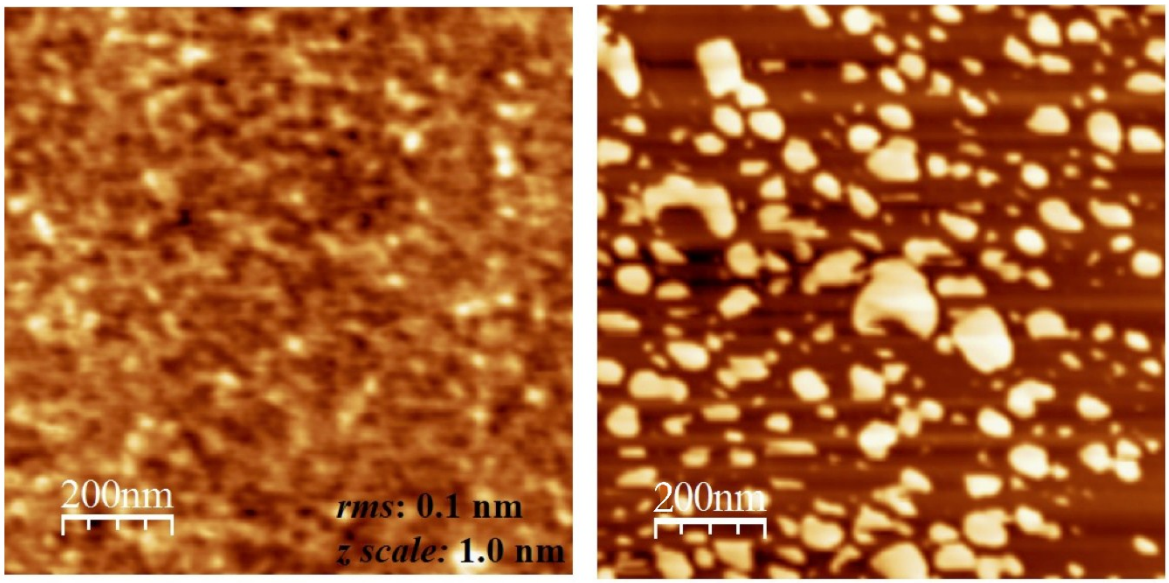

Figure S3. AFM image. (a) Pristine surface taken as background. (b) Coated with S-GQDs

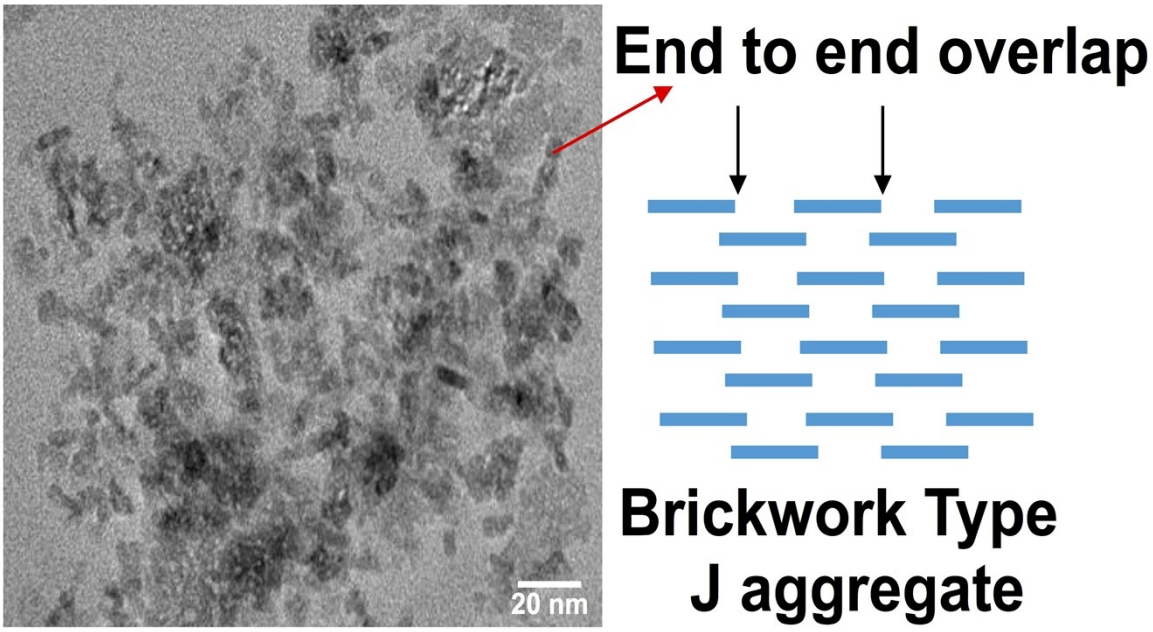

Figure S4. Schematic representation of the brickwork type J aggregation of S-GQD

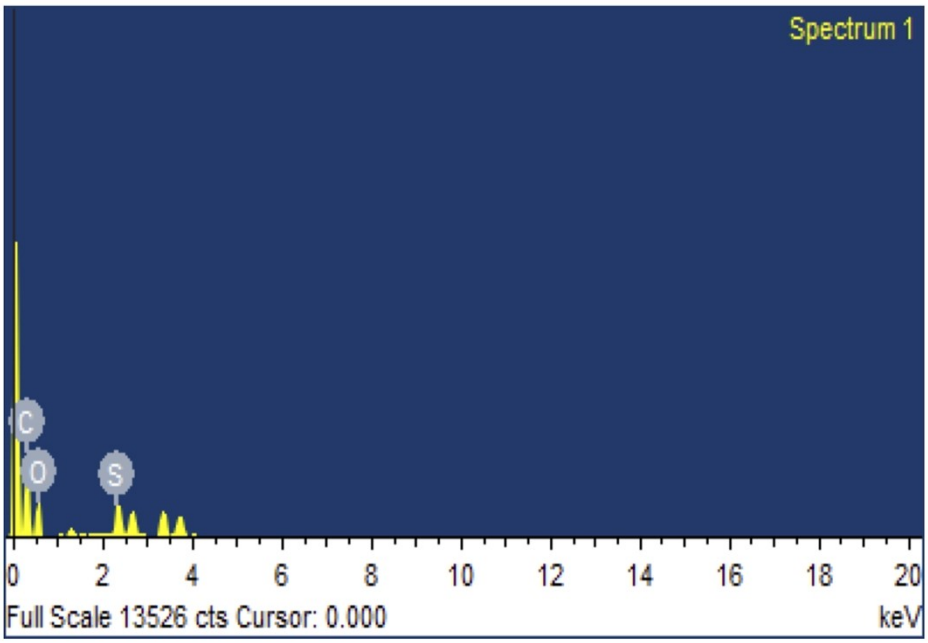

Figure S5. EDX spectra of S-GQDs showing the presence of C, O and S

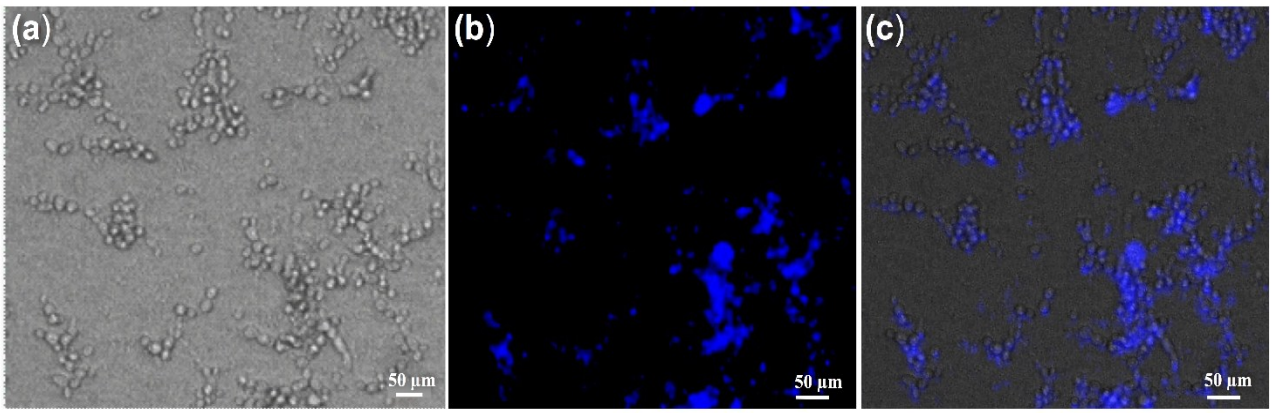

Figure S6. DIC image (a) DF-1 cells, (b) DF-1 cells treated with S-GQDs, (c) Overlay image of (a) and (b)

MTT Assay

For MTT assay, absorbance was taken at 570nm with a reference wavelength higher than 650nm as specific absorbance. The viable quantity of cells was calculated by the following equations:

$$\%\, Viablity = \frac{Specificabsorbance(test)}{SpecificAbsorbance(control)} \times 100$$

.....(1)

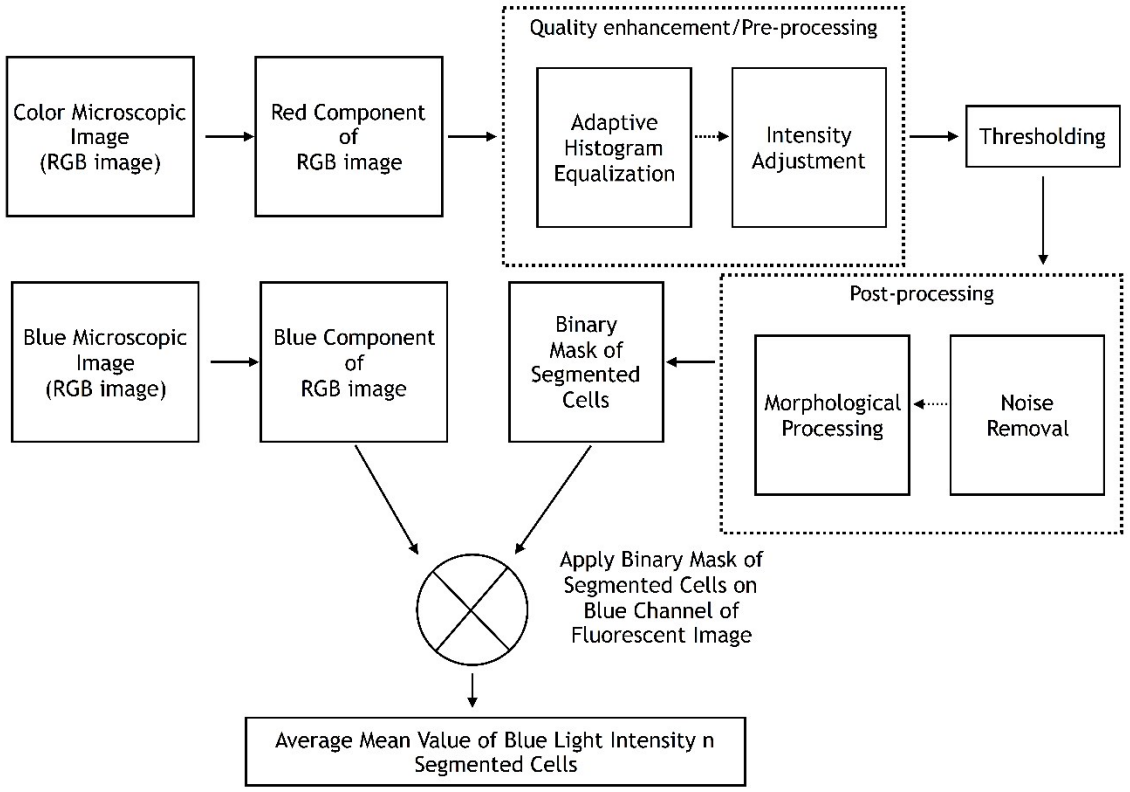

Figure S7. Schematic representation of calculation of intensity of blue light inside the cells in fluorescent microscopic images.

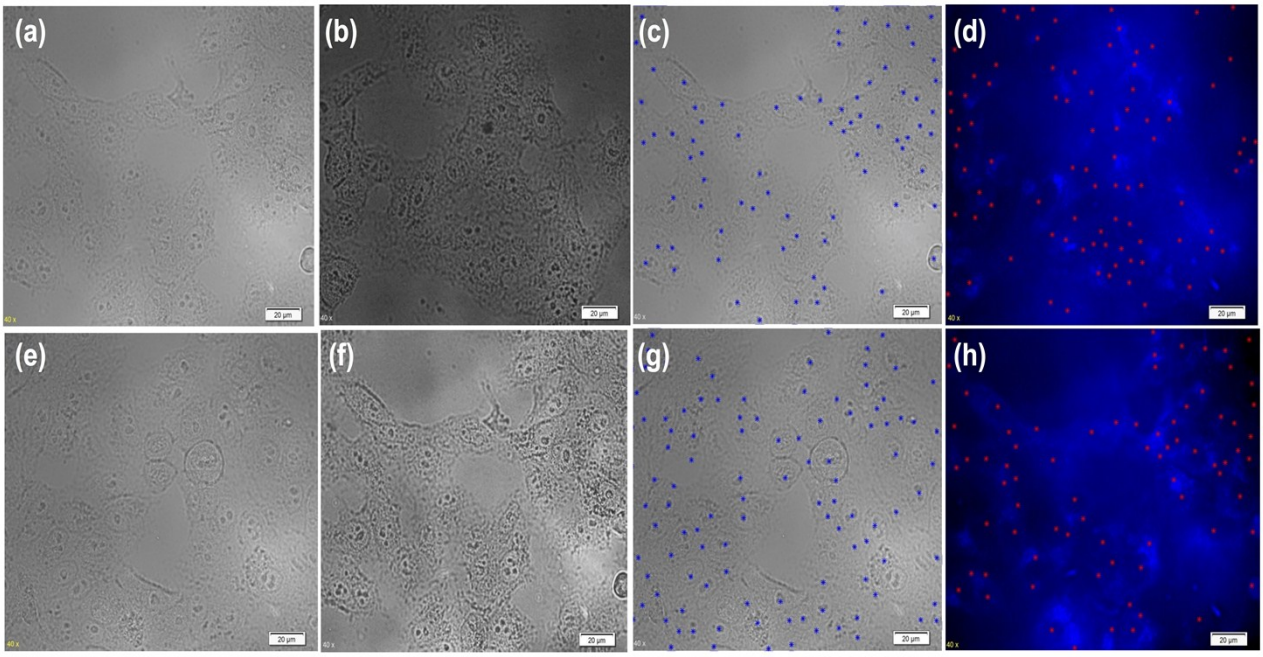

Figure S8. (a) and (e) Input DIC image of HepG2 cells, (b) and (f) Image after pre-processing, (c) and (g) Marked nuclei, (d) and (h) Segmented nuclei marked on DAPI-FITC filtered image and fluorescence intensity calculated from the cell

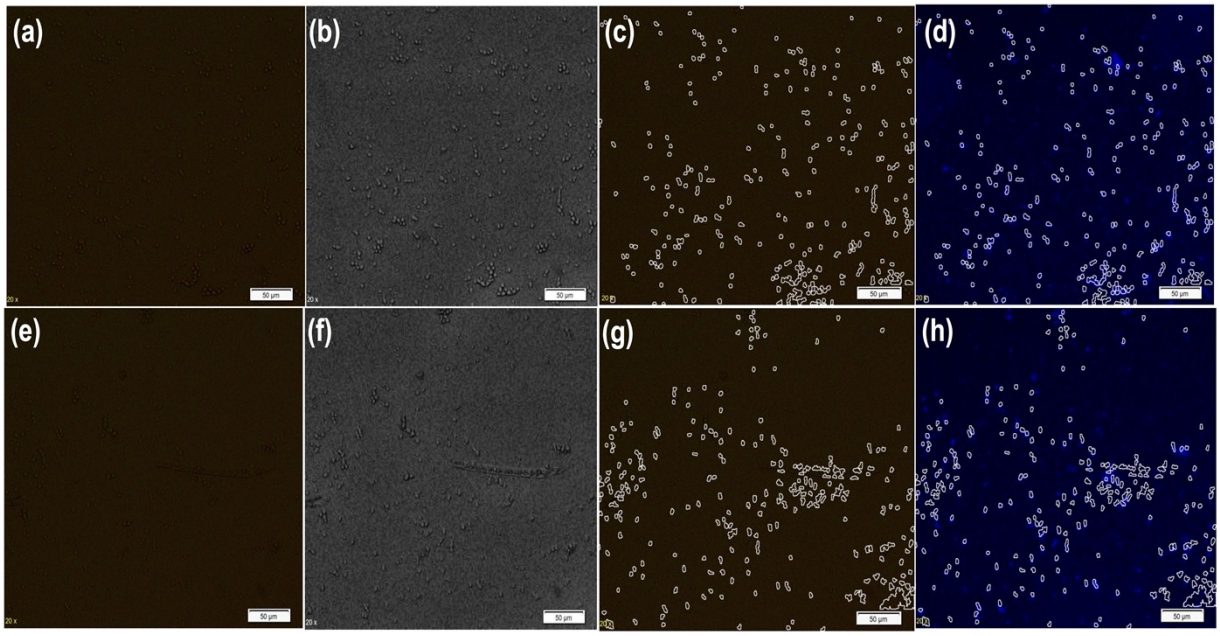

Figure S9. (a) and (e) Input DIC image of DF-1, (b) and (f) Image after pre-processing, (c) and (g) Segmented cells, (d) and (h) Segmented cells marked on DAPI-FITC filtered image and fluorescence intensity calculated from the cell.

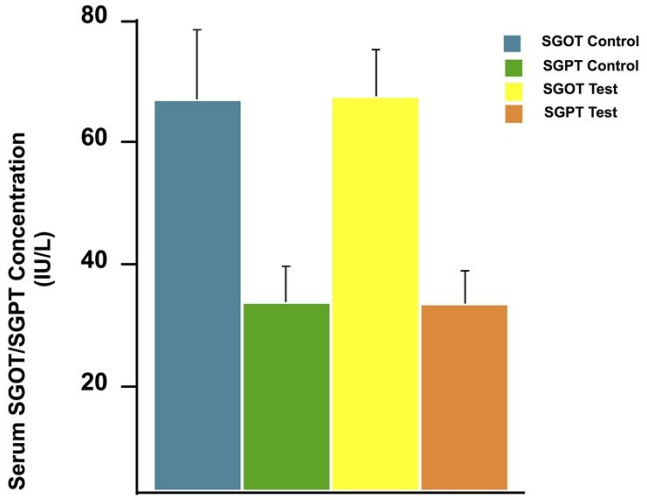

Figure S10. SGOT/SGPT analysis of Wistar Rats after S-GQD administration.

Table S1. Calculation of Quantum Yield of S-GQDs

Sample	Emission Intensity (I)	Abs at 365 nm Wavelength	Refractive index of solvent (n)	Quantum Yield	Quantum Yield (%)
Quinine sulphate	51.47	0.002	1.37	0.54	54%
GQDs	605.31	0.0258	1.34	0.4709	47.09%

Calculation of Quantum Yield (QY) of S-GQDs

Quinine sulphate taken as reference

Equation for the calculation of Quantum Yield.

$$\phi_x = \phi_y \times \frac{A_s}{A_x} \times \frac{\eta_{2x}}{\eta_{2s}} \times \frac{I_x}{I_s} \quad \text{.....(2)}$$

$$\phi_x = 0.54 \times \frac{0.002}{0.0258} \times \frac{1.34}{1.37} \times \frac{605.31}{51.47} \quad \text{.....}$$

.(3)

$$\phi_x = 0.4709 \quad \text{.....(4)}$$

Where the subscripts 'x' and 'y' denote the standard and tested samples, respectively. Φ refers to QY, and I is the measured integrated emission intensity, whereas, η and A refers to the refractive index of the solvent and optical density, respectively. The subscript "s" refers to standard with known QY. In order to minimize the re-absorption effects, absorption in the 10 mm fluorescence cuvette was kept below 0.10 at the excitation wavelength (344-360 nm).

Table S2. EDAX analysis of S-GQDs

Element	Weight %	Atomic %
C K	59.53	66.08
O K	37.73	32.32
S K	2.74	1.60

Table S3. Recovered excited state intensity decay parameters along with the goodness-of-the-fit (χ^2) for S-GQDs dissolved in water. Excitation at 340 nm using NanoLED. Error in recovered decay times is ≤ 5%. τ's are the recovered decay times and α's are the corresponding pre-exponential factors and τ_{avg} indicates an average lifetime.

$\lambda_{em}/\lambda_{ex}$	τ_1/ns (a_1)	τ_2/ns (a_1)	τ_3/ns (a_1)	τ_{avg}/ns	χ^2
425/340	1.51 (a_1= 0.77)	7.65 (a_2= 0.23)	……..	3.1	1.00
450/340	1.49 (a_1= 0.76)	7.55 (a_2= 0.24)	……..	3.1	0.98
425/340	1.42 (a_1= 0.63)	2.71 (a_2= 0.28)	8.95 (a_3= 0.09)	1.86	1.00
450/340	0.28 (a_1= 0.70)	2.47 (a_1= 0.22)	8.57 (a_1= 0.08)	1.42	1.06

Table S4. Various behavioural patterns in Functional observation battery (FOB)

Home Cage Observation		**Home Cage Removal and Handling**	**Open Field Activity**		
Spontaneous Activity [A]	Clonus	Excitation [C]	Spontaneous Activity [F]	Stereotypy	Palpebral Reflex
Posture [A]	Vocalization	Salivation [D]	Gait [G]	Diarrhoea	Visual Placing
Respiration [B]	Straubs Tail	Lacrimation [E]	Posture [A]	Auditory Response [I]	Surface and Aerial Righting
Convulsions	Writhing	Piloerection	Arousal [H]	Somatosensory Respose [J]	Pupil Reaction
Tremors	Retropulsion	Fur Appearance	Convulsions	Visual Approach	Tail Pinch Response
Fasiculations	Diarrhoea	Ptosis	Straubs Tail	Olfactory Response	Urination Spots
Tonus		Exophthalmia	Writhing	Pinna Reflex	Hind Limb Foot Splay
			Retropulsion	Extensor Reflex	Muscle Tone

[A:]1 = No activity (animal may be asleep or sitting motionless); 2 = Slight (animal moves its head or body, just a very few times); 3 = Moderate (animal moves about some); 4 = Active (animal moves more actively around cage); 5 = High activity (mice moves about rapidly)

[B:]1 = low (very slight respiration; apnea); 2 = Moderately low (slight respiration; Dyspnea); 3 = Moderately high (normal breathing); 4 = High (Hyperpnea); 5 = Very high (abnormal respiration; Tachypnea)

[C:] 1 = low (no resistance, easy to hold or prick up); 2 = moderately low (slight resistance); 3 = moderately high (some squirming or moving around); 4 = High (excited, squirming, twisting); 5 = Very high (aggressive actions, e.g., biting, tail and throat rattling).

[D:] -1 = Decreased salivation (Dryness of mouth); 0 = Normal salivation; 1 = Wetness around the mouth and chineyes; 2 = Oozing out of saliva.

[E:] -1 = Dryness of eye; 0 = Normal eye; 1 = Wetness around the eyes; 2 = Tears (Clear or tinged red)

[F:]1 = No body movement; 2 = Low (somewhat sluggish, little movement); 3 = Somewhat low (some exploratory movements); 4 = Low but active (mostly walking with very little or no running); 5 = Clearly active (exploratory movements, includes walking and running); 6 = High (very active, darting or running)

[G:]1 = low (no resistance, easy to hold or prick up); 2 = Moderately low (slight resistance); 3 = Moderately high (some squirming or moving around); 4 = High (excited, squirming, twisting); 5 = Very high (aggressive actions, e.g., biting, tail and throat rattling)

[H:]1 = Very low (stupor, coma, or prostrate); 2 = Low (sluggish, only some movements); 3 = Somewhat low (slightly sluggish, some exploratory movements); 4 = Moderate (alert, exploratory behavior); 5 = Somewhat high (slight excitement, tenseness) 6 = Very high (very alert, very excited or tense, sudden running or movements)

[I:]1 = No activity (animal may be asleep or sitting motionless); 2 = Slight (animal moves its head or body, just a very few times); 3 = Moderate (mice moves about some); 4 = Active (animal moves more actively around cage); 5 = High activity (animal moves about rapidly)

[J:]1 = No reaction or response; 2 = Slight or sluggish reaction (flinch or startle as evidence of perception); 3 = Obvious reaction (locomotor orientation as evidence of perception); 4 = Clear reaction or response (more intense startle or locomotion); 5 = Exaggerated reaction (may jump, bite, or attack).

The observation for spontaneous activity for 5, 10 and 60 mins was made for the existence of any unconventional behavior being present or absent (SI Table 2). For posture, the focus was made on the positions of the back, the belly, and the sagittal plane of the body. Presence of abnormal urination/diarrhea was observed by counting the spot/stool characteristics, respectively, on paper kept below the bedding.

In handheld position, level of excitability or resistance during handling and/or removal from the home-cage was monitored.

Table S5. Behavioral results after treatment with S-GQDs

Home Cage Observation						**Home Cage Removal & Handling**			**Open Field Activity**								
Spontaneous Activity			Clonus			Excitation			Spontaneous Activity			Stereotypy			Palpebral Reflex		
3(3,3)	2(2,2.7)	2(2,2.7)	A	A	A	2(2,2)	2(2,2)	2(1.3,2)	3.5(3,3)	3(3,3.7)	3(3,3)	A	A	A	P	P	P

Posture			Vocalization			Salivation			Gait			Diarrhoea			Visual Placing		
2(2,2)	2(2,2)	2(2,2)	A	A	A	0(0,0)	0(0,0)	0(0,0)	1(1,1)	1(1,1)	1(1,1.7)	A	A	A	P	P	P
Respiration			**Straubs Tail**			**Lacrimation**			**Posture**			**Auditory Response**			**Surface and Aerial Righting**		
3(3,3)	3(3,3)	3(2.3,3)	A	A	A	0(0,0)	0(0,0)	0(0,0)	2(2,2)	2(2,2)	2(2,2)	3(3,3)	3(2.3,3)	3(2.3,3)	P	P	P
Convulsions			**Writhing**			**Piloerection**			**Arousal**			**Somatosensory Response**			**Pupil Reaction**		
A	A	A	A	A	A	A	A	A	4(4,4)	3(3,3.7)	3(3,3.7)	3(2.3,3)	3(2.3,3)	3(2.3,3)	P	P	P
Tremors			**Retropulsion**			**Fur Appearance**			**Convulsions**			**Visual Approach**			**Tail Pinch Response**		
A	A	A	A	A	A	A	A	A	A	A	A	P	P	P	P	P	P
Fasiculations			**Diarrhoea**			**Ptosis**			**Straubs Tail**			**Olfactory Response**			**Urination Spots**		
A	A	A	A	A	A	A	A	A	A	A	A	P	P	P	A	A	A
Tonus			**Gait**			**Exophthalmia**			**Writhing**			**Pinna Reflex**			**Hind Limb Foot Splay (cm)**		
A	A	A	1(1,1)	1(1,1)	1(1,1)	A	A	A	A	A	A	P	P	P	9.7±0.2	10.0±0.3	9.6±0.2
Colour coding for different groups						Control	po	ip	3(3,3)=Median Score (25th , 75th)			cm=centimeter					

Functional observation battery (FOB)

FOB[1] was carried out to detect instant undesirable effects of S-GQDs on the central nervous system. Rats (n = 6/group) were treated with either saline or S-GQDs (10 mg/kg, with *p.o*/ *i.p* route) to observe FOB parameters after 30 mins of treatment. The scoring was done on a 5 point scale (1- lowest activity to 5- highest activity). Briefly, rats were acclimatized and observed individually for various observations categorized as home cage observation, home cage removal and handling, and open field activity (Table S4 and S5). In the open field activity, the rat was placed in the center of an open field and immediately observed for arousal and spontaneous activity. Same time observation was made for presence or absence of convulsions or any bizarre or stereotypic behavior. Surface writhing was computed by holding the animal in a supine position on a surface, releasing quickly and noted down the final position of the limbs. Aerial righting reflex was measured by holding animal in supine position 30 cm above the surface, with hands under the back and shoulders and releasing quickly. Palpebral reflex was measured by touching the eye with the sharp pointed end of the cotton ball while pinna reflex was studied by touching the skin/hair inside the ear. Visual placing response was observed by positioning the animal at the edge of an inclined table. As the animal moved down slowly toward the surface, the manner in which it extended its head and neck and reach towards the edge with the forelimbs was observed. Tail pinch response was seen by pinching the animal tail (~2 to 3 cm) from the tip using a metal clip for measuring the nociceptive behavior. Visual approach response was noted by holding a blunt object at a distance of around 3cm from the face of a rat for few seconds to see the response. The somatosensory response was examined by keeping the rat at the edge of an open field and the rump was nudged gently with a blunt object for few seconds. The auditory response was studied by a sudden sound produced by snapping the finger. Next, a petri dish containing garlic pieces was kept inside the open field and the olfactory response was examined for the perception of the stimulus. Landing hind limb foot splay test was performed by swabbing a small amount of tempera paint on the outer portion of the hind feet of Wister rats. It was held in a prone position at a standard height, typically 30 cm above a piece of paper and dropped. The distance between the centers of the marks was measured and repeated two or three times. The average of readings has been taken into account.

Image analysis

Image processing was used to calculate the intensity of light inside the cells in the fluorescent image to analyze discriminating behavior in the microscopic image of normal/cancer cells. The color microscopic image is considered as the input image for segmentation of cells in microscopic images. In the terms of image processing, the color image is the combination of three independent primary color components as red, green and blue. It was observed that cells are more distinct in red component hence the same was used for segmentation of cells. Adaptive histogram equalization followed by intensity adjustment was applied to enhance the quality of red component.[2] Cells were segmented from pre-processed red components by thresholding method[3] in which threshold parameter was empirically selected. Morphological processing[2] was applied to cells segmented image to remove noisy pixels present during thresholding. The binary mask of segmented cells was applied to blue component of the fluorescent image to calculate the average intensity of blue light inside the cells. Image processing steps followed in the proposed work are represented in the Figures S7 - S9.

Statistical analysis:

Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed using one-way analysis of variance (ANOVA) and post-hoc Dunnett's test. Two groups were compared using student t-test. $p<0.05$ were considered as statistically significant. All statistical analysis was performed using Jandel Sigma Stat 2.0 statistical software. Median was determined in FOB and statistical significance was assessed by Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunnett'stest.'p' value of less than 0.05 was considered statistically significant.

1. S. Irwin, *Psychopharmacologia,* 1968, **3**, 3, 222

2. P. Soille, *Morphological image analysis*, Springer, Berlin, 1999.