Supplementary Information

Engineering Colloidally Stable, Highly Fluorescent and Non-toxic Cu Nanoclusters via Reaction Parameter Optimization

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A) Experimental section

**Materials:** All reagents were purchased and used directly without purification. Bovine serum albumin (BSA, \(\geq 96\%\)), Human serum albumin (HSA), Copper (II) sulphate pentahydrate (CuSO\(_4\).5H\(_2\)O, 98.5 to 102.00\%), Fetal bovine serum (FBS), RPMI 1640, Phosphate-buffered saline (PBS) was purchased from HIMEDIA. Sodium hydroxide pellets (NaOH, 97\%) were purchased from FINAR. Hydrazine hydrate (N\(_2\)H\(_4\).H\(_2\)O, 80\%), 2-Mercaptomidazole (98\%), m-Phenylenediamine (98\%), 5-Fluorodeoxyuridine (FUDR), paraformaldehyde (P-00635, OXFORD LAB FINE CHEMICALS LLP) purchased from Sisco research laboratories. Sodium hypochlorite (NaOCl, 9-12\%), Cell culture materials were acquired from Gibco, USA and Hi-media, India. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fluoro-shield mounting medium, 4% formaldehyde solution were procured from Sigma Aldrich. Milli Q-double distilled water was used for all experiments.

B) Instrumental details:

**UV-Visible spectroscopy:** Absorption and PL measurements were performed through TECAN Spark M model microplate reader in absorbance and fluorescence intensity scan modes with Greiner-96 well plate (200 \(\mu\)L volume) by using XENON lamp source.

**Fourier Transform Infrared Spectroscopy:** FTIR measurements were performed using attenuated total reflectance (ATR) mode in ALPHAOPTII, Bruker Optik GmbH instrument. The electromagnetic spectrum was created using the Globar IR source (silicon carbide) detector. The samples were prepared as a thin film (10-15 \(\mu\)L) on the glass substrate by using a simple drop-casting technique and were characterized by IR spectroscopy between 4000 cm\(^{-1}\) to 400 cm\(^{-1}\). Further, the spectral analysis was examined using the OPUS/IR software.
Transmission Electron Microscope: Bright field TEM images were captured from JEOL-JEM 2100 high-resolution transmission electron microscope under high voltage 200 kV. 5-10 µL of BSA-Cu NCs were drop-casted on the copper grid (200 mesh, from TED PELLA) and allowed to dry overnight at room temperature. TEM images further analysis was done with the help of ImageJ software.

Time-Resolved Photoluminescence: TRPL measurements were carried out by using time-correlated single-photon counting (TCSPC - Horiba Jobin Yvon IBH) spectrometer. The laser diode (Delta Diode – 425L) peak power 230 mW, and the standard optical pulse-width ~100 ps can be generated at repetition rates up to 100 MHz. The sample lifetime measurement range is 6500 ns and the excitation laser source peak wavelength at 420 nm. IBH DAS6 software was used for further analysis of PL decay curves.

X-ray Photon Spectroscopy: The surface elemental analysis of the sample was determined by X-ray photon spectroscopy (XPS) measurements which were conducted by using Mg Kα (1253.6 eV) radiation (PHI VersaProbe III). The measurement was carried out with a detection angle of 45 °C, using pass energies at the analyzer for survey spectra as 55 eV and detailed spectra as 280 eV, respectively. The exact surface spot size of the sample (5 × 5 µm) was selected for the XPS analysis. The samples were neutralized with electrons from a flood gun (current 20 μA) to compensate for the charging effects of the surface. After the Cu NCs sample was sputter cleaned. The energy resolution for the instrument and for calibrating the binding energy for Cu peaks used at 932.6 eV was ≤0.5 eV.

DLS and Zeta Potential: Zeta Potentcolorasurements were taken in electrical double-layered cells and the hydrodynamic size measurements were taken in disposable sizing cuvette at 25 °C by using Malvern Zetasizer Nano ZS.
Fluorescence Quantum Yield

Red colour emitting Cu NCs sample and reference solutions were optically matched at 480 nm and then used to estimate fluorescence quantum yield (QY) measurements. Rhodamine 6G (QY 95%) power a pinch was dissolved in ethanol solvent and used as the reference. The following formula was used to calculate the relative fluorescence QY of Cu NCs.

\[ Q = \frac{Q_{\text{ref}} \times I \times A_{\text{sample}} \times \eta_2}{I_{\text{ref}} \times A_{\text{ref}} \times \eta_2_{\text{ref}}} \]

\( Q, Q_{\text{ref}} \): quantum yield of the sample, reference, \( I, I_{\text{ref}} \): integrated emission intensity area in the sample, reference, \( A_{\text{ref}}, A \): absorbance of reference, sample, \( \eta, \eta_{\text{ref}} \): refractive index of sample, reference.\(^1,2\)

Computational Methodology

Geometry optimizations of Cu\(_{38}\) cluster and hydrazine and alkyl chains functionalized Cu\(_{38}\) are carried out using the B3LYP method. Two different basis sets viz., LANL2DZ basis set for Cu atoms and 6-31g* basis set for C, H, N, and O atoms are used. Binding energies between Cu clusters and alkyl chains are calculated by the supermolecular approach used to calculate counterpoise corrected interaction energy,\(^3\)

\[ \Delta E_{\text{int}}(AB) = [E(AB)_{\text{Complex}} - (E(A) + E(B))] \] (1)

All calculations are performed using Gaussian 16 package.\(^4\)

Cu NCs – In vitro studies

Cell Culture

Cancerous cells MDA-MB 231 (Human mammary gland adenocarcinoma cell line) and non-cancerous cells HEK 293 (Human embryonic kidney epithelial cells) were acquired from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown in DMEM
(Dulbecco Modified Eagle Medium) media (Gibco) and RPMI-1640 (Roswell Park Memorial Institute) media (Hi-media) supplemented with 10% (v/v) fetal bovine serum, 1% L-glutamine, 1% non-essential amino acid, 1% penicillin, and 1% streptomycin. All cells were kept at 37 °C in a humidified 5% CO2 incubator.

**Cytotoxicity studies using MTT assay**

The MTT (3-(4,5-dimethylthiazole-2-y)-2,5–diphenyltetrazolium bromide reagent) test was used to assess the cytotoxicity of Cu NCs compounds in MDA-MB 231 malignant cells and HEK 293 normal cells. The cells were seeded and cultivated in triplicate in 96-well plates for 24 hours at 0.5x10⁴ cells per well. After that, the compounds were treated for 72 hours at varied doses (0.5, 1, 2.5, 5, 10, 25, 50 & 100 uM). The cells were then rinsed twice with 1XPBS before being treated for 4 hours with 450 mL (1 g/mL) of MTT solutions at 37 °C. The formazan crystals were then dissolved using the MTT solubilizing buffer. Using a Multimode microplate reader, the absorbance was determined at 570 nm (Varioskan, Thermo Fisher Scientific).

**Intra-cellular uptake study using confocal microscopy:**

A fluorescent con-focal microscope was used to examine the cellular morphology and uptake of MDA-MB 231 adenocarcinoma cells treated with Cu NCs sample at 10 uM concentration. The morphology of the MDA-MB 231 cells did not change significantly following the above treatments as compared to the control (untreated-UT) cells, as shown in the figure. It is obvious from the photos that the Cu NCs were efficiently localized within the cell. At the 24-hour time-point, the red fluorescence signal was determined to be stable but relatively less. These findings strongly suggest that the Cu NCs compound can be used as a fluorescence cell marker and delivery agent in future studies due to its excellent biocompatibility.
Cu NCs – *In vivo studies on C. elegans*

**Distribution study:** Embryos were shaken at 200 rpm in a complete M9 buffer at 20 °C. Worms in the L1 stage were grown on agar plates with *E.coli* ht115 at 20 °C. FUDR was added to nematode growth medium (NGM) plates to prevent offspring from nematodes understudy from reaching adulthood. Animals were washed three times with M9 buffer once they reached the late L4 stage. L4 stage worms were incubated with optimized Cu NCs (25 μL, N₂H₄H₂O), BSA (30 mg/mL, as control) at different incubation time points such as 1 hr, 2 hr, 4 hr, and overnight at 20°C, performed centrifugation for spinning down at 3,200 rpm at 4°C for 2 minutes. After that, Worms were treated with 4% paraformaldehyde for further distribution study. Then, worms were placed on a sliding glass with a 2 % agarose pad and soaked in a drop of M9 buffer. Following the application of a coverslip to the agarose pad, images were taken with Optika microscope ITALY 40x0.75 lens, excitation channel 405 nm. The images were processed with the ImageJ software.

**Lifespan Assay:** L4 stage nematodes were exposed to optimized Cu NCs for overnight at 20°C before being transferred to fresh 60 mm NGM FUDR and *E.coli* ht115 seeded plates (lifespan experiment "Day 0"). FUDR was added to NGM plates to prevent offspring from nematodes understudy from reaching adulthood. A total of 120 nematodes were investigated on six 35 mm NGM plates, one for each condition. Poking nematodes lightly with a platinum wire (three times) and monitoring them for at least 5 minutes confirmed mortality; nematodes that did not move after stimulation were scored as dead and removed from the plate. Censored nematodes died as a result of protruding/bursting vulva, bagging, or crawling off the agar. Each condition's median, mean, and maximal lifespan were calculated in comparison to wild-type nematodes that were also studied at the same time. Statistical analysis was performed using Oasis2 lifespan survival analysis, each experiment was repeated at least 3 times and normalized to unexposed groups.
Acute Toxicity Assay: Synchronized worm population was obtained by bleaching. To bleach the gravid hermaphrodites, a solution of NaOCl (9-12 %) was mixed with 1M NaOH and H₂O in a 1:2:4 ratio. Gravid adult Worms were washed from NGM plates with M9 buffer, incubated in bleaching solution for 3 minutes in a 1:1 ratio, vortexed, and eggs were washed three times with M9 buffer following worm lysis. Embryos were shaken at 200 rpm at 20 °C in M9 buffer. L1 stage nematodes were raised at 20 °C on NGM plates with FUDR and seeded with E.coli ht115. A toxicity Assay was performed on L4 nematodes. Synchronized L4 stage animals have been exposed to Cu nanoclusters of two different concentrations (Cu NCs of 25 µL N₂H₄ and Cu NCs of 1 mL N₂H₄), BSA (30 mg/mL) and for overnight at 20°C, then hundred and twenty nematodes were studied per condition, divided on six 35 mm NGM plates. Animals were counted every six hours for the first three days, then once every twenty-four hours until the twenty-first day of the assay. Missing nematodes and worms that died as a result of vulval bursting were censored. The Kaplan-Meier curves were generated using the online program Oasis2, and log-rank analysis was used to compare the significance of the survival curves using median lifespan. Experiments were carried out three times with consistent results.

Cu NCs – Antimicrobial assay on E. coli

Disc diffusion study: LB-Agar plates are used in the disc diffusion assay, which is carried out in laminar airflow. Whatman filter paper with pore size 11 µm was punched and the discs were sterilized with 100% ethanol. After drying, the discs were loaded with Cu NCs (20 µL) and left to dry. 200 µL of freshly prepared bacterial culture was spread over the plate with the help of an L-shape spreader and left for a few minutes. After loading the samples onto the discs, they were transferred to the plates using forceps that had already been labeled. To avoid contamination, forceps are treated with 100% ethanol and heated each time a new disc is held. Discs-loaded plates were placed inside the incubator at 37 °C without disturbing the plates for 48 hrs. We observed no zone of inhibition formed in the negative control due to the absence of
other antibacterial agents. Positive control, kanamycin (0.5 mg/mL) formed inhibition zone around 2 cm. The Cu NC composite was used at a concentration of 0.6-2.48 µg/mL (Cu 1x – 2.48 µg, Cu 0.5x – 1.24 µg, Cu 0.25x – 0.6 µg). It should be noted here that the Cu is present in the composite, excluding BSA because the control experiment (30 mg/mL) clearly demonstrates that there is no zone of inhibition. At higher concentrations of Cu NCs at 2.48 µg/mL forming the zone of inhibition ~2.5 cm comparatively more than the positive control. Digital images have been captured and added below.8
C) Additional Results

1. UV-Vis Absorbance measurement of larger Cu nanoparticles

![UV-Vis Absorbance Spectrum](image)

**Figure S1:** A typical UV-Vis absorbance spectrum of Cu NCs synthesized with the strong reducing agent NaBH4, allowing for the formation of larger size particles with a surface plasmon resonance peak at 540 nm in a few minutes.

2. BSA-Cu NCs – XPS analysis of functional groups
**Figure S2:** The surface analysis of different elements of BSA-mediated Cu NCs through XPS. (a) C1s (b) N1s (c) O1s.
3. Cu NCs – FTIR analysis

**Figure S3:** FTIR spectrum of as-prepared BSA and the BSA-Cu NCs. Due to their ultrasmall size, the as-synthesized BSA-Cu NCs did not show much observable difference when compared to the control BSA protein matrix.

4. Optimization of Cu NCs at the different reaction temperature
**Figure S4:** The PL intensity measured at different reaction temperatures from 35 °C to 75 °C and the maximum PL intensity observed at 55 °C.

5. Optimization of Cu NCs at different reaction times

**Figure S5:** The PL intensity was measured at different reaction times from 15 minutes to 4 hrs and the maximum PL intensity was observed at 75 minutes.
6. Optimization of Cu NCs at different pH

**Figure S6:** (a) The PL intensity spectrum of Cu NCs at different pH from 2-12, and at pH 9 Cu NCs produced high emission observed in the red region at 650 nm. (b) Digital photographs of corresponding Cu NCs from pH 2 to pH 12.
7. Optimization of Cu NCs at different reducing agent concentrations

**Figure S7:** (a) The PL intensity spectrum of Cu NCs at different concentrations of second reducing agent N$_2$H$_4$ and the high PL intensity observed at 0.025 mL concentration.
8. Optimization of Cu NCs with BSA capping agent

Figure S8: (a) The PL emission profile of Cu NCs at different concentrations of surface capping agent BSA and the maximum PL intensity observed at 30 mg/mL concentration.

9. PL Lifetime analysis of 1 mL reducing agent Cu NCs
Figure S9: The decreased QY corresponds to the time-resolved PL lifetime decay patterns of 1 mL reducing agent used controlled reaction condition of BSA-Cu NCs.

10. PL stability measurement of colloidal Cu NCs

![Figure S10: The representative PL intensity spectrum of optimized BSA-Cu NCs demonstrated exceptional colloidal stability for up to 6 months. But the PL intensity of Cu NCs showed a slight decrease in the QY after 10 months up to 9%. However, the Cu NCs still have high PL QY compared to the 4.1% reported in the literature so far.](image)

11. PL stability study at the different biological environment
**Figure S11:** The BSA-Cu NCs showed excellent stability as measured by PL intensity, and the Cu NCs were incubated for 6 hours at room temperature in different biological media such as FBS, PBS, and RPMI 1640 in Eppendorf tubes with a 1:1 ratio of Cu NCs under continuous thermoshaking.

12. Intra-cellular morphology & uptake study

**Figure S12:** Confocal images of fixed MDA-MB 231 human adenocarcinoma cells incubated Cu NCs at 40X magnification, time points (4h and 24h) and 40X+ 2.5X zoom images.
13. Acute toxicity study of Cu NCs-II on *C. elegans*

(Video) attached

**Figure S13:** Compared to animals incubated in Cu NCs generated using the reported methodology, nematodes treated with Cu NCs-II synthesized in a higher concentration reducing agent died instantly.²

14. Acute toxicity study of Cu NCs-I on *C. elegans*

(Video) attached

**Figure S14:** *C. elegans* were treated to a solution of Cu NCs-I synthesized in reducing agent of optimized concentration exhibited non-toxicity compared to animals incubated in Cu NCs-I without disturbing the normal survival of *C. elegans*.

(a) ![Image](image1)
(b) ![Image](image2)

15. Control experiment of BSA inside *C. elegans*

**Figure S15:** Control experiment: (a) *C. elegans* incubated with 30 mg/mL concentration of BSA and no significant variations were observed in the normal health span of nematodes. (b) Absence fluorescence observed from BSA inside the *C. elegans* in the
red region (OPTIKA B1000 fluorescent microscope ITALY 40×0.75 lens, excitation channel 405 nm).

16. Antimicrobial studies of Cu NCs on E. coli

![Figure S16](image)

**Figure S16:** Inhibition zone formation study with Cu NCs has been studied by using disc diffusion method: (a) BSA was used as a control, and no zone of inhibition was identified in solid culture medium at any of the three concentrations. (b) Cu NCs added as an antimicrobial agent, we have noticed that at higher concentrations inhibition zone (2.5 cm) formed more than the positive control (2 cm). At lower concentrations of Cu NCs were unable to show good antimicrobial behavior due to their low toxic property.

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