

Research Article

Synergistic in-vitro inhibition of lung cancer cells using L-carnosine-capped copper nanoclusters

Ritika Mittal^a, Neetu Kumari^b, Nitish Kumar Singh^{a,c}, Mulaka Maruthi^b, Nancy Gupta^{a,*}^a Department of Chemistry, Netaji Subhas University of Technology, Dwarka Sector-3, Dwarka, Delhi, 110078, India^b Department of Biochemistry, Central University of Haryana, Mahendergarh, Haryana 123031, India^c Department of Chemistry, Indian Institute of Technology, Roorkee, Uttarakhand 247667, India

ARTICLE INFO

Keywords:

Copper nanoclusters

L-carnosine

Green synthesis

Glutathione sensing

Anti-cancer properties

ABSTRACT

Traditional cancer treatments are often associated with high toxicity and significant side effects, limiting the safe dosage range for patients. To address these challenges, there is a critical need for specific medications with reduced toxicity and enhanced efficacy. This study combined the unique anticancer properties of L-carnosine, a dipeptide, and copper to synthesize L-carnosine-capped copper nanoclusters (Cu₄L₅). The nanoclusters were characterized using UV–vis spectroscopy, fluorescence spectroscopy, FT-IR spectroscopy, HR-TEM, HR-MS, and EDX mapping. The synthesized Cu₄L₅ exhibited synergistic anticancer effects against A549 lung cancer cells, as evidenced by MTT assay results showing significantly higher cytotoxicity toward cancer cells while demonstrating minimal toxicity to normal Vero-2 cells, as indicated by IC₅₀ values. Their anticancer properties were compared to those of L-carnosine alone and copper nanoclusters stabilized with L-histidine. Cu₄L₅ exhibited approximately twofold greater anticancer activity, confirming the enhanced efficacy of the L-carnosine-CuNCs combination. Additionally, CuNCs enable the detection of GSH in solution within the nanomolar concentration range using the Stern-Volmer equation. Computational studies of nanoclusters further revealed that copper atoms bind with ligands through its N of imidazole ring and C=O group, leaving carboxylate and NH₂ sites available for interacting with cancer cells. This dual functionality of Cu₄L₅, combining therapeutic and diagnostic capabilities, highlights its potential as a promising candidate for targeted cancer treatment with minimal off-target effects.

1. Introduction

Cancer remains one of the leading causes of mortality worldwide, posing significant challenges to public health and emphasizing the need for advanced and targeted therapeutic approaches to improve treatment outcomes [1]. Traditional chemotherapy, which utilizes cytotoxic drugs to target and destroy rapidly dividing cancer cells, has been complemented by advancements such as targeted therapy, immunotherapy, and modern chemotherapy. While these approaches have significantly improved cancer treatment, challenges such as severe side effects, non-specificity, toxicity to healthy cells, and drug resistance persist, emphasizing the necessity for more precise and effective solutions [2,3]. Nanotechnology has emerged as a transformative approach to cancer treatment, addressing many of the limitations of traditional therapies. Engineered nanoparticles offer targeted drug delivery to cancer cells, minimizing off-target effects and reducing harm to healthy tissues. They also enhance drug solubility, improve therapeutic efficacy, and help

overcome drug resistance mechanisms, making them a promising innovation in the fight against cancer [4].

Various theragnostic agents have been developed using a diverse range of nanomaterials, including carbon nanotubes (CNT) [5], quantum dots [6], Fe₂O₃ NPs [7], and silica NPs [8]. Among the nanomaterials, metal nanoparticles and clusters exhibit impressive anticancer properties while being less poisonous. Their easy surface modification, inherent advantages in geometrical control, and considerable water solubility have the potential to contribute to their precisely controllable interactions with cancer cells [9,10]. Notable examples include kaempferol-AuNCs synthesized by Yun and co-workers, DNA-AgNCs synthesized by Shi and co-workers, and Au₂₅(Capt)₁₈ synthesized by Bhattacharya and co-workers. These metal nanoclusters exhibit selective cytotoxicity, showing reduced toxicity to normal human cells while effectively damaging cancer cell nuclei, demonstrating their potential as promising anticancer agents [11–13].

In addition to their therapeutic properties, MNCs have found utility

* Corresponding author.

E-mail address: nancy@nsut.ac.in (N. Gupta).<https://doi.org/10.1016/j.inoche.2025.114278>

Received 22 October 2024; Received in revised form 16 January 2025; Accepted 4 March 2025

Available online 7 March 2025

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as fluorescent probes for biochemical sensing, attributed to their small size, unique core-shell geometry, strong fluorescence, and superior biocompatibility [14,15]. For example, MNCs have been employed to sense glutathione (GSH), a tripeptide comprising glutamine, cysteine, and glycine. GSH contains a crucial sulfhydryl ($-SH$) functional group that acts as the metal binding site, making it generally a suitable ligand for synthesizing metal nanoclusters [16–19]. However, sensing glutathione levels is also crucial, especially in cancer research, as the cancer cells, including A549 often exhibit higher levels of glutathione than normal cells. This increased level of glutathione helps cancer cells to survive and proliferate by protecting them from oxidative stress and promoting their resistance to chemotherapy and radiation therapy [20,21]. Sensing glutathione levels before and during treatment can provide insights into the anticipated response of cancer cells to therapy. Various types of sensors have been reported for detecting and measuring GSH in samples, including electrochemical and optical methods. Optical sensors, including fluorescence, surface plasmon resonance (SPR), and absorbance-based sensors, each offer unique advantages depending on the specific application, contributing to their widespread use across various analytical fields [22]. The detection of GSH using Cu-nanoclusters follows a fluorescence quenching-based method, which offers several advantages over other analytical techniques. Fluorescence sensors are known for their high sensitivity and selectivity, enabling the detection of low concentrations of analytes [23].

Nanoclusters are generally synthesized by stabilizing the metal core with ligands. A diverse range of ligands has been investigated, including amino acids, thiol, DNA, proteins, enzymes, and peptides [24]. The selection of an appropriate ligand is critical and often driven by specific objectives, such as improving solubility in particular solvents, enhancing optical and electronic properties, or achieving synergistic effects that make the nanoclusters suitable for targeted applications [25–27]. Among these, peptides emerge as highly promising ligands due to their non-toxic nature, excellent water solubility, and ability to interact effectively with metal surfaces through multiple functional groups [28]. L-carnosine (Fig. 1) is a naturally occurring dipeptide that serves as a significant supplier of β -alanine in the human body [29]. In addition to its physiological functions, L-carnosine demonstrates significant anticancer properties [30]. Its unique molecular characteristics, when combined with metal atoms in nanoclusters, offer a promising avenue for developing novel anticancer therapeutics. Among various metal nanoclusters, gold and silver nanoclusters have long been the focus of research due to their exceptional optical properties and high stability. However, the growing interest in sustainability and cost-effective materials has shifted attention toward copper nanoclusters (CuNCs). Copper, being more abundant and significantly cheaper than gold and silver, offers an economical alternative, making it suitable for large-scale applications [31,32].

In this study, we synergistically combined the anticancer properties of copper at the nanoscale with L-carnosine by synthesizing L-carnosine-stabilized copper nanoclusters (L-carnosine-CuNCs). The synthesized nanoclusters were thoroughly characterized and evaluated for their anticancer efficacy through *in vitro* experiments using the A549 lung cancer cell line (Scheme 1). The anticancer activity of L-carnosine-CuNCs was compared with L-carnosine alone and copper nanoclusters

stabilized with L-histidine to determine if the L-carnosine-CuNCs combination offers superior efficacy. Their safety and toxicity were further assessed on Vero-2 normal cells. Additionally, since the cancer cells, including A549, often exhibit elevated levels of glutathione (GSH) compared to normal cells, these CuNCs were also utilized for sensing GSH, demonstrating their dual functionality. Computational studies provided insights into the specific ligand sites interacting with the metal clusters.

2. Materials and methods

2.1. Materials

L-carnosine was purchased from Sigma-Aldrich, Ascorbic Acid (AA) from Thomas Baker Chemicals, and Copper Nitrate ($Cu(NO_3)_2$) from Thermo Fisher Scientific. L-histidine was purchased from Sisco Research Laboratories (SRL) Pvt. Ltd (SRL). Glutathione (GSH) was purchased from Central Drug House Pvt. Ltd. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco, Pen-Strep and Trypsin-EDTA from Himedia, Fetal Bovine Serum (FBS) from HyClone, DMSO from Sisco Research Laboratories Pvt. Ltd (SRL), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen. Ultrapure deionized water was used for all synthesis and characterization. All the chemicals were used as purchased and were not subjected to any further treatment or purification before utilizing in the experimental procedures.

2.2. Synthesis of L-carnosine capped copper nanoclusters

Synthesis was performed by mixing 60 mL of 0.1 M L-carnosine, 100 μ L of 0.1 M cupric nitrate, and 1 mL of 0.1 M ascorbic acid in a 100 mL beaker and keeping the mixture covered under continuous stirring at 60 °C for 4 h for complete growth and stabilization of nanoclusters. Turning of colorless solution to yellow colored solution indicated the formation of copper nanoclusters in the presence of L-carnosine as a capping agent. Obtained CuNCs were purified twice using an Amicon Ultra-15 Millipore filter membrane of cellulose with 10 kDa (kDa) molecular weight to remove dissolved reagents [25]. After the purification, to get rid of any large particles, the supernatant was pipetted off, and its diluted solution was centrifuged for 30 min at 6000 rpm. The resultant L-carnosine-capped CuNCs supernatant solution showed yellow color under day and light and appeared blue under UV light exposure and it was stored at 4 °C for further usage. Also, L-histidine-capped nanoclusters were synthesized by following the procedure published elsewhere [33].

2.3. Characterization

Perkin Elmer Lambda 365+ instrument was used to record absorption spectra. The Steady-state excitation (ex) and emission (em) spectra of the synthesized CuNCs were recorded on an FL 8500 Perkin Elmer fluorescence spectrofluorometer in a 1 cm $\times$ 1 cm $\times$ 3 cm quartz cuvette. Excitation and emission spectra were recorded by keeping the slit width of 5 nm slit and 0.6 s integration time. FL 8500 Integrating Sphere accessories were used for measuring the absolute quantum yield (QY). G6530AA (LC-HRMS-Q-TOF) was utilized to record the mass spectra using a smart beam laser operating in linear positive mode. Copper grids coated with a layer of carbon were used for sample preparation. HR-TEM images were recorded using a TECNAI G20 microscope. ImageJ software was used to calculate the average size of MNCs and a histogram was plotted to present the data. Elemental analysis was performed using EDX (TECNAI G20). Attenuated Total Reflection (ATR) mode was used to record the 500–4000 cm^{-1} range infrared spectra using a Bruker Alpha II FTIR spectrophotometer.

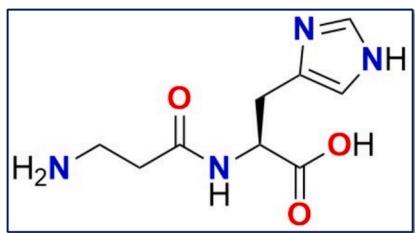
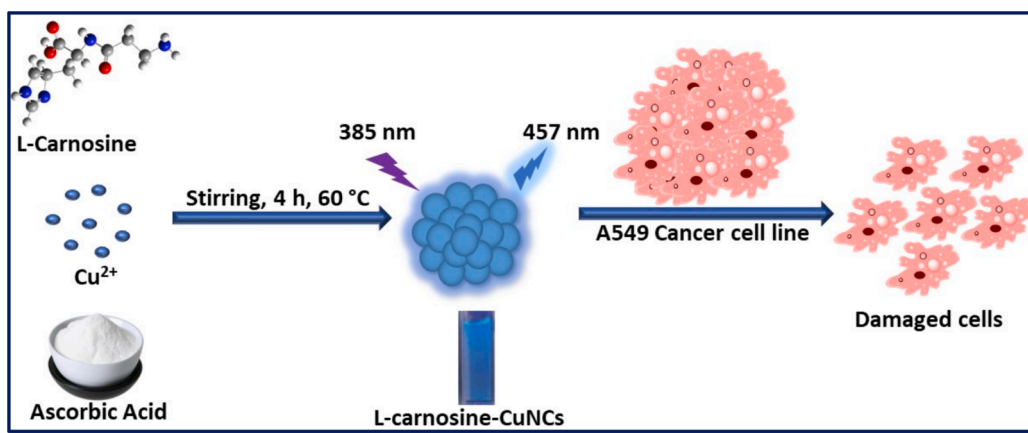


Fig. 1. Structure of L-carnosine.



Scheme 1. Schematics of the synthesis of water-soluble L-carnosine-CuNCs and their anticancer activity against A549 lung cancer cells.

2.4. Detection of GSH

A 1 μM stock solution of GSH was prepared in deionized water by diluting a 1 mM solution. Nanocluster solution was prepared by mixing 600 μL of purified clusters in 1.5 ml of deionized water. The stock solution of the GSH was added to the NCs in increments in the 5–80 μL range. The solution was made homogeneous by thorough mixing, it was then incubated at room temperature for one minute. Fluorescence spectra were then recorded in the absence (F_0) and increasing concentration of GSH (F).

2.5. Cell culture

The DMEM added with 10 % FBS and 1 % antibiotics (10,000 U/mL penicillin and 10,000 g streptomycin) at 37 °C with 5 % CO_2 , was used to cultivate Vero-2 and A549 cells in a controlled environment. Every 24 h, the medium was changed, cell confluency was monitored under a microscope, and cell detachment was accomplished using 0.25 % Trypsin-EDTA.

2.6. Development of MTT assay

For the development of the MTT assay, old media was replaced with fresh media, and then cells were treated with L-carnosine-CuNCs, L-histidine-CuNCs, and L-carnosine. The samples were dispersed in an appropriate quantity of DMEM media and applied to the cells at varying concentrations (31.25, 62.5, 125, 250, 500 μM , and 1000 μM). All sets of both Vero-2 cells and A549 cells, with and without the samples, were incubated for 48 h at 37 °C in 5 % CO_2 .

After the incubation period, the medium from the wells was removed, and they were washed with 1X PBS (Phosphate buffer saline), then 10 μL of MTT (5 mg/mL in PBS), and 90 μL of DMEM were added in each well. The plates were incubated for three to four hours at 37 °C in an incubator with 5 % CO_2 . After that, media containing MTT was removed from the wells. Using a microplate reader, the plates were read at 492 nm to determine the absorbance. The samples' 50 % inhibitory concentration (IC_{50}) was calculated using GraphPad Prism software.

2.7. Computational details

All calculations were done using the Gaussian 16 suite of quantum chemical program package. The Møller–Plesset (MP2) theory was used for geometry optimization with the LanL2dz basis set for all atoms to ensure uniformity across our study [34]. For metal nanoclusters, MP2 correlation energy corrections tend to yield more precise outcomes compared to alternative methods [35] and its combination with the LanL2dz basis set has been reported to yield reliable energy estimates

[36]. First, the initial structures of Cu_4 and L-carnosine were optimized individually. Since including five ligands with four metal atoms results in a large system that is impractical for geometrical optimization using MP2 methods, only one ligand with four Cu atoms was considered in the input file for optimization. Then, following optimization, an analytical frequency calculation was executed, confirming that all calculated frequencies were positive, thus affirming the authenticity of the reported structures as true minima. To elucidate the nature of the interaction between the Cu_4 cluster and the L-carnosine ligand, an integrated gradient model (IGM) analysis was performed using the Multiwfn software [37], and the resulting isosurfaces were visualized with the VMD software [38].

3. Results and discussion

3.1. Synthesis and characterization

The one-pot green synthesis method was used for producing fluorescent L-carnosine stabilized CuNCs utilizing ascorbic acid as a moderate reductant. Due to the bio-origin of the ligand and reducing agent and the utilization of an aqueous medium in its synthesis, it can be appropriately characterized as a green synthesis [24]. Under short UV illumination, the L-carnosine-CuNCs aqueous solution showed bright blue luminescence, while it appeared yellow in the daylight. The fluorescence spectra of CuNCs showed blue emission at 457 nm having 7.4 % quantum yield when excited at 385 nm (Fig. 2A) with the stoke shift difference of 72 nm to prevent the overlap between the excitation and emission peak. The absence of a broad peak due to SPR in the visible range of the absorption spectrum rules out the possibility of the formation of bigger nanoparticles. Turning of non-fluorescent reaction mixture (Fig. 2B) into a fluorescent product also confirms the formation of nanoclusters.

Various reaction conditions are generally found to affect the quality of synthesized nanoclusters, therefore, the reaction was optimized by using different concentrations of ligand (i.e. different metal–ligand ratios), reaction time, reaction temperature, and pH of the reaction mixture [39]. Reaction conditions resulting in the maximum quantum yield were further used for all the studies and applications. (Fig. S1, Table 1). Reactions performed at various ratios of the amount of metal ion precursor and ligand in the reaction mixture result in a maximum quantum yield at M:L of 1:600 (Fig. S1A). Certainly, the presence of an adequate amount of L-carnosine is vital for the generation of CuNCs, directly impacting their fluorescence intensity. Higher L-carnosine concentrations significantly raise the intensity of CuNCs. However, an excess of L-carnosine has the opposite effect, leading to a sharp decrease in fluorescence intensity. The effect of ligand concentration concerning the metal ion precursor has been found to have a similar effect on the

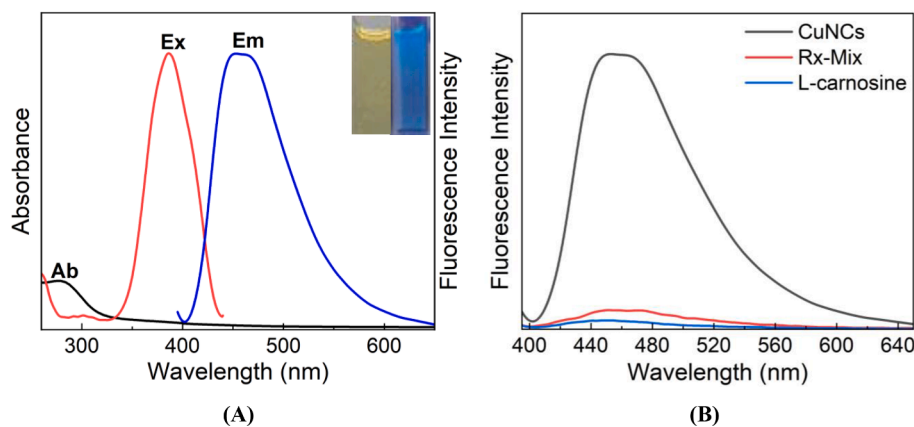


Fig. 2. (A) Absorption (Ab), Excitation (Ex), and Emission (Em) spectra of L-carnosine-CuNCs. (B) Emission spectra of synthesized nanoclusters (black), the reaction mixture (red), and L-carnosine (blue). Synthesized nanoclusters show blue fluorescence.

Table 1

Summary of the effect of reaction conditions such as metal-ligand ratio, reaction time, and reaction temperature on the fluorescence intensities of synthesized nanoclusters.

M: L	QY (%)	Time (h)	QY (%)	Temperature (°C)	QY (%)
1:100	2.1	2	1.9	30	2.9
1:200	2.6	3	2.8	40	6.2
1:400	3.4	4	6.5	50	6.8
1:600	6.5	5	3.2	60	7.4
1:800	2.4	6	2.9	70	7.2
1:1000	1.9	–	–	–	–

fluorescence intensity of synthesized nicotinamide-gold nanoclusters synthesized by Csapó and co-workers [40]. Prolonging the reaction time led to a gradual enhancement in fluorescence intensity (Fig. S1B) peaking at 4 h, after which it declined with further extension of the reaction period. Similar dependence on time was observed by Shao and co-workers also for L-carnosine-protected gold nanoclusters [41].

Similarly, increasing the temperature exhibits a substantial rise in emission intensity until reaching the 60 °C. However, beyond this point, further heating leads to a rapid decline in fluorescence intensity (Fig. S1C). This drop can be attributed to fluorescence quenching caused by the vigorous movement of particles [42]. L-carnosine does not support the formation of Cu nanoclusters at acidic pH, whereas some other ligands such as histidine, pepsin, and trypsin, have been found to stabilize the different number of metal atoms in the synthesized clusters at various pH levels [33,43,44]. On the other hand, at basic conditions, the reaction happens at all pH values and yields overlapping emission maximum (Fig. S1D).

When excited at various wavelengths, the emission maximum remains unchanged, indicating that the dispersion of nanoclusters has nearly a uniform size distribution. The same is true for excitation spectra, where the excitation maximum is independent of the wavelength of emission further supporting the clusters' uniform size distribution. Samples excited at 385 nm have the highest corrected emission intensities, making it the best wavelength for further excitation of all the samples (Fig. S2). Without a noticeable decline in fluorescence intensity, the fluorescent supernatant solution remained stable for three months at 4 °C (Fig. S3).

High-resolution mass spectrometry (HR-MS) was pivotal in accurately determining the number of metal atoms and ligands within the clusters. Linear positive-ion mode was utilized to measure both the ligand and the synthesized nanoclusters' masses. Peaks at m/z 227.11 ($n = 1$) and 453.22 ($n = 2$) are identified as monomeric and dimeric forms of L-carnosine. An additional peak at m/z 1389.79 is attributed to the

nanoclusters. By considering the molecular weight of L-carnosine and the atomic weight of Cu as 227.11 and 63.5, respectively, the most probable chemical composition of these well-dispersed Cu nanoclusters is determined to be Cu_4L_5 . This indicates that one nanocluster consists of 4 Cu atoms and 5 L-carnosine molecules in a combination of monomer and dimer (Fig. 3).

The HR-TEM image reveals a nearly consistent size distribution of nanoclusters. An average diameter of 2.2 nm was calculated using ImageJ software for analysis (Fig. 4). EDX spectra analysis validated the presence of Cu, O, N, and C in the sample (Fig. S4).

The synthesized L-carnosine-CuNCs were structurally investigated by FT-IR spectroscopy. Peaks in the IR spectrum located at 2922 cm^{-1} and 2855 cm^{-1} can be attributed to the C–H stretching vibration, the peak at 1740 cm^{-1} reveals the presence of COOH groups in L-carnosine [45]. The emergence of a novel peak at 717 cm^{-1} in the FT-IR spectra of L-carnosine-CuNCs confirms the creation of nanoclusters. This peak signifies the copper metal's interaction with the ligand (Fig. 5) [46].

3.2. Quantification of GSH using L-carnosine capped copper nanocluster

When varying concentrations of a 1 μM glutathione (GSH) solution were introduced into copper nanocluster (CuNCs) solutions, a proportional decrease in photoluminescence (PL) intensity was observed. This decline in intensity follows the Stern-Volmer Eq. (1) [47], demonstrating a linear correlation between intensity alteration and the quencher concentration.

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

Here, F_0 and F denote the emission intensity of CuNCs in the absence and presence of GSH, respectively, K_{sv} represents the Stern-Volmer quenching constant, and Q is the quencher concentration. A linear fit of the data yields an R^2 value of 0.99762, a K_{sv} value of $2.19 \times 10^7\text{ M}^{-1}$, and the detection limit for GSH is determined to be 3.1 nM by applying the formula $3\sigma/\text{slope}$, where σ denotes the standard deviation of the intercept. This remarkable sensitivity of CuNCs as a fluorescent probe underscores their efficacy in detecting even minute concentrations of GSH, particularly within the nanomolar range, thereby highlighting their superior sensing capabilities (Fig. 6). The limit of detection obtained here is significantly lower than the reported values for glutathione sensing using copper nanoclusters [48,49]. Fluorescent Au and Ag nanoclusters have also been reported for use in sensing GSH, for example, silver nanoclusters (AgNCs) synthesized by Shuang and co-workers [50] and amylase-gold nanoclusters (AuNCs) synthesized by Kailasa and coworkers [51] display higher limits of detection compared to what we observed with our synthesized copper nanoclusters.

Also, the binding constant of GSH was calculated using a modified

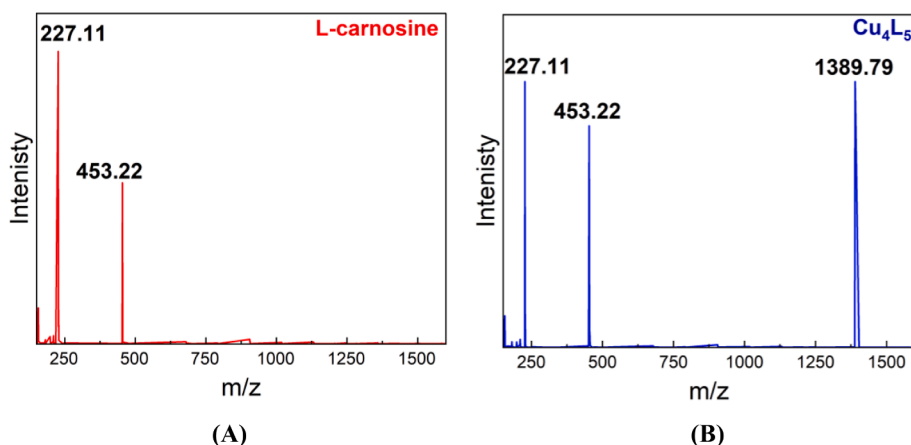


Fig. 3. (A) HR-MS spectrum of ligand L-carnosine and (B) L-carnosine-CuNCs. Peaks of nanoclusters correspond to Cu_4L_5 composition.

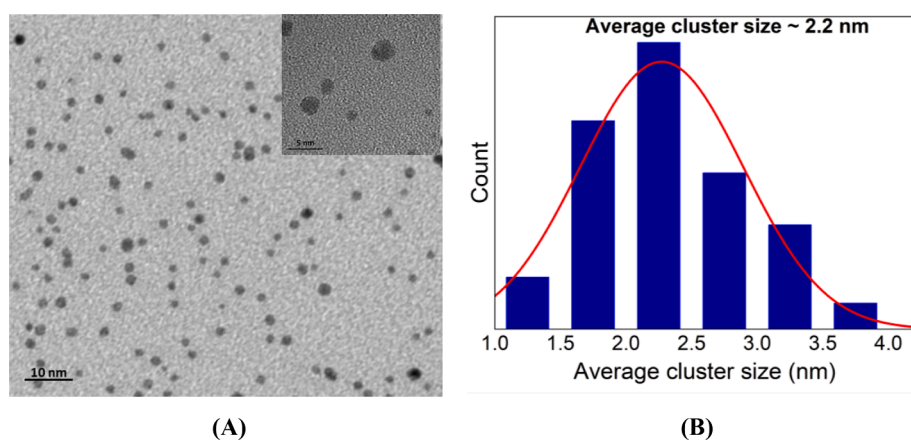


Fig. 4. (A) HR-TEM image and (B) the size distribution of L-carnosine-CuNCs. The histogram illustrates the size distribution of the clusters, with an average diameter of approximately 2.2 nm.

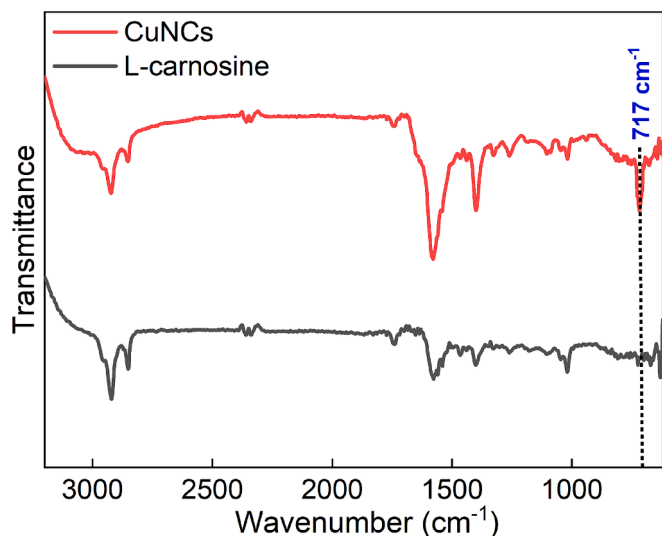


Fig. 5. FT-IR spectra of L-carnosine-CuNCs (Red) compared with ligand L-carnosine (Black). The additional peak at 717 cm^{-1} confirms the interaction of Cu metal with ligand L-carnosine.

Stern-Volmer Eq. (2) [52].

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n \log[Q] \quad (2)$$

Where K_b represents the binding constant of GSH, n is no. of binding sites of CuNCs. A linear fit of the double logarithm plot yields an R^2 value of 0.99549, a K_b value of $9.27 \times 10^7\text{ M}^{-1}$, and the n is determined to be 0.9836 (Fig. S5).

Analysis of the Stern-Volmer and modified Stern-Volmer plots reveals that the calculated K_{sv} and K_b values are of the same order, with $n \approx 1$. This strongly indicates that the quenching mechanism follows a static quenching model [53].

3.3. Cytotoxicity assay of L-carnosine capped copper nanoclusters against normal and cancer cell line

The cytotoxicity of the L-carnosine capped copper nanoclusters (assuming total conversion) was evaluated by an MTT assay in the A549 (lung cancer cells) cell line and Vero-2 normal kidney cell line. L-carnosine and Cu nanoclusters are individually known to show anticancer activities based on a previous report that L-carnosine is cytotoxic to cancer cells [54]. Therefore, they may produce a synergistic platform that can target cancer cells by combatting oxidative stress and disrupting vital biological processes linked to cancer development [32,55]. Their anticancer activities were evaluated in comparison to L-histidine-Cu nanoclusters and L-carnosine independently to study the performance of synthesized nanoclusters compared with their components. Various

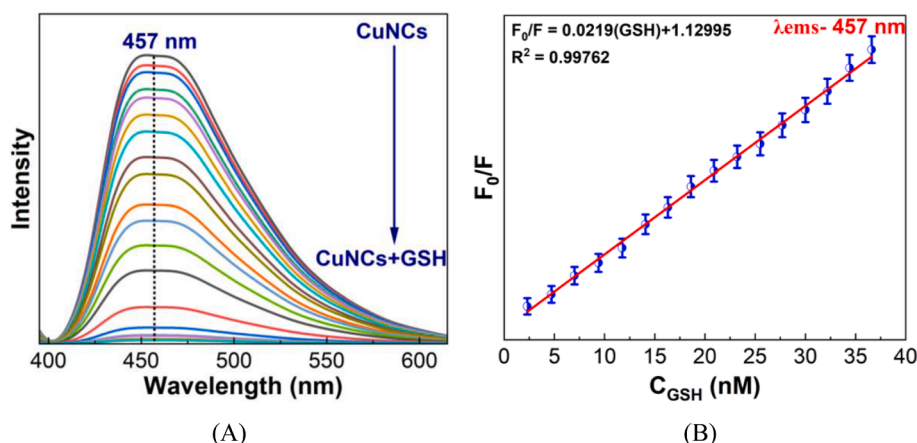


Fig. 6. (A) Emission spectra of CuNCs recorded in the presence of increasing concentrations of GSH (B) The Stern-Volmer plot for fluorescence quenching of nanoclusters by GSH.

concentrations of the CuNCs (ranging from 1000 to 31.25 μM) were incubated with normal cells and cancer cells, cell viability was assessed by MTT assay using absorbance values read from a microplate reader. Using GraphPad Prism software and a nonlinear regression (4 parameters) model, IC_{50} values were determined based on the viability percentages obtained. L-carnosine exerts its anticancer effects through a variety of mechanisms. These include the induction of apoptosis, inhibition of cell proliferation, and immune modulation. The collective action of these mechanisms contributes to carnosine's potential as a therapeutic agent in cancer treatment [56].

The MTT assay results indicate that all the samples show anti-proliferative activity against the human lung cancer cells i.e. A549 (Table 2). Among the tested samples, L-carnosine-CuNCs display the highest efficacy with lower IC_{50} values of $89.11 \pm 1.84 \mu\text{M}$, as compared to the higher IC_{50} values displayed by L-carnosine alone & L-histidine-CuNCs, which are $187.3 \pm 11 \mu\text{M}$ and $142.4 \pm 1.3 \mu\text{M}$, respectively (Fig. 7, Table 2). L-carnosine-CuNCs are more than twice as effective as L-carnosine alone and significantly outperform L-histidine-CuNCs, indicating that the combination of L-carnosine and Cu nanoclusters synergistically enhances anticancer activity.

Furthermore, L-carnosine-CuNCs require the highest concentration to inhibit 50 % of normal cell growth, followed by L-histidine-CuNCs and L-carnosine alone. Among all the formulations, L-carnosine-CuNCs exhibit the least toxicity to normal cells. The IC_{50} value of $1602.5 \pm 2.5 \mu\text{M}$ for L-carnosine-CuNCs on Vero-2 normal cells is approximately 18 times higher than that of the cancer cells, suggesting that normal cells are far less affected. This selective cytotoxicity profile, where L-carnosine-CuNCs are more potent against A549 cancer cells but less harmful to normal cells, makes them a promising candidate for cancer therapy with a favorable therapeutic index.

3.4. Computational analysis of the electronic structure of the L-carnosine capped copper nanoclusters

The structure of L-carnosine capped nanoclusters was optimized at the MP2 level of theory with LanL2dz basis set. Initially, L-carnosine and Cu_4 metal clusters were optimized separately to identify the minimum energy structures for subsequent L-carnosine-Cu nanocluster

Table 2

IC_{50} values for L-carnosine-CuNCs, L-histidine-CuNCs & L-carnosine for normal cell and lung cancer cell.

	L-carnosine-CuNCs	L-histidine-CuNCs	L-carnosine
IC_{50} (Vero-2) (μM)	1602.5 ± 2.5	1340 ± 1.0	1277.5 ± 1.5
IC_{50} (A549) (μM)	89.11 ± 1.84	142.4 ± 1.3	187.3 ± 11

optimization. The optimized structure of L-carnosine, Cu_4 , and L-carnosine- Cu_4 are shown in Fig. 8. To create suitable input structures for placing the ligand moiety and metal clusters, Mulliken charges obtained from the individual optimizations were used (Fig. S6). Four input configurations (Fig. S7) were generated by placing positively charged Cu atoms near the four negatively charged sites of L-carnosine (Table ST1), based on the expected electrostatic interactions between them. Electrostatic interactions between Cu_4 and four distinct sites within carnosine, with the optimized structures, are depicted in Fig. S8. The optimized structures revealed significant rearrangements during the optimization process. The structure of Cu atoms placed near the NH_2 nitrogen having the most negative Mulliken charge is expected to have the lowest energy. Whereas, surprisingly lowest energy structure corresponded to the one where imidazole nitrogen, with a lower negative charge, interacted with Cu_4 . During optimization, copper atoms not only approached the imidazole ring but also came into proximity with the $\text{C}=\text{O}$ group, reaching a distance of 2.15 Å (Fig. 8C). This suggests interactions not only between the Cu atom and the imidazole nitrogen but also involving the $\text{C}=\text{O}$ group. Similar electrostatic interactions between amino acids as ligands and metal clusters in the core have been studied by researchers using computational methods [34,57]. The energy differences of all structures from the most stable structure (Fig. 8C) have been calculated and provided in Table ST2 revealing a considerable energy difference between the different optimized structures. After all optimizations, the lowest-energy structure is the one in which Cu_4 interacts specifically with the oxygen (O2) and nitrogen (N6) sites of the ligand.

The optimized structure revealed interactions between copper atoms and specific functional groups of the L-carnosine ligand, namely the nitrogen of the imidazole ring and the oxygen of the carbonyl group. To classify these interactions, an Independent Gradient Model (IGM) analysis was conducted [58–60]. The nanocluster was divided into two components: the Cu_4 core and the L-carnosine ligand. The IGM analysis identified interactions between Cu(1) and the imidazole nitrogen, as well as between Cu(3) and the carbonyl oxygen (Fig. 9). The δ_g peak values for both Cu(1)–N and Cu(3)–O interactions were approximately 0.4, indicating characteristics consistent with coordination bonding.

To further explore the electronic structure of the lowest-energy Cu_4 -L-carnosine nanocluster, its HOMO–LUMO orbitals were also analyzed (Fig. S9). The calculated energy gap was 5.18 eV, with the electron density in both the HOMO and LUMO primarily localized on the four Cu atoms. A detailed examination of the molecular orbitals revealed two critical orbitals where the d-like orbitals of copper interact with the lone pairs or π -cloud of the ligand at the O(2) and N(6) sites (Fig. S10). In the optimized minimum-energy structure, the carboxylic and NH_2 groups remain fully accessible for interactions with cancer cells, while

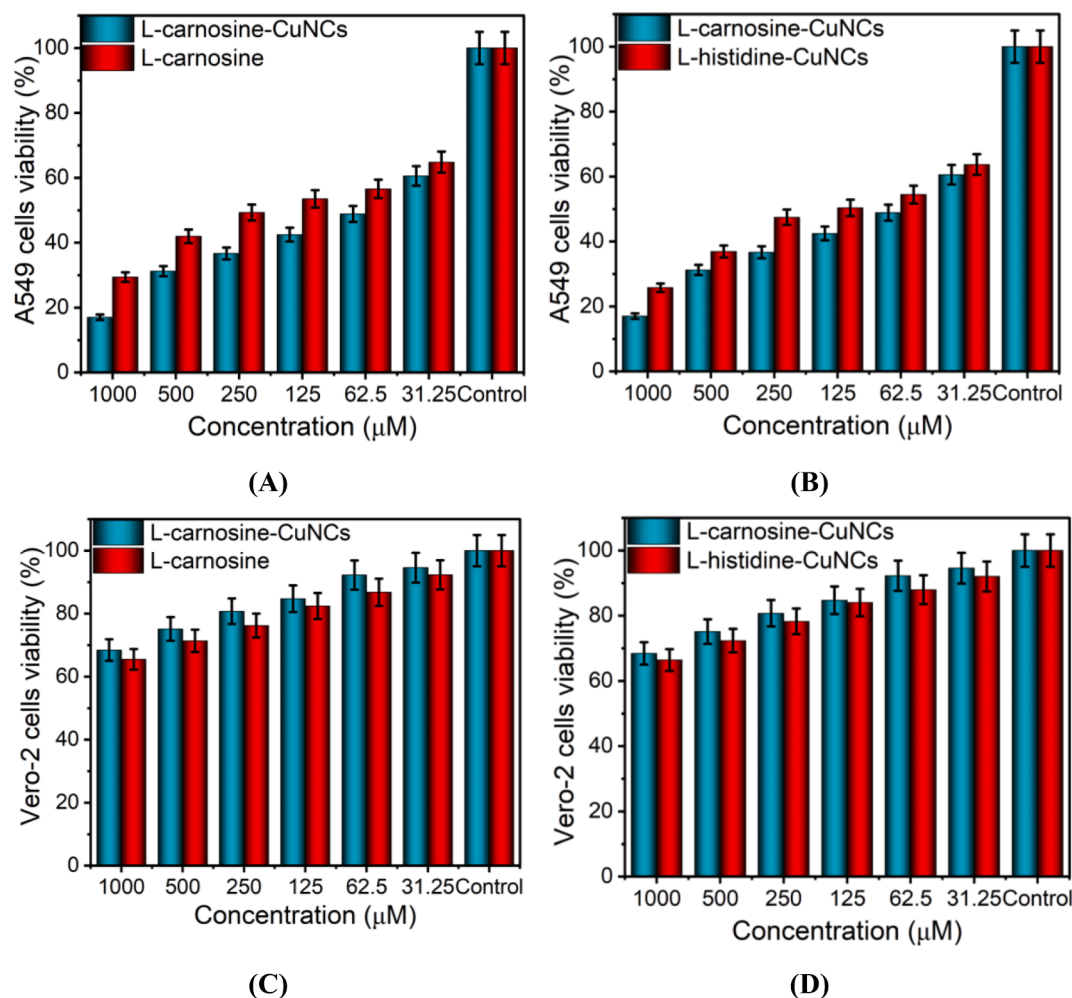


Fig. 7. Percentage of A549 viable cells on treatment with different concentrations of L-carnosine-CuNCs compared with L-carnosine (A) and compared with L-histidine-CuNCs (B). The percentage of Vero-2 viable cells on treatment with different concentrations of L-carnosine-CuNCs compared with L-carnosine (C) and L-histidine-CuNCs (D).

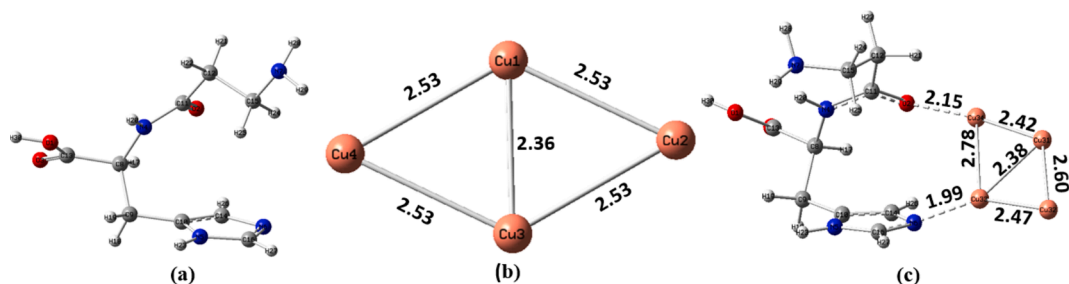


Fig. 8. Optimized structures of (a) L-carnosine, (b) Cu_4 cluster, (c) Cu_4 -L-carnosine, with MP2 - LANL2DZ.

the C=O and imidazole sites are only partially exposed.

4. Conclusion

Blue-emitting copper nanoclusters stabilized by the naturally occurring dipeptide L-carnosine were synthesized successfully for the first time. These nanoclusters exhibit strong blue fluorescence and remain stable for at least 3 months. TEM and HR-MS characterization revealed that the nanoclusters, with a molecular formula of Cu_4L_5 , possess a size of approximately 2.2 nm and they display adequate monodispersity. The fluorescent nanoclusters were utilized for sensing GSH concentrations in the nanomolar range using Stern-Volmer plot

analysis. Additionally, their anticancer properties were investigated against the A549 lung cancer cell line using an MTT assay and compared with L-carnosine and L-histidine-capped copper nanoclusters. Efficient conjugation of L-carnosine to the CuNCs was achieved, and the conjugate exhibited higher toxicity against A549 lung cancer cells compared to both L-carnosine and L-histidine-capped Cu nanoclusters. Better cytotoxicity against cancer cells is reflected in their lower IC_{50} values, as shown in Table 2. Computational analysis using MP2 theory combined with LanL2dz basis set revealed the binding sites of Cu clusters with L-carnosine as the N of imidazole ring and C=O group, leaving carboxylate and NH_2 groups free for interaction with cancerous cells. This work highlights the potential of L-carnosine-capped CuNCs as a promising

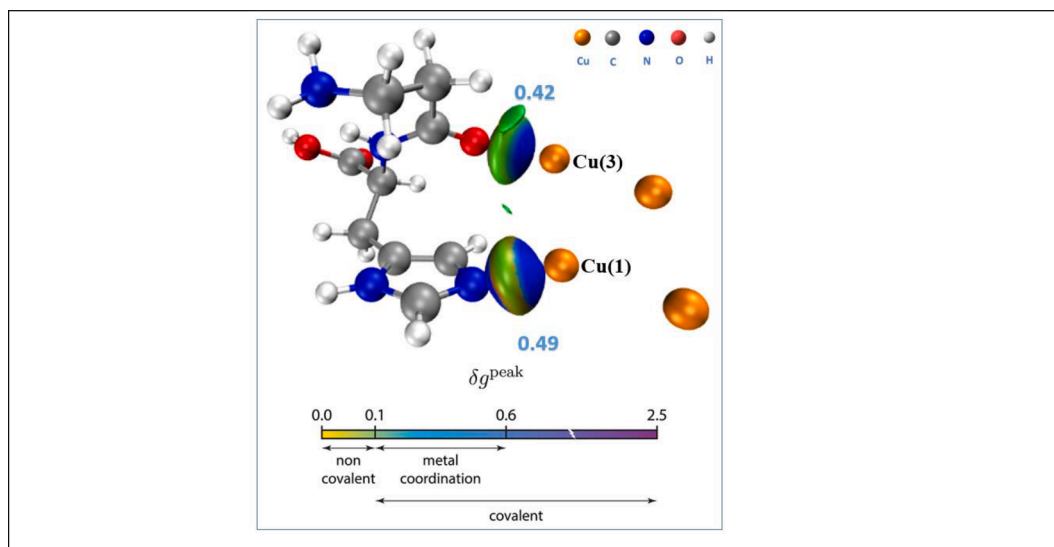


Fig. 9. IGM interaction visualization and δg^{peak} value.

candidate for cancer therapy and advances research in nanomaterial-based anticancer agents.

5. Consent to publish

Not applicable.

6. Consent to participate

Not applicable.

Ethical approval

Not applicable.

CRediT authorship contribution statement

Ritika Mittal: Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Neetu Kumari:** Writing – original draft, Methodology, Investigation. **Nitish Kumar Singh:** Software, Conceptualization. **Mulaka Maruthi:** Supervision, Formal analysis. **Nancy Gupta:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Funding

Author R.M. has received research support from the Council of Scientific & Industrial Research (CSIR), India (Grant number – CSIR-WARD/JRF-NET2021/113630). The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

R.M. thanks the Council of Scientific & Industrial Research (CSIR), India, for providing a fellowship. We thank the Department of Chemistry, Netaji Subash University of Technology (NSUT) for providing

infrastructure. N.K. and M.M. thank the Department of Chemistry, Central University of Haryana for providing infrastructure. We thank Dr. Yatender Kumar from the Department of Biological Sciences & Engineering, NSUT for the valuable discussion on anticancer studies. We also thank Dr. Harsh Yadav, Department of Physics, NSUT, for his support in computational studies.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.inoche.2025.114278>.

Data availability

Data will be made available on request.

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Ritika Mittal received her BSc (2017, Kurukshetra University, Kurukshetra, India), MSc (2019, Chaudhary Devi Lal University, Sirsa India). She is currently a research scholar in the Department of Chemistry, Netaji Subash University of Technology, New Delhi, India.



Neetu Kumari received her BSc(H) Zoology (2017, Hindu college, Delhi University, India), MSc (2019, Department of Zoology, Delhi University, India). She is currently a research scholar in the Department of Biochemistry, Central University of Haryana, Mahendergarh, India.



Nitish Kumar Singh received his BSc (2022, Deen Dayal Upadhyaya College, Delhi University, India), MSc (2024, Netaji Subash University of Technology, New Delhi, India)



Mulaka Maruthi received his Master of Science degree from the Department of Biochemistry and Molecular Biology, Pondicherry University, Pondicherry (2008), India and his doctorate from the Department of Animal Biology, University of Hyderabad, Hyderabad, India in 2016 for the study of “Modulation of host cell sumoylation facilitates efficient development of *Plasmodium berghei* and *Toxoplasma gondii* & Functional characterization of *Plasmodium berghei* proteasome inhibitor (PI31) through reverse genetics approach”. He then completed his postdoctoral research at, the Institute of Molecular Medicine and Infectious Diseases, Drexel University College of Medicine, USA (2018-2020). He works as an Assistant Professor, at the Department of Biochemistry, School of Interdisciplinary and Applied Life Sciences, Central University of Haryana, Mahendergarh.



Nancy Gupta received her Master of Science degree from Indian Institute of Technology (IIT) Roorkee, India in 2010 and doctorate from IIT Bombay (supervisor, Professor Anindya Datta) in 2016 for the spectroscopic studies of Nafion thin films and membranes. She then completed her postdoctoral research at IISER Pune working with Prof. Angshuman Nag in 2019. She then joined an accelerator program Entrepreneur First in Bangalore to explore the market size and utility of her ideas which could be converted to potential start-up. After finding the ideas are in nascent state of research and need some years of intense research to bring them in the market, she joined Thermo Fisher Scientific to lead the content marketing and aggregator business of the organization. In June, 2021, she joined Netaji Subhas University of Technology as Assistant Professor.