Chapter-2

2 Methodology and Instrumentation

2.1 Materials Used for the Synthesis and Other Experiments of CQDs

The precursor (*Plumeria rubra* plant leaves) was collected from Dr. C.P.R. Aiyer Hostel BHU Varanasi Uttar Pradesh. Metal salts like KCl, NH_4Cl, $CaCl_2$, NaCl, $MgCl_2$, $CuCl_2$ were bought from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). $BaCl_2$ and $HgCl_2$ were purchased from Fisher Scientific Pvt. Ltd. (Mumbai, India); the $AgNO_3$ received from Merck, $NaAsO_2$, Na_2HAsO_4 picked up from SD Fine Chemical Ltd. (Mumbai, India). The solvent was used here 1- propanol, taken from Merck Life Science Pvt. Ltd. (Mumbai, India) and as well as distilled water (ultrapure type II with resistivity 11MΩ cm). We use the following starting materials for the WLEDs application, Terbium acetate hydrate (99.99%), Di-ionised water, 1-propanol, and fresh *Plumeria* plant leaves. All materials were used further without purification. These materials are purchased from Sigma Aldrich and *Plumeria* plant leaves taken from IIT(BHU) campus. All the purchased chemicals were used as received without further purification. However, for the tumor regression application the starting materials as gold salt ($HAuCl_4$) was obtained from Sigma-Aldrich (st. Louis, MO, USA). Tulsi and Vinca plant leaves were collected from Ayurvedic department of IMS BHU, India. Sodium bicarbonate and HEPES (Hydroxy ethyl piperazine ethane sulphonic acid), 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), 1-Chloro-2,4-dinitrobenzene (CDNB), Triton-X, (3-(4,5-dimethyathiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Bovine Serum Albumin (BSA), and Dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (USA). β- nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase (GR) and oxidized glutathione (GSSG) were purchased from MP Biomedicals, India. Trichloroacetic acid (TCA), Sodium pyrophosphate, Nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), sulphanilamide, N-1-nepthylethyldiamine dihydrochloride (NEDD), ortho-phosphoric acid,

Tris-HCl, Ethanol, Sodium pyuruvate, Sodium chloride, Ethylene diamine tetra-acetic acid (EDTA), were purchased from SRL, India. All the chemicals were used without further purification. All the experiments were done using double-distilled and deionized water.

2.2 Preparation of CQDs

2.2.1 *Plumeria* derived CQDs for Heavy Metal Ions Detection

The leaves of the plant were gathered from the Aiyer hostel at BHU, washed multiple times thorough DI water, and allowed to air dry overnight before being used in the one-step solvothermal process to the synthesis of CQDs. First, small pieces of leaves from the Plumeria rubra plant were collected and finely chopped before being added to popanol-1 as a solvent at a ratio of 1:2 w/v and maintained at a temperature of 100ºC for about 180 minutes[133], [134]. The prepared hot extraction was collected and transferred to a 200ml Teflon-lined stainless steel autoclave chamber, where it was heated in a muffle furnace at different temperatures (160ºC, and 220ºC) for the same amount of time (8 hours). After solvothermal treatment, it allows to cool naturally at room temperature and collected dark brown solution. Additionally, centrifugation at 11000RPM for 15 minutes at 25ºC and collect supernatant and filtered through a 0.2mm syringe filter (Pall-Gelman Supor Acrodisc®) for removing big particles. The filter CQDs were kept at 4ºC for further characterization. The stock solution of CQDs had a concentration of 3.5µg/ml, however for all other characterization techniques, CQDs were employed at a concentration of 0.012µg/ml in DI water. For discussion purpose the CQDs synthesized at 160°C and 220°C will be labelled as CQD160 and CQD220 in the following text.

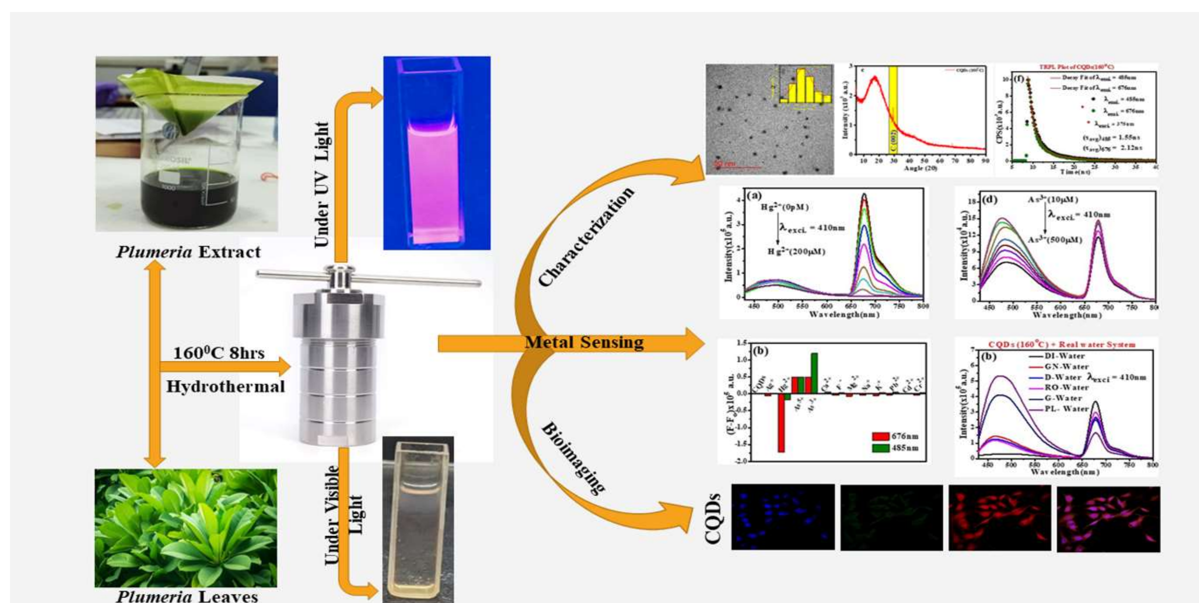

Figure 2. 1 Solvothermal Synthesis of CQDs using Plumeria plant leaves and its applications in metal detection and bioimaging.

2.2.2 *Plumeria* derived CQDs for WLEDs Application

Using the hydrothermal approach, we must prepare carbon quantum dots (CQDs) from leaves of the plumeria plant. First, 38 grammes of neat, clean plumeria plant leaves were taken and cut into small pieces. They were then added to 100 millilitres of 1-propanol and heated at 100^0C for three hours before being stored and safely kept in a hydrothermal autoclave. This autoclave was then placed inside a muffle furnace for 8 hours at 160^0C, cooled naturally at room temperature, and then the resulting solution was centrifuged at 10000 rpm for 15 minutes and filtered through a 0.22mm. This solution stored at 4^0C for the further application. Second, make a 10 millimolar solution of the readily soluble Terbium acetate in DI water for use in the processing of samples. At various volume ratios, terbium acetate and CQDs were mixed for the optimization.

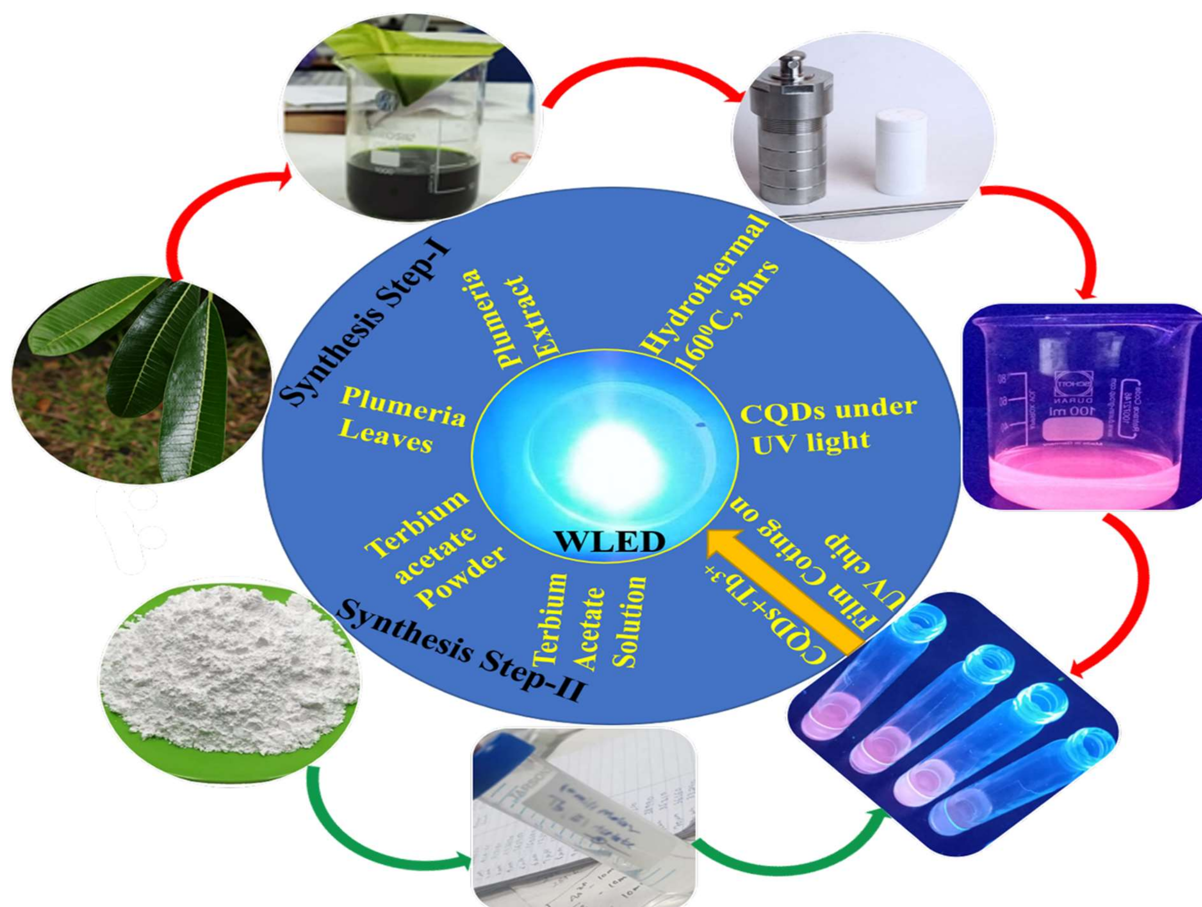

Figure 2. 2 Schematic diagram of synthesis and fabrication of WLEDs materials.

2.2.3 Preparation of plant extract and Nanoparticles

The plant extract was obtained by using cold extraction methods[23]. Tulsi (*Ocimum tenuiflorum*) and *Vinca* leaves were used for preparation of plant extract. Fresh plant leaves were collected and washed three times by using distilled water (DI) and dried in ambient environment. 20 g of small plant leaf pieces were added to 100 ml DI in a separate conical flask for the Tulsi and Vinca extract, while 10 g of plant leaves were added to 100 ml DI for the Tulsi and Vinca combined extract. This mixture was kept at room temperature over magnetic stirrer for 24 hours, a magnetic bead was used to properly mixed DI and plant leaves. After 24 hours, obtained solution was filtered with 0.2 μm Whatman filter paper. Filtered solutions were stored at 4 °C. However, the gold nanoparticles (GNPs) were synthesized using Tulsi, *Vinca*, and 1:1 mixing of Tulsi and *Vinca* plant leaves extract. For biologically active synthesis of GNPs, gold

salt (stock solution 1 mM) at 37 °C was added to the plant extracts (Tulsi, *Vinca*, and mixed samples) in different V/V (Au salt/plant extract) ratio. The optimized concentration (V/V) for gold salt and Tulsi, *Vinca*, and mixed (Tulsi and Vinca) were obtained as 7:3, 9:1, and 8:2, respectively. At optimized volume ratios, the colour of gold salt changes from yellow to dark red in all the solutions indicating the reduction of gold salt and formation of nanoparticles. All these experiments were performed at 37 °C kept in water bath to maintain uniform temperature environment. The synthesized GNPs with addition of Tulsi, *Vinca*, and mixed extract, were labelled as T-Gold, V-Gold, and T+V-Gold stored at 4 °C.

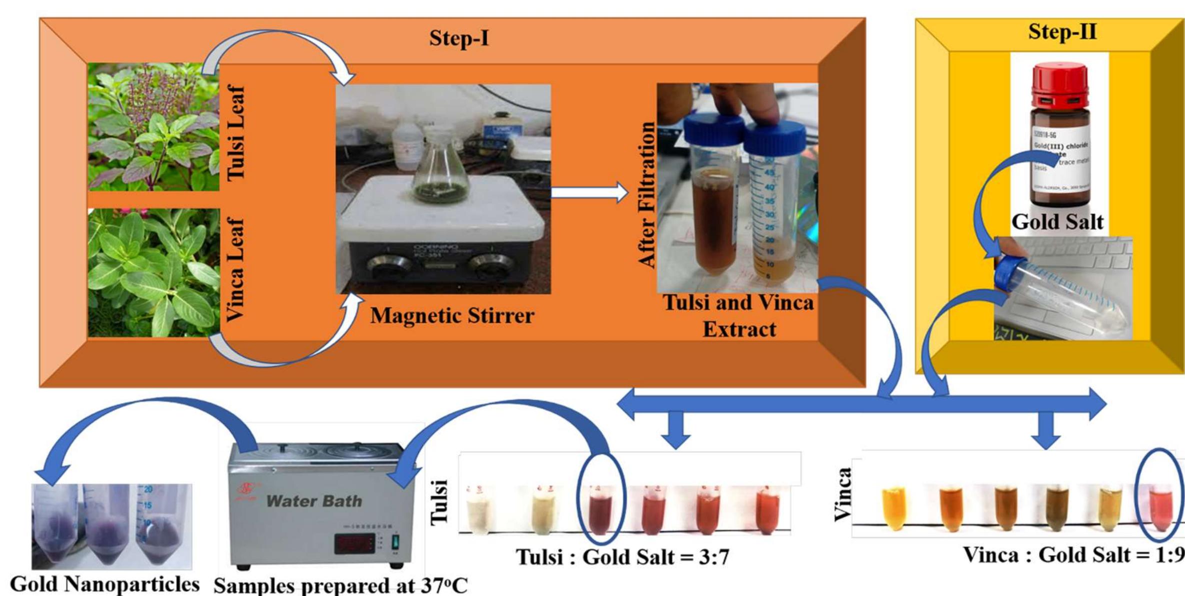

Figure 2. 3 Schematic diagram of green synthesis method of gold nanoparticles (GNPs) using Tulsi and Vinca plant leaves.

2.2.4 BN derived CQDs for detection of PNP

The present study reports the synthesis of boron nitride quantum dots (BNQDs) using a facile, low-cost, and chemical-free hydrothermal method. Boron nitride nanoparticles (BNNPs) were employed as the precursor material for the synthesis. Initially, a 1 mM (0.13 mg/mL) stock solution of BNNPs was prepared in a solution of deionized (DI) water induced with ethanol,

with a water to ethanol ratio of 80:20. The stock solution was sonicated using an ultrasensitive sonication method and stabilized by adding citric acid at a concentration of 3.3 mg/mL, which served as a nitrogen dopant and provided stability to the solution. The resulting homogeneous, stable, and white-coloured solution was then subjected to solvothermal treatment in an autoclave at 180°C for 6 hours in a muffle furnace. The heated autoclave was allowed to cool down naturally to room temperature. Subsequently, the supernatant was separated by centrifugation at 3500 RPM for 15 minutes, and both solid and liquid samples were collected. The samples were stored at 4°C in a freeze for further characterization. The stored solution named as BNQDs.

2.3 Apparatus Used in the Experiments

The HAADF Detector, running at 200kV, was used to take pictures with the FEI TECNAI G2 20 TWIN USA transmission electron microscope (TEM). We used the software ImageJ to analyze high resolution images. Using a Bio-Spectrophotometer (Eppendorf), the UV-Vis absorption was measured in the 200nm to 800nm range. Using a Fluorolog-Horiba fluorescence spectrophotometer, the PL emission spectra of CQDs was carried out (HORIBA Jobin Yvon, France). Making KBr pellets in the frequency range of 4000-400 cm-1 allowed researchers to determine the surface functional groups of CQDs using a Fourier transform infrared (FT-IR) spectrophotometer (Thermo Scientific Nicolet iS5 FT-IR). Using a monochromatic, micro-focused K-Alpha (thermo fisher scientific) X-ray source (Al K-Alpha 1486.6 eV) operating range from10 mA and 15 kV. CasaXPS software was used to analyse the XPS data (Casa Software Ltd., U.K.). Using Origin2019b, we analysed the CIE coordinate and CCT of the emitting white LED, and we conducted EL experiments with QEpro (Ocean optics). The quantum yield (QY) of functionalized CQDs was achieved by using a comparative approach in accordance with a predetermined protocol [135]. Relative quantum yield (QY) of the CQDs

was also evaluated by comparing with a reference fluorophore, Rhodamine 6G, to determine the QY of the functionalized CQD solution (quantum yield of Rhodamine 6G is 95%). Zetasizer Pro measured the charge stability and hydrodynamic radius in the water dispersion medium (Malvern Panalytical). CuK radiation at 1.54, 40 kV, and 15 mA were used to provide information about the nature of crystallinity to the X-ray diffractometer (Rigaku Miniflex 600 Desktop X-Ray Diffraction System, Tokyo, Japan). Additionally, confocal microscopy was used to monitor cellular uptake of the CQDs (Carl Zeiss 780 LSM laser scanning confocal microscopy system, Germany). TRPL was measured using Edinburgh instruments with picosecond pulsed diode LASER as a source and quantum yield measured by using Integrating sphere Horiba pti.400 spectrometer. confocal microscopy (Carl Zeiss 780 LSM laser scanning, Germany) was used to monitor cellular uptake.

2.4 Experiments Related to CQDs as Detection and Bioimaging

2.4.1 Selectivity and Sensitivity Measurements

We were able to detect the presence of heavy toxic metal ions using the highly sensitivity florescence probe method, and we were able to distinguish between the selectivity of Hg^{2+} and As^{3+}in different aqueous solutions of metallic ions (Mg^{2+}, Cr^{2+}, Ag^{+}, Hg^{2+}, Pb^{2+}, As^{3+}, As^{5+}, Cd^{2+}, Ca^{2+}, K^{+}, and Na^{+}) that were made from the corresponding salts and kept at 100µM For the purpose of measuring selectivity, the different molarity of Hg^{2+} and As^{3+}were now added to a predetermined amount of CQD160 to determine sensitivity. The prepared samples were incubated for 5min before being subjected to 410nm and 350nm excitations to record fluorescence at various concentrations.

2.4.2 Sensing of Hg^{2+} and As^{3+} ions

The final CQD160 stock were prepared by addition of 10 µl (3.5µg/ml) CQD160 in 3ml DI water with maintaining pH around 7. The ability of metal ions sensing was followed by

Sachdev et al. with certain modifications[136]. Herein, we were fixed the total volume 4ml, accurately 2ml of metal solution with different molarity concentration mixed with 2ml of diluted CQD160, but the control solution was prepared by addition of 2ml diluted CQD160 in 2ml of DI water. All the prepared solutions were incubated about 5min at the room temperature, after that recorded their fluorescence intensity, excited by 410nm and 350nm. The heavy metals (Hg^{2+} and As^{3+}) were greatly interacted with CQD160, given change in intensity simultaneously at different emission peaks (485nm & 676nm). As^{3+}enhanced the intensity of blue green emissive peak (485nm) while Hg^{2+} quenched the emissive intensity of red emissive peak(676nm). The experiment was performed with respect to Hg^{2+} and As^{3+}as 100pM to 200µM and 10µM to 500µM. The efficiency of quenching phenomenon of the CQDs was calculated by using Stern–Volmer relation shown in equation (2.1)[137].

$$\frac{F_0}{F} = 1 + K_{sv}\,[Q] \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. (2.1)$$

Where F_o and F are represented the PL intensities of CQDs in the solvent at the absence and presence of metal ions respectively, K_{sv} is the quenching constant of Stern–Volmer, and Q is corresponding to the concentration of metal ions. Then we are able to calculate the limit of detection (LOD) of the prepared CQDs with different concentration of metals in DI water were calculated by using equation (2.2)[137].

$$LOD = \frac{3\sigma}{s} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots..\ldots\ldots\ldots\ldots (2.2)$$

Where, "σ" is correspond to standard deviation of y-intercept (F_o/F values), and "s" is denoted the slope of the linear line.

2.4.3 In Vitro Cytotoxicity Assay

The biocompatibility of CQD160 was evaluated in NIH-3T3 cell line at concentrations of 80, 160, 240, and 320 µg/ml. To perform the assay, 5x103 cells/well were seeded in a 96-well microtiter plate and incubated with 10% FBS for 24 hrs at 37°C in 5% CO_2. The cells were then treated with the CQD160 samples for 24, 48, and 72 hrs. After the respective time points, MTT solution was added to each well, followed by incubation for 4 hours at 37°C to allow for formazan crystal formation. The formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm on an ELISA reader plate[138], [139]. The percentage of cytotoxicity was calculated using equation (2.3), and the percentage of viability was obtained by subtracting the percentage of cytotoxicity from 100. All measurements were performed in triplicate, and the colour developed was directly proportional to the number of viable cells. This study provides important insights into the biocompatibility of CQD160 in NIH-3T3 cells, which may have implications for their potential use in biomedical applications.

$$\%\,Cytotoxicity = \frac{[A]_c - [A]_t}{[A]_c} X100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. (2.3)$$

$$\%\,Viability = (100 - \%\,cytotoxicity)$$

Where, $[A]_t$ = Absorbance of the sample to be test

$[A]_c$ = Absorbance of the control sample

2.4.4 *Ex-vivo* Hemolysis Assay

In order to assess the hemolytic activity of CQD160, human blood was collected in an EDTA tube. The whole blood was mixed with an equal volume of PBS at pH 7.4 and then centrifuged at 1500 rpm for 5 minutes, after which the plasma was removed. The collected blood cells were washed with PBS (pH-7.4) and the supernatant was discarded. The washed red blood cells were

mixed with an equal volume of PBS (7.4), and 200 μL of these cells were placed in each micro-centrifuge tube, to which CQD160 were added. All micro-centrifuge tubes were kept at room temperature, under continuous stirring on a shaker for 2 and 4 hours. After 2 and 4 hours of incubation, the tubes were centrifuged at 11,000 rpm for 5 minutes, and the supernatant was transferred to a 96 well plate. Absorbance was measured at 540 nm. A positive control was included, which was Triton-X (0.1%), and a negative control was included which was PBS pH 7.4 hemolysis was calculated by the formula:

$$\%\, Hemolysis = \frac{[A_{sample} - A_{saline}]}{[A^{+/-}{}_{control} - A_{saline}]} X100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots..(2.4)$$

Where, A_{sample} = Absorbance of the sample

A_{saline} = Absorbance of the control sample

$A^{+}{}_{control}$ = Absorbance of positive control

The results of this experiment will provide insights into the hemolytic activity of CQD160 on human red blood cells[140]. It is expected that the absorbance measurements will differ between the CQD-treated samples and the negative control samples, but will be similar to the positive control samples. By comparing the results of this experiment with previous research on the hemolytic activity of CQDs, it will be possible to further understand the potential toxicity of these nanoparticles on human cells.

2.4.5 Fluorescence based assay protocol:

NIH-3T3 (50,000 cells/ well) were inoculated on 12mm round glass cover slip in a 24 well microplate in complete media. Next day change the media and cells were pre-treated with 100uM of As, Hg for 30 min and then CQD160 was added. After 2hrs of incubation, the supernatant was removed and the cells were washed thrice with chilled PBS. The cells were

then fixed with 4% paraformaldehyde for 30 min. After that again wash the cells with PBS to remove traces of paraformaldehyde, DAPI (1µg/ml) was added to the treated cells to mark the nucleus. Again, wash with chilled PBS to remove excess DAPI. Cover slips were mounted in DPX on the slides and were visualized under confocal microscope at a magnification of 63x. Excitation for DAPI and quantum dot was 359nm and 410nm respectively.

2.5 Experiments Related to WLEDs

2.5.1 WLEDs Demonstration

We created a white LED using the obtained CQD+Tb^{3+}(25%) film used as a single generator for white light, effectively converting UV light into white light, taking into account the excellent qualities the synthesised functionalized CQDs exhibit, such as strong PL intensity, high QY value, and good film-forming ability[141]. This white LED has a fairly broad emission spectrum that covers practically the whole visible spectrum (400 to 800 nm), which has a wider emission of CQDs under UV stimulation. The behaviour differs from earlier results where a strong UV light from the chip was present[142], [143]. After absorbing greater energies, fluorescence often manifests itself at lower energies or longer wavelengths. Additionally, fluorophores may cause additional Stokes Shifts as a result of environmental factors such complex formation, solvent effects, or energy transfer[135]. When compared to the PL spectra of CQD solution, the LED using CQDs as the phosphor exhibits two distinct peaks at 448 and 580 nm. This effect may be explained by the solid-state CQDs integrated in the LED lens transferring energy through light reabsorption[144], [145]. Some CQDs are excited by the UV chip to produce light with a shorter wavelength (448 nm). The light is then partially reabsorbed

by the CQDs in the area, and longer wavelength light is released from those CQDs. This offers an adequate justification for the peak centred at 580 nm.

2.6 Experiment Related to GNP as Tumor Regression

2.6.1 Cell Culture

Murine 4T1 cells were a kind gift from the Regional Centre of biotechnology (RCB), Faridabad, India. The cells were cultivated in RPMI-1640 Medium containing 10% FBS and 1% antibiotics at 5% CO_2 atmosphere at 37 °C. The medium was changed every alternate day. Cells were passaged when they reached 70% confluence and used later for experiments.

2.6.2 Cell cytotoxicity assay

The cytotoxicity activity of the T-Gold, V-Gold, and T+V-Gold against 4T1 cells was tested using the colorimetric method with minor modifications as previously reported[26]. MTT assay was used to determine viability or cytotoxicity of cells. Exponential-phase cells that were 70% to 80% confluent were collected from maintenance cultures and counted using a haemocytometer. Cells were dispensed in triplicates in 96-well culture plates at an optimal cell density of 5000 cells/well. After 24 hours of incubation at 37 °C, cells were exposed to various concentrations of T-Gold, V-Gold, and T+V-Gold (200, 100, 50, 25, 12.5 and 6.25 µg/ml) for 24, 48 and 72 hours. 20 µl of MTT prepared at a concentration of 5 mg/ml and incubated the plate at 37 °C for 4 hours, allowing the formation of purple precipitate of formazan crystals. Then, 200 µl of DMSO was added to each well in order to dissolve the formazan crystals. The optical density (OD) of the contents of the plate was quantified in the ELISA reader (Synergy HT, BioTek, USA) at 570 nm. The developed colour in each well was closely proportional to the viability of the cells present in that particular well. Following the determination of the 50% inhibitory concentration (IC_{50}) after 24 hours, 48 hours, and 72 hours,

the percentage of cytotoxicity was computed using the provided formula (Equation (2.5)). All experiments were performed in triplicates.

$$\% \ Cytotoxicity = \frac{[A_c - A_t]}{A_c} X100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. (2.5)$$

Where, $[A]_t$ = Absorbance of the sample to be test

$[A]_c$ = Absorbance of the control sample

The dose of GNPs required to reduce cell growth by 50%, from which the IC_{50} values were deduced using a dose-response curve (Graph pad prisms).

2.6.3 Cellular uptake studies

Confocal microscopy was used to examine the cellular uptake of T-Gold, V-Gold, and T+V-Gold on the 4T1 cell line. Suspension of T-Gold, V-Gold, and T+V-Gold were prepared based upon the IC_{50} dose by mixing at the time of preparation. For this study, a circular glass coverslip 12 mm in size was placed in the 24-well microplate and 4T1 cells were seeded at a density of 1×10^4 cells/well. Cells were treated with GNPs suspension and incubated for 2 and 4 hours after reaching 70% confluency. To remove uninternalized GNPs, the cells were rinsed twice with ice cold phosphate buffer saline (PBS) after the incubation. The cells were subsequently fixed using 4% formaldehyde and mounted the cells with DPX mounting medium. The fluorescently-stained cells were observed with Leica TCS SP8 (AOBS- Acousto Optical Beam Splitter based) Confocal laser-scanning microscope with emission wavelength of 491 nm (Green) and 457 nm (Blue). For DAPI and FITC, blue and green filter were used. Quantitative analyses of the mean fluorescence intensity emitted by the cells were estimated using Image J Software Pro Plus 6.0.

2.6.4 *in viv*o Evaluation of the Nano-formulation (T-Gold, V-Gold, and T+V-Gold) as metastatic Breast Cancer Therapeutic Agent.

We evaluated the probable *in vivo* oppressive effects of T-Gold, V-Gold, and T+V-Gold NPs in a subcutaneous 4T1 mammary carcinoma mouse model. 1 x 10^6 4T1 cells suspended in 50 µL of PBS (1X, pH 7.4) were injected into the right lower flank of total 15 BALB/c mice. The therapeutic response was assessed every 10 days after tumour induction, when the tumours reached an average volume of 80–100 mm^3, they were randomised into four treatment groups (n = 4 per group): Group (1) = PBS (1X, pH 7.4) used as a Control; Group (2) = V (350 mg/kg), Group (3) = T (350 mg/kg) and Group (4) = T+V (350 mg/kg) were used as a test. PBS and different nano-formulations were then injected intraperitoneal (i.p) on alternate days for 21 days with the total of 11 doses.

The tumour size/growth and body weight of each group of mice were measured every other day for 21 days using digital Vernier callipers and an analytical weighing scale, respectively, to assess the therapeutic response of nano-formulation. Finally, the formula was used to determine the tumour volume (Equation (6)).

$$Tumor\ Volume = \frac{Length\ X\ Width^2}{2} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots..(6)$$

At the completion of the experiment, 21 days later, all the mice were sacrificed simultaneously by cervical dislocation[146]. Then, tumours from each group were removed, weighed, and taken in pictures frame. Further, biochemical assay was investigated in tumour, serum and spleen.

2.6.5 Histopathological Assessment of Liver section

For histological examination, appropriately sized pieces of the liver fixed in 10% neutral buffered formalin. The liver fixed specimens underwent overnight dehydration, clarifying, and impregnation procedures. Using an embedding appartus, the specimens were embedded n paraffin blocks and serial sections with a thickness of 4 m were cut by microtome (ModelRM2245, Leica Biosystems, Wetzlar, Germany). Further, stain the sections with

Hematoxylin and Eosin. The mounted specimens section were observed under light microscopy to examine the abnormalities under the effect of various treatments.

Oxidative Stress Assessment

2.6.6 Estimation of Nitric Oxide (NO)

The Griess Reagent System is based on the chemical reaction between sulphanilamide and NEDD under mild acidic (orthophosphoric acid - 88% purity) conditions[147]. This assay was based on a diazotization reaction to detect the nitric oxide (NO). Reagents A and B were combined in equal parts (1:1), with Reagent A containing 1% sulphanilamide in 2.5% orthophosphoric acid and Reagent B containing 0.1% NEDD in the same solution. The equal ratio (1:1) of mitochondrial fraction and Griess Reagent were added to the 200 µl final assay mixture.

2.6.7 Estimation of Superoxide dismutase (SOD) activity

The Kakkar et al., 1984 method was used to assess the Superoxide Dismutase activity[148]. The reaction mixture contained 180 µM phenazine methosulfate, 300 µM NBT, and 0.025 M sodium pyrophosphate (pH 8). The reaction was initiated by adding 20 µl of sample and 60 µl of reaction mixture to the 20 µl of 780 µM NADH. The absorbance of all the sample against blank was measured kinetically for 5 minutes at a wavelength of 560 nm. SOD activity was measured in unit/mg of protein.

2.6.8 Estimation of lactate dehydrogenase (LDH) release

Estimation of lactate dehydrogenase (LDH) release involves measuring the reduction in β-NADH activity of the LDH by converting sodium pyruvate to free lactate. Briefly, 160 µl freshly prepared β-NADH (0.28 mM) was added to 20 µl of tissue homogenate samples along with 20 µl of sodium pyruvate. After incubation of 10 min absorbance was measured spectrophotometrically at 340 nm against blank containing phosphate buffer in place of the tissue homogenate sample. Data representation for LDH activity was expressed as nM/mg of protein[149].

2.6.9 Estimation of Glutathione s-transferase (GST) activity

The Habig et al. method was used to assess the GST activity. The reaction was calculated by monitoring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH)[150]. Using the substrate CDNB, the activity for GST was detected spectrophotometrically. This was achieved by detecting a rise in absorbance at 340 nm. One unit of GST enzyme activity was defined as the conjugation of 1 μmol of CDNB with reduced glutathione per minute (1 μmol product min^{-1}) at 25 ºC. The final assay mixture, 180 μl of reaction cocktail and 20 μl of mitochondrial fraction, was added to each well. The absorbance of all the samples was measured at 340 nm for 20 minutes against a blank containing phosphate buffer in place of the sample.

2.6.10 Estimation of Reduced GSH activity

Similarly, Reduced GSH levels were also estimated, 100 μl of test sample was initially precipitated with 5% trichloroacetic acid (TCA) at 1200 rpm for 5 min at RT. 45 μl of the centrifuged supernatant was added to wells with reaction mixture of 45 μl of sodium phosphate buffer (0.2 M, pH 8) along with 20 μl of DTNB (10 mM) reagent. After 10 min of incubation the absorbance was recorded at 412 nm against a blank containing TCA instead of the sample. The amount of reduced GSH was expressed as nM/mg protein of the sample[149].

2.6.11 Statistical Analysis

The entire set of experiments were done in triplicates. The standard software: Origin were used to make the graphs and for statistical analysis. The data sets were compared using One Way ANOVA under Tukey's test. Significance level was accepted at $P \leq 0.05$ level.