Chapter 2:

Methodology and Instrumentation

2.1 Materials Used for the Synthesis and Other Experiments of CQDs

Fresh, healthy and green Tulsi leaves were obtained from Botanical garden (Banaras Hindu University campus). Fresh bark derived from *Arjuna terminalia* was procured from the Botanical garden (Banaras Hindu University campus). Fresh marigold flowers were bought from local vendors on the day of the hydrothermal treatment. Type-I collagen derived from bovine calf skin (C8919), amino acids and proteins was purchased from Sigma-Aldrich. 100% pure sandalwood powder was obtained from a local vendor (Krishna Traders). Graphene oxide was purchased from Graphene Supermarket. MG solutions used during the experiments were prepared daily by adequate dilution of the stock solution with water. All the chemicals used in the present report were of analytical grade and used without any further chemical modification. Ultrapure Type II water with a resistivity of 10-15 MΩ.cm from a Milli-Q ultrapure water purifier (Millipore, Billerica, MA) was used throughout for all the experiments.

2.2 Preparation of CQDs

2.2.1 *Tulsi*-derived CQDs

60 grams of fresh and green Tulsi leaves were plucked and washed with water. They were briefly dipped in ethanol for 5 seconds for sterilization and left on filter paper for complete drying. Leaves were crushed using sterilized mortar and pestle and filtered with muslin cloth. The collected dark brown filtrate was centrifuged at 15,000 rpm for 35 minutes. The supernatant was mixed with water in a 1:2 ratio and heated in

a 200 ml Teflon-lined cylinder in a stainless steel hydrothermal autoclave at 200°C for 12 hours. After cooling down to room temperature, the solution was dialized against ultrapure water to obtain highly fluorescent carbon dots which were used for subsequent experiments.

2.2.2 *Arjuna-terminalia* derived CQDs

The CQDs were made by using the hydrothermal method. The bark of *Arjuna terminalia* was washed thoroughly with 0.5% sodium hypochlorite (NaOCl) solution for sterilization and chopped into small pieces. The pieces were dried and ground into a fine powder using a household mixer grinder and used as such as a reaction precursor for synthesizing carbon quantum dots. 0.2 g of the bark powder was dissolved in 20 ml each of two separate solvents, namely water and ethanol at room temperature. To remove any insoluble substances, the brown mixtures were centrifuged at 3500 rpm for 5 minutes. Each solvent-based mixture was treated at two temperatures, 160°C and 200°C thus resulting in four different conditions. For each condition, 20 ml of the supernatant obtained was heated in a 200 ml Teflon-lined cylinder in a stainless steel hydrothermal autoclave for 8 hours. After cooling down naturally to room temperature, the solution was again centrifuged at 15,000 rpm for 20 minutes to remove the less fluorescent deposits. Finally, it was passed through a 0.22 µm membrane filter to obtain fluorescent carbon dots synthesized in four different conditions denoted as E160, E200, W160 and W200. The CQD solutions were stored at 4°C for subsequent use.

2.2.3 Sandalwood-Derived CQDs

Fluorescent CQDs were synthesized using hydrothermal carbonization method and sandalwood powder as the carbon precursor source. Pure sandalwood powder was

purchased from a local vendor (Krishna Traders). 0.5 grams of pure sandalwood powder was added into 50 ml of deionised water. The mixture was stirred continuously for 10 minutes followed by briefly filtering the solution. The prepared solution was heated in a 200 ml polytetrafluoroethylene-lined cylinder in a stainless-steel hydrothermal autoclave at a constant temperature of 200°C for 8 hours. After naturally cooling down to room temperature, the light brown aqueous solution was centrifuged at 15,000 rpm for 20 minutes and passed through a 0.22μ filter to remove any solid residues. Highly fluorescent CQDs were obtained which were stored at 4°C and used as such for subsequent experiments.

2.2.4 Marigold-derived CQDs

Three cultivars of marigold- orange, yellow and brown were selected as the starting material for the hydrothermal series of reactions for the synthesis of CQDs. Different reaction parameters were applied like temperature (120°C and 200°C), addition of ions (hydrogen and hydroxide) and different solvents (DMSO, PEG, isopropanol, water, acetone and ethanol). The petals were broken, thoroughly washed with distilled water and dried on filter paper for 15 minutes for complete drying. 5 grams of the selected petals were immersed in the solvent of choice and the solution was covered to prevent evaporation. The solution-containing beaker was heated for 1 hour till the extract from the the solid residues was separated into the solvent. 200 μl of 0.2 M HCl/0.1 M NaCl was added to the respective filtrate before centrifuging at 7,500 rpm for 10 minutes. The supernatant was heated in a 200 ml Teflon-lined cylinder in a stainless steel hydrothermal autoclave at 120°C/200°C for 4 hours. After cooling down to room temperature, the solution was dialized against ultrapure water to obtain highly fluorescent carbon dots which were used for subsequent experiments.

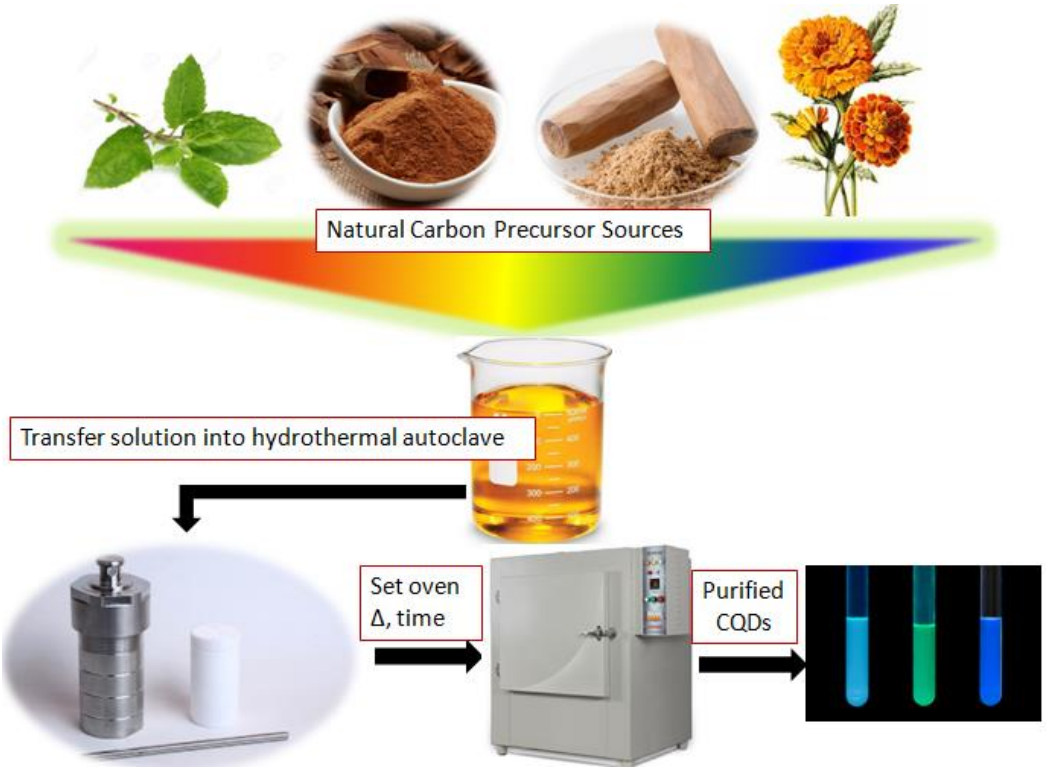

Figure 2.1: General Schematic to Illustrate a Typical Hydrothermal CQD Synthesis Process

2.3 Apparatus Used for the Experiments

The absorption and fluorescence studies were carried out using a kinetic UV-visible spectrophotometer (Eppendorf kinetic BioSpectrophotometer; Eppendorf AG, Germany) and a fluorescence steady-state spectrometer (Fluorolog FL3C-21, Horiba), respectively with excitation slit width set at 5 nm band pass and emission at 5 nm band pass in 1 cmx1 cm quartz cell. Dynamic Light Scattering (Nanopartica SZ 100 series, Horiba Scientific) was used to measure the hydrodynamic size and zeta potential. The high resolution transmission electron microscopy (HRTEM) images were obtained using a TEM microscope machine (Type FEI Tecnai™ G2 Transmission Electron Microscope) operated at 200 kV accelerating voltage, equipped with liquid nitrogen cooled sample-holder. Samples for TEM were prepared by dropping the sample solution onto a 300-mesh copper grid coated with carbon film. AFM measurements

were conducted using NTEGRA Prima, (NT-MDT Service & Logistics Ltd.). Elemental and functional group analyses were obtained by ESCALAB 250 X-ray photoelectron spectrometer and Fourier transform infrared spectrometer (Jasco FR/IR-4600), respectively. The resolution of the XPS system used for the measurement was 0.1 eV. Kratos Axis Ultra DLD.1486.4 eV Al k alpha line was used for the measurement. Fluorescence decay profile was obtained using a time-resolved fluorescence spectrophotometer (Edinburg FLS 900) using the time correlated single photon counting (TCSPC) method and milk powder as Instrument Response Function (IRF). The X-ray diffraction (XRD) pattern of carbon dots was obtained using a bench-top X-ray diffractometer (Rigaku Miniflex 600 Desktop X-Ray Diffraction System, RIGAKU Corporation,). Scanning Electron Microscopy images were obtained using Nova Nano SEM 450 (FEI Company of USA (S.E.A.) PTE, LTD) with an accelerating voltage of 10.0 kV. Bright-Field Microscopy images were captured using Radical RXLr-5 Pol optical microscope. The data recorded in the CD machine (Jasco, J-1100) can be converted to mean residue ellipticity as:

Ellipticity [Θ], in deg cm^2 $dmol^{-1}$= (millidegrees*mean residue weight)/(pathlength in millimeters*concentration in mg ml^{-1})

The biological assays were performed using Laminar Air Flow (Toshiba, Thermo Scientific 1300 series A2), standard Autoclave (Benchmark Scientific), table-top centrifuge (REMI RM-12C, Tarsons Spinwin MC-00), UV-Vis double beam spectrophotometer (Systronic 2202) and microplate absorbance reader (Biorad iMark^{TM}). Incubation of samples was done in BOD Cooling Incubator (REMI CI 10), Orbital Shaking Incubator (REMI RM-12C) humidified Thermo Scientific Hera cell vios 160i CO_2 incubator with 5% CO_2 and temperature 37°C. Bright-Field Microscopy images were captured using Radical RXLr-5 Pol optical microscope.

Tissue and cell-imaging were carried out by using Nikon LV100ND fluorescence microscope and Carl Zeiss 780 LSM laser scanning confocal microscope (Germany).

2.4 Fluorescence Quantum Yield

The quantum yield of the synthesized CQDs was estimated by the golden standard method using quinine sulphate dissolved in 0.1M H_2SO_4 solution as reference [1,2,3]. The quantum yield was calculated by using the equation given below-

$$\emptyset_{CD} = \emptyset_R \left[\frac{I_{CD}}{I_R}\right].\left[\frac{A_R}{A_{CD}}\right].\left[\frac{\eta_{CD}^2}{\eta_R^2}\right]$$

where Ø stands for the quantum yield, *I* represents the integrated emission intensity, *A* represents the observed absorbance values and η is the refractive index of the solvent. The subscripts R and CD stand for reference fluorophore (QY=0.54 for quinine sulphate) and CQDs respectively. In order to minimise re-absorption effects, the absorbance values of both solutions in cuvette were adjusted to below 0.1 (0.089) by dilution at the excitation wavelength (320 nm). The photoluminescence intensity was calculated by keeping the excitation wavelength at 320 nm to get the PL emission intensity at the same excitation wavelength, maintaining the same measured absorbance intensity for both the reference and carbon dot samples at 0.1 to avoid inner filter effect.

2.5 Experiments Related to Tulsi-derived CQDs for sensing Malachite Green

2.5.1 Free Radical Scavenging Activity

The free radical scavenging activity of the carbon dots was evaluated by the gold standard DPPH scavenging method.[4] Dose-dependent study was carried out in which carbon quantum dots (106.3-531.5µg/ml) were incubated with 0.2mM DPPH in

methanol and the mixture was shaken vigorously, allowed to stand at room temperature for 30 min, and the absorbance was measured at 517 nm. Lower the absorbance of the reaction mixture, higher is the free radical scavenging activity. The percent DPPH·scavenging effect was calculated from the following equation where A_{cont} is the absorbance of the control sample and A_{test} is the absorbance in the presence of carbon dots:

DPPH scavenging effect=$[(A_{cont}–A_{test})/A_{cont}]$x100.

2.5.2 Antibacterial Assay

Four bacterial species *Pseudomonas aeruginosa* (Gram negative), *Escherichia coli* (Gram negative), *Staphylococcus aureus* (Gram positive), *Bacillus subtilis* (Gram positive) were used as model bacteria to evaluate the antibacterial activity of carbon dots. The carbon dots were added to the bacterial culture media at various concentrations (50, 150 and 250 µg/ml), and the bacterial growth was determined by measuring the optical density of the sample measured at a wavelength of 600 nm (OD_{600}).[5,6] Moreover, the cytotoxicity of carbon dots was investigated by the standard MTT assay [1] at the same concentrations. Bacterial cells were incubated for 24 hours in the culture medium and then the cells were pelleted down by centrifuging at 8000 rpm for 5 minutes. Briefly, the cells were exposed to three different concentrations of carbon dots (50, 150 and 250 µg/ml) incubated in fresh culture media for 24h in culture medium. At the end of the treatment, 200 µL fresh culture medium containing 5 µl MTT (5mg/ml) was added and incubated for another 4 hours. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100 µL) and incubated in dark for an hour by shaking at room temperature. The absorbance at 570 nm of the solution was recorded, which indicated the intensity of the colour developed. Each sample was

prepared in triplicate. The number of viable cells was expressed as a percentage of control cells cultured in carbon dot-free medium. Cell viability in control medium without any treatment was represented as 100%. The untreated cells were used as controls for calculating the relative percentage cell viability from the following equation:

% cell viability = [A_{570} nm of treated cells/A_{570} nm of control cells] × 100

Similarly, cell growth was also expressed as a percentage of control cells cultured in carbon dot-free medium, represented as 100%. The following equation was used:

% cell viability = [OD_{600} of treated cells/OD_{600} of control cells] × 100

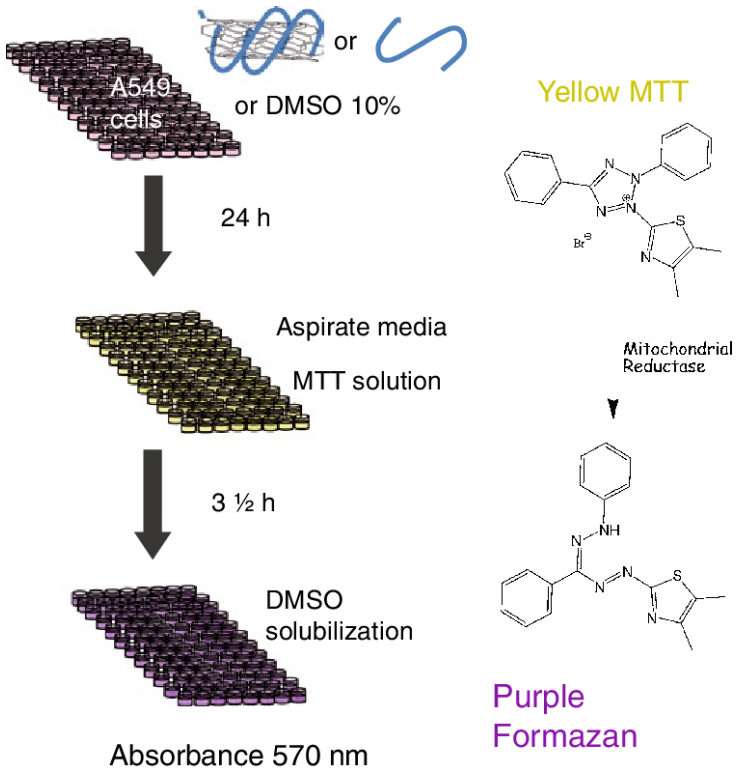

Figure 2.2: Schematic Illustrating a Typical Protocol for MTT assay (source: *Methods in molecular biology* 726 (2011) 299-312)

2.5.3 Antifungal Assay

All the fungal strains (*Candida albicans, Aspergillus niger, Aspergillus terreus, Colletotrichum falcatum*) were maintained at 4°C. The antifungal activity of bio-synthesized carbon dots was determined by disk diffusion method. Muller Hinton agar plates were prepared for determining the antifungal activity and fungal strains were grown in fresh culture broth. 100 µl of the 24 h fungal culture broth was applied on the surface of the solidified agar plates and uniformly spread by using sterilized L-shaped glass rod and was allowed to set for ten minutes. The Whatman No. 1 filter paper disks were sterilized and dipped in different concentrations of quantum dots (1.67, 5 and 8.3 mg/ml) and then dried in an oven at 30–40°C for 24 hours. For antifungal activity, carbon dot loaded disks were placed in the agar plates using sterile forceps and ultrapure water was taken as control. The plates were left for 10-15 minutes in LAF and then sealed and incubated at 27ºC for 2 days. Experiments were done in triplicate and mean values of zone diameter were taken.

2.5.4 Sensing of Malachite Green Using CQDs

Stock solution of malachite green (MG) was prepared in deionized water at 1mg/ml concentration. MG was gradually added to the aqueous solution of monodisperse carbon dots (35 µg/ml) at ambient temperature in an increasing concentration ranging from 9.1-118.3 nM.The solutions as mentioned above were transferred into a quartz cuvette, and then their fluorescence spectra were recorded at an excitation wavelength of 320 nm. For control studies, volume of MG added was replaced by ultrapure deionized water. To check the effect of ionic strength on the fluorescence stability of carbon dots, NaCl was also added in the solution containing carbon dots with sequential increment of its concentration (333 µN-4.67 mN).

2.5.5 Details of Computational Analysis Methodology

To understand the interaction between malachite green (MG) with carbon quantum dots, *in silico* analysis was done using Gaussian 09 system. A 5x5 single layer of fused benzene rings was considered as a model of carbon quantum dots. The molecules such as MG cation and model of carbon dots were fully optimized separately then their complex at M06-2X/6-31G(d,p) level of density functional theory in gas phase followed by optimization in aqueous media employing SMD solvation model at the same level of theory. To understand the effect of functionalisation in carbon quantum dots, an epoxy (-O-), hydroxyl anion (OH-), cyano (-CN) and carboxylic (-COOH) groups were added to the optimised model of carbon dots. The functionalised carbon quantum dots, then its complex with MG were also optimised at M06-2X/6-31G(d,p) level of density functional theory in aqueous media employing SMD solvation model.

2.5.6 Detection of Malachite Green in Aquaculture

The next step involved evaluation of whether carbon quantum dot based detection system could be used for practical applications to sense trace amounts of malachite green in aquaculture. Therefore, two water samples from IIT(BHU) aquaculture fish ponds were procured and centrifuged at 15,000 rpm at 25°C for 15 minutes. Then the samples were filtered by passing through a 0.22 syringe filter. Carbon dots were added to make the final concentration 35 µg/ml. Subsequently, they were spiked with six concentrations of malachite green (9.1-54.6 nM). These test samples were then tested for fluorescence quenching of carbon dots for detection of malachite green in fisheries.

2.5.7 Detection of Malachite Green in Green Leafy Vegetables

For rapid and easy detection of the presence of malachite green in green vegetables as adulterant, leaves of common garden plants were taken as test material. Two sets of the leaves were taken, one set was used as control and one set was immersed in 1 mg/ml malachite green solution followed by complete drying. The leaf sets were immersed in 35 μg/ml carbon dot aqueous solution in petri dishes. Next, both the sets were observed under UV irradiation to visually assess the optical difference in the carbon dot solution of the two sets.

2.5.8 Biosensor Design for Easy Calorimetric Detection of Malachite Green

Based on above result, a very simple prototype calorimetric sensor was designed for fast and easy detection of malachite green in sample green vegetables. The body composed of four quadrants made from flexible glasssheet assembled by chloroform. A sieve was mounted in the test quadrant for trapping test vegetable sample. A basic circuit was made with UV source (395 nm) in the two left quadrants. Carbon dot solution (1 mg/ml) was filled fully in the lower right quadrant (control) and partially in the upper right quadrant (test). To test the applicability of the sensor, green vegetable material (pea/chilly/capsicum) coated with malachite green was dipped in the test quadrant trapped by the sieve. The instantaneous visual difference is taken as observation on illuminating the two quadrants with fitted switch operable UV light source.

2.6 Experiments Related to *Arjuna terminalia*-derived CQDs for the Synthesis of Gold Nanoparticles

2.6.1 Preparation of AuNPs

Gold nanoparticles (AuNPs) were prepared by the spontaneous reduction of $HAuCl_4$ with CQDs as a reducing agent.[7] $HAuCl_4$ (0.5 ml, 1.5 mM) and 0.5 ml of diluted CQD solution were mixed thoroughly in equal proportion. The quantity of CQDs was varied from 0 to 500 µL. No additional boiling is required for the reaction carried out at room temperature. The change in colour of the solution from colourless to different shades of pink/purple indicated the rapid materialisation of the reduction reaction within 5 minutes.

2.6.2 MTT Assay

To evaluate the biocompatibility of the AuNPs, each AuNP sample was subjected to MTT assay using MG-63 human bone osteosarcoma cell line. Cells were incubated for 24 hours in a 96-well plate using the DMEM culture medium. 50 µl of sample was mixed with 50 µl of fresh medium in a 1:1 ratio. 5 mg of MTT reagent was dissolved in 1 ml of PBS buffer. 10^4 cells were plated into 96-well plate in serum free culture medium overnight. After 24 hours of cell incubation, 100 µl of MTT was added into the solution. The media was incubated again at 37°C for 2 hours. 100 µl of DMSO was added into the solution to dissolve the formazon crystals and incubated in dark for an hour by shaking at room temperature. The absorbance at 595 nm was recorded. Each sample was prepared in triplicate. Cell viability was expressed as a percentage of control cells which were represented as 100% viable without treatment, using the following formula-

% cell viability = [A595 nm of treated cells- A595 nm of negative control /A595 nm of control cells- A595 nm of negative control] × 100

2.6.3 Computational Studies of AuNP-CQD Interaction

A 5x5 monolayer model of fused benzene rings is chosen as a backbone for modeling the carbon-quantum dots. To this, functional groups are added by taking hints from the FTIR study. The compound sheet is then optimized using the M06-2X/6-311G (d,p) level.[8] An eight-atom complex (Au_8) as a model for the AuNPs has been chosen which is optimized using the LANL2DZ basis set. In order to figure out the interaction energy of the CQD-AuNP complex, the optimized structures of the AuNP cluster and the CQD model are placed in close proximity and optimized at the M06-2X level with their corresponding basis sets, and the interaction energy is calculated using the energy of the complex with respect to the individual fragments.

2.6.4 Collagen Stabilisation of AuNPs

AuNPs reduced by W160 and W200 were less stable. To increase their stability, 1mg/ml stock solution of Type-I collagen derived from bovine calf skin was added in the solutions to act as an additional stabilizer. 0.5 ml of 1.5mM $HAuCl_4$ solution, 0.3 ml of deionised water containing (25/50/75/100 µl) collagen (1mg/ml stock) and 0.2 ml of W160/W200 were added and mixed together. Absorbance spectra were recorded every 24 hours for 7 days. The hypochromic shift of the peak was analysed for assessing stability. Microscopy images were examined to investigate how the structures were stabilised.

2.6.5 Snowing of AuNPs by β-mercaptoethanol

20 μL of β-mercaptoethanol was added to 1 ml AuNP (W160-AuNP) solution resulting in spontaneous snowing of AuNPs to the base of the micro-centrifuge tube. β-mercaptoethanol was added into two solutions- only $HAuCl_4$ and AuNP-CQD solutions respectively, resulting in a similar pattern of snowing. SEM and microscopy images of these precipitates were studied to gain better insight into the self-assembly and interaction mechanism.

2.7 Experiments Related to Sandalwood-derived CQDs for the Bioimaging of the Adverse Effects of Malachite Green on Plant and Animal Models

2.7.1 Mung Bean Sprout Cultivation

Mung beans (*Vigna radiata*) were selected to have uniform sizes and plumpness followed by a brief surface sterilization step of soaking in 10% NaClO solution for 10 minutes followed by thorough washing with deionised water three times. 15 seeds for each group (total 7 groups) were selected. For the control group, deionized water was used as medium while the rest of the groups had different dilutions of MG (10, 200, 400, 600, 800 and 1000 μM) as the sole growth medium to avoid the effect of additional salts, ions and other growth promoters. The seeds were cultivated in partially covered beakers at room temperature. After 5 days, the mung bean sprouts were harvested for further evaluation. The sprouts were immersed in sandalwood CQDs (0.33 mg/ml) for 20 hours as new culture medium after which they were thoroughly rinsed with deionized water to remove any adsorbed CQDs and contaminants before further characterization.

2.7.2 Mung Bean Sprout Characterization

The root and shoot length of the sprouts and their ratios were measured to assess the growth conditions. Longitudinal leaf diameter, fresh biomass and dry weight were taken as additional parameters for plant health assessment. To measure the dry weight, the plants were dessicated in an oven at 80°C for 20 hours. In order to ensure the accuracy of test data, all the experiments were repeated in triplicates. To observe any anatomical changes, the stem/root was cut into a thin transverse section before viewing under a light microscope. The sections were also analysed a fluorescence microscope and confocal microscope. EPA-Ecological effects test guidelines (OPPTS 850.4200) and International Rules for Seed Testing 2019 were followed for seed and plant assessment.

2.7.3 Onion Cell Analysis

Fresh onion bulb was selected and peeled. Using forceps, the epidermal layer from the concave side of the peel was carefully extracted and immediately placed in solution (0, 400, 1000 µM MG). The peels were allowed to rest and absorb the dye for 12 hours after which they were seen under the microscope. For fluorescence microscopy, the peels were gently lifted and rinsed with deionised water before placing them in CQD solution (0.33 mg/ml) for 2 hours before viewing.

2.7.4 Cell culture

Human MG-63 osteosarcoma cell line was acquired from the National Centre for Cell Science (NCCS), Pune. The obtained cell line was further raised within T-25 flasks with vented caps (Genetix) in 10% Fetal Calf Serum (FCS) containing Dulbecco's Modified Eagle Medium (DMEM, High Glucose) complemented with

2mM L-glutamine, 0.1 mM Minimum Essential Medium (MEM), Non-Essential Amino Acid (NEAA) and 1% penicillin-streptomycin. All the cell cultures were maintained in humidified atmosphere (Thermo Fisher Scientific, Heracel vios 160i) at 37°C with 5% CO_2. Before experiments, no contamination was observed in the cell line.

2.7.5 Cell viability test

1 X 10^4 MG-63 cells were seeded for 24 hours in 96-well plate (flat-bottomed) before the treatments with complete media containing 10% FCS. Cells were plated into nine groups including control and with eight different concentrations from 0.1 µM- 100 µM of MG for 24 hours. Further the half maximal inhibitory concentration IC50 was determined from these concentrations and the same was used for further studies in further experiments.[9] To find out the working concentration of sandalwood-derived CQD, dose-dependent study was performed with three different concentrations (0, 0.17, 0.33 mg/ml) CQD. The cytotoxicity of MG was determined by performing standard MTT assay by monitoring the formation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt to the coloured formazan products.[10] The optical density (OD) was measured at 595 nm using microplate reader (Biorad). Experiment assays were performed in triplicates for each sample and control.

2.7.6 Animal ethical treatment

Laboratory-bred strain golden hamsters were obtained from the Central Drug Research Institute (CDRI), Lucknow. All animals were maintained at 12h light: 12h dark light: dark cycle with 25 ± 2°C temperature in animal house and provided *ad libitum* water and food for 2 weeks for acclimatization. All the experimentations were conducted according to the institutional practices and approved by the support of the

revised Animals Act of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal), Government of India, 2007 (CPCSEA Registration No.-1802/GO/Re/S/15/ CPCSEA). The golden hamster models (N=20) were primarily divided into two groups (n=10 per group). Groups were divided as:- Group1- Control (oral normal saline) and Group 2 - MG treated (oral 13.5 mg/kg/day, at 11 am). After 14 days of treatment, animals of each of the groups were again subdivided into two sub-groups (n=5), one group was treated with normal saline via intravenous injection and the other group animals were treated with CQD (0.33 mg/ml) through intravenous injection.

2.7.7 Animal Sample Collection and Morphometric analysis

All the animals were first weighed and then sacrificed after two weeks of treatment at forenoon under deep ether anaesthesia. Trunk blood was collected in a heparinized tube. Serum was separated and stocked up at -80°C. The liver, spleen, kidney, adrenal and testes tissues were immediately removed, blotted dry, weighed and kept in 10% buffered formalin for further morphometric analysis. After fixation in 10% buffered formalin for 48 hours, extra fixative was removed from tissues under running tap-water followed by tissue dehydration, tissue clearing, tissue-paraffin block formation and sectioning (7 μm). This was followed by tissue stretching on pre-coated (1% gelatin) slides and finally hematoxylin and eosin staining was done following laboratory standard protocol.[11] Histoarchitecture of the tissues was observed under inverted bright field microscope (Nikon E 200, Japan) in randomly selected sections.

2.7.8 Colorimetric analysis of reactive oxygen species (ROS) assay

Total serum ROS of all groups (control and treated) were assessed with the fluorescent probe dichloro-dihydro-fluorescein diacetate H2DCFDA.[12,13] In the serum of all groups H2DCFDA was added and following spectrophotometric method, optical densities were measured. Free radical activity was expressed as relative fluorescence intensity (RFI).

2.7.9 Colorimetric analysis of superoxide dismutase assay (SOD)

The serum of all groups (control and treated) was processed for total Cu, Zn, Mn-Superoxide dismutase (SOD) activity. The reaction was stopped by adding freshly prepared Griess Reagent (1% sulphanilamide, 5% orthophosphoric acid, 0.1% N-1-napthylethylenediamine dihydrochloride) and absorbance was measured at 543 nm. SOD activity was expressed as specific activity (units/mg protein).[14]

2.7.10 Colorimetric analysis of lipid peroxidation assay (LPO)

The serum of all groups (control and treated) were subjected to thiobarbituric acid (TBA) assay by mixing it with 8.1% sodium dodecyl sulphate (SDS), 20% acetic acid, 0.8% TBA and boiling for 1 h at 95ºC. The reaction mixture was immediately cooled in running water and vigorously shaken with n-butanol and pyridine reagent (15:1) and centrifuged for 10 min. at 1500 g.[15] The absorbance of upper phase was measured at 534 nm. LPO was expressed as TBARS in nmol/g tissue weight using 1,1,3,3-tetraethoxy propane (TEP) as standard. The standard curve was calibrated using 10 nM concentration of TEP.

2.7.11 Cellular and tissue uptake studies by confocal and fluorescence microscopy

For cellular uptake study and cellular morphometric analysis first MG-63 cells were seeded at a concentration of 6000 cells per well upon the cover slides placed inside the 24-well culture plates one day prior to treatment. The culture plates were divided into three different groups, Group 1- only CQD (0.33 mg/ml), Group 2- CQD (0.33 mg/ml) with 4.5 μM MG exposure and Group-3 CQD (0.33 mg/ml) with 1 μM MG exposure. After treatment MG-63 cells were cultured for 24 h at 37°C. Finally after 24 hours of cell culture 4′,6-diamidino-2-phenylindole, (DAPI, fluorescent nuclear stain) was added to the cells at 1:5000 concentration and images were captured by confocal microscope. For *in vivo* MG-caused tissue morphometric analysis, all tissue sections of both external (as stain) and internal (intravenous delivery) CQD espoused groups were visualized by fluorescence microscopy.

2.7.12 Statistical analysis

Using one-way ANOVA and Tukey post hoc test in the SPSS software, all data was analyzed and all graphs were generated using Microsoft Office Excel 2007 and Origin software. Values were accepted as significant when $p < 0.05$. Results are stated as the mean ± SEM.

2.8 Experiments Related to the Self-Assembly of Amino Acids/Peptides/Proteins and GO

2.8.1 Interaction of Amino Acids/Peptides/Proteins with GO

The GO suspension with concentrations ranging from 50-150 μg/mL was thoroughly mixed with amino acid/peptide/protein at room temperature in a suitable buffer. The resultant GO-protein complex was formed at lower pH buffers below the

pKa/pI of the protein/amino acid moieties. The amino acid/peptide/protein concentrations used in this work were in the range 0.5-1 mg/mL. For conjugation, the GO and protein samples were heated at 80°C for BSA and 90°C for lysozyme for 30-45 minutes. Glycine-HCl buffer was used for buffer pH 2, phosphate citrate for pH 2, 5 and 6, sodium acetate for pH 4 and Sorensen's phosphate buffer was used for pH 7 and 8.

2.8.2 Gel Electrophoresis Procedure

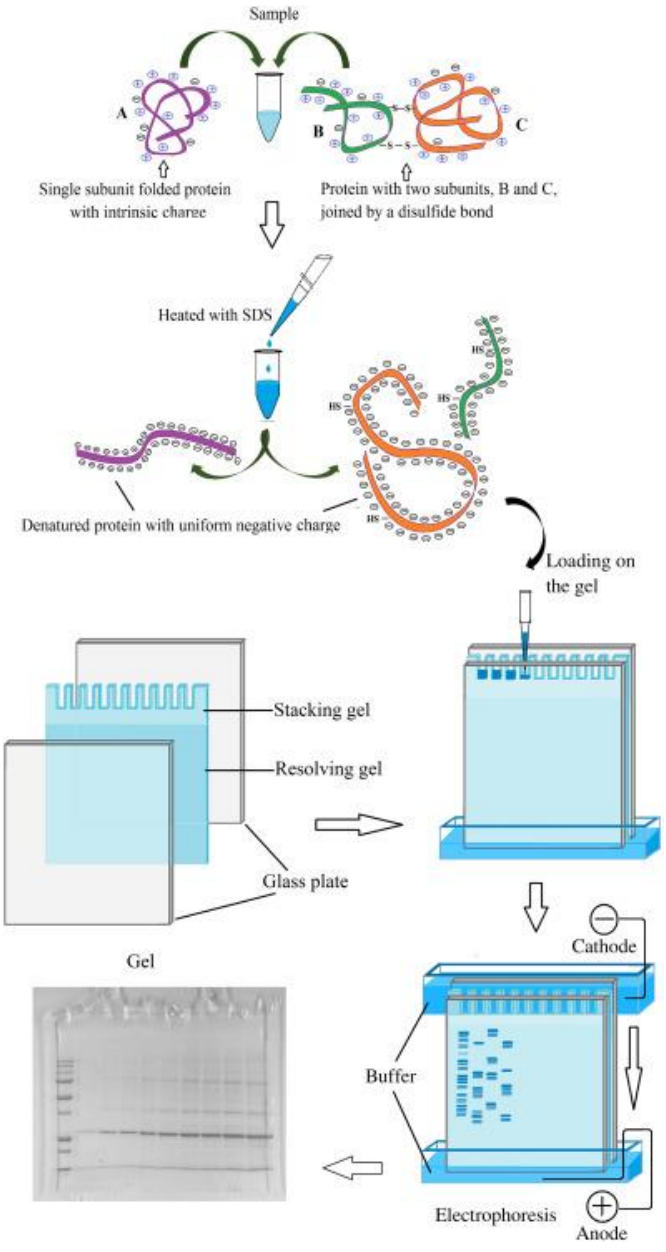

Figure 2.3: A Typical Schematic of SDS Gel Polyacrylamide Process Cycle

Pepsin digestion was carried out with enzyme concentration of 1 mg/ml at 37°C for 2 hours. After digestion, the samples were prepared for SDS-PAGE by heating in Laemmli Buffer at 85°C for 10 minutes. The composition of 1X Laemmli buffer was taken as 60 mM Tris HCl (pH 6.8), 10% (v/v) glycerol, 0.001% (w/v) SDS and .001% Bromophenol Blue. Bromophenol Blue was obtained from Sisco Research Lab and all the other reagents were from Sigma Aldrich. Analytical Poly acrylamide gel electrophoresis was carried out in SDS PAGE setup by Balaji Scientific, under denaturing conditions at 25°C in 10% acrylamide gels in 25 mM Tris (pH 8.8), 200 mM Glycine and 0.1% (w/v) SDS, at a voltage of 100 mV. The stacking gel and resolving gel were made according to standard protocol.[16] Fixing of the Gel was done in 50% Methanol and 10% (v/v) Glacial Acetic acid, and staining was done with Commassie Brilliant Blue R-250. Final destaining was done with 40% methanol and 10% glacial acetic acid.

2.9 Experiments Related to the Marigold-derived CQDs for the Detection of Adulterant Dyes

2.9.1 Complexation of Malachite Green with G-Quadruplex DNA sequences

Stock solution of malachite green dye was prepared in deionized water at a concentration of 1 mg/ml (1 M). Four DNA sequences were synthesized in a DNA synthesizer having 18 base pairs each. Each individual sequence was capable of forming a quadruplex secondary structure in a suitable phosphate buffer. Sequentially, MG was added into each DNA solution (25 mM) in 20 mM increments. The solution was mixed vigourously before observing the photoluminescence spectra.[17]

2.9.2 Construction of a Multi-Detection Device for the Sensitive Sensing of Common Food Adulterants using CQDs as Tools

For the detection of multiple adulterants using a facile and cost-effective device, the four selected CQDs were incorporated in separate partitions in two rows each. One row was designated as control while the second row was used for testing the adulterants in question.[18] For this purpose the cubic exterior body and simple circuit were built as additional components. This prototype sensor is small and easily scalable requiring very less material and monetary resources. It is made using a mould to set high density polyethylene (HDPE) into the desired structured panel. CQDs at a concentration of 5 mg/ml are filled in the eight compartments having a small nozzle for the addition of the adulterant solution through a syringe. A reference label was attached on the exterior body for comparison and rough estimation of the dye concentration. UV source with complete circuit was attached at the back of the device for the excitation incidence on the CQDs. Colorimetrically, the difference is optically palpable in the presence of an adulterant which can be compared with the reference chart.

2.10 References

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