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Lysozyme coated copper nanoclusters for green fluorescence and their utility in cell imaging

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Abstract

Fluorescent, pH dependent and water soluble copper nanoclusters (CuNCs) were synthesized using lysozyme (lyz) as the stabilizing agent to give lysozyme coated copper nanoclusters, viz., lyz–CuNCs. The lyz–CuNCs were 3-5 nm in size at neutral pH and exhibit green fluorescence ($\lambda_{em}$ ~510 nm) when excited at 490 nm and exhibited maximum quantum yield of 18%. However, under the basic conditions, aggregates of lyz–CuNCs were seen with a particle size of ~100 nm. The emission of lyz–CuNCs observed at 510 nm compliments that from the blue ($\lambda_{em}$ = 450 nm) and the red ($\lambda_{em}$ = 650 nm) nanoclusters and thus bridges the gap. On the other hand, under acidic conditions, these show a size of 5-10 nm but are weakly fluorescing. The particle size and aggregations were monitored by TEM studies carried out using the samples prepared both from the acidic and the basic conditions. The lyz–CuNCs prepared at neutral pH shows >90% cell viability and hence can be used as probe for cellular imaging. The imaging was carried out with both healthy and cancer cell lines, viz., NIH3T3 cells (Mouse embryonic fibroblast cell), MCF7 cells (human breast cancer cells) and MDA-MB-231 cells (human estrogen negative breast cancer cells). The Z-stack study suggested the presence of lyz–CuNCs in the cells in cytoplasm. Thus, the green fluorescent lyz–CuNCs can be an alternate to green fluorescent protein (GFP) that is used for cell imaging purpose, since the latter needs tedious procedure to express, purify and to conjugate.

Key words: lysozyme coated copper nanoclusters (lyz–CuNCs); green synthesis; cell imaging; biocompatibility; green fluorescence; pH dependent emission.
Introduction

Metal nanoparticles of 3-5 nm shows new physical and chemical properties owing to their nano-metric size\(^1\) and hence can be used in a broad range of applications, including, cell labeling,\(^2\) ion sensing,\(^3\) catalysis\(^4\) and anti-cancer activity.\(^5\) The recent literature primarily deals with the luminescent nanoclusters of Au\(^6,7\) and Ag\(^8\) stabilized by a variety of small chemical species as well as large biological molecules. Till date, no abundant information is available regarding the synthesis of copper nanoparticles (CuNPs) and nanoclusters (CuNCs) due to the problems arising from the stability and the size of such nanoparticles.\(^9-11\)

However, the CuNPs have recently attracted special attention because of their low cost and novel optical, electrical, mechanical and thermal properties.\(^5\) The CuNPs are used in textiles, paints, plastics and food industries\(^3,4\) due to their antimicrobial property. The protein coated CuNPs are expected to exhibit anticancer activity owing to their redox ability.\(^5\) Recent studies showed that the fluorescent CuNPs are stabilized by proteins, such as, BSA, which gives red fluorescence, and lysozyme, which gives both red and blue fluorescence.\(^12-18\) Therefore, the stabilization of fluorescent nanoclusters of copper emitting in the visible region is still a challenging task. Proteins have been widely used in directing the synthesis of functional nanomaterials owing to the coordinating ability of –NH\(_2\), -COOH, -CONH\(-\), -OH and –SH groups present as side chains in proteins.\(^18\) The use of lysozyme in the present study derives its impetus from, (i) high pH stability due to the presence of six Lys and eleven Arg (pKa = 10) residues, (ii) coordination ability towards metal ions through the carboxylates (Asp and Glu) and imidazole (His), (iii)
ability to reduce metal ion such as Cu\(^{2+}\), Ag\(^{+}\), Au\(^{+}\) using Tyr side chains\(^{19}\) and (iv) its well-known antibacterial property.\(^{20}\) Hence, Lysozyme has been widely used for the synthesis of metal nanoclusters, such as copper,\(^{13}\) gold\(^{21, 22}\) and silver\(^{23}\) nanoclusters. Herein, we report the lysozyme (\textit{lyz}) directed synthesis of stable, water-soluble and green fluorescent copper nanoclusters \textit{lyz–CuNCs}, with excitation (\(\lambda_{\text{ex}}\)) at 490 nm and the emission (\(\lambda_{\text{em}}\)) at 510 nm and demonstrated their utility in cell imaging.

**Experimental**

**Preparation of copper nanoclusters coated with lysozyme (\textit{lyz–CuNCs})**

Thirty mg of lysozyme was added to 3 ml of water. To this 100 \(\mu\)l of 100 mM CuCl\(_2\) was added. The solution was stirred for 30 min and to this a 100 \(\mu\)l of 1 M NaOH was added as 10 \(\mu\)l portion each time for ten times over a period of 10 min and stirred for another 30 min. Then 100 \(\mu\)l of hydrazine was similarly added over a period of 10 min and stirred for one day. The pH of the resultant solution was 12. The pH of the solution was brought down to 7, 5 and 3 in different vials by adding requisite volume of dil HCl and the final volume was maintained at 10 ml and the solutions were stirred for one day. At pH = 7, the \textit{lyz–CuNCs} solutions are stable for more than a month as confirmed by fluorescence spectral measurements. In order to remove the un-reacted small molecular components, the resultant solutions were dialyzed against water for 24 hrs by replacing the medium three times intermittently. The dialyzed samples showed similar characteristics as that of the un-dialyzed ones as proven by fluorescence and TEM studies. The dialyzed \textit{lyz–CuNCs} were used for cell imaging studies.
Quantum yield (QY) measurement

The fluorescence quantum yields of lyz–CuNCs were determined using the equation,

$$\Phi_X = (\Phi_{ST} \times A_{ST} \times F_X \times \eta_X^2) / (A_X \times F_{ST} \times \eta_{ST}^2),$$

where \(\Phi\), \(A\) and \(F\) refers to the quantum yield, absorbance and fluorescence intensity in the solvent having a refractive index of ‘\(\eta\)’. The subscripts, ST and X refers to standard and unknown respectively. In the present case, \(\Phi\)’s were measured in water with \(\eta = 1.33\). The emission spectra for the lyz–CuNCs samples were recorded at the excitation wavelength of 490 nm at pH values of 3, 5, 7, 10 and 12, and keeping the slit width at 3 nm. The quinine hemisulfate in 0.1 M H\(_2\)SO\(_4\) was used as reference having a quantum yield of 0.546 (\(\lambda_{ex} = 310 \ & \lambda_{em} = 455\) nm).

Sample preparations and studies

Fluorescence studies were performed on Perkin Elmer LS55 Fluorescence Spectrometer. The lyz–CuNCs solutions were used for the fluorescence studies by exciting these at 490 nm and measuring the spectra from 500 to 700 nm using a 5 nm slit width for the excitation and emission gates at a scan speed of 300 nm/min. UV–visible absorption studies were performed on a Shimadzu’s UV-2101 PC. One ml solutions were used for collecting the spectrum using a faster scan speed and slit width of 5 nm in the range of 200 – 800 nm. Far- UV CD spectra were collected using a Jasco J-815 CD spectrometer. The path length of the cuvette was 0.1 cm and the band width of the light used was 1 nm. Three scans were recorded with a scan speed of 100 nm/min and the scans were averaged.

Spectroscopy and microscopy studies
The matrix assisted laser desorption ionization (MALDI) studies were performed on AutoflexIII TOF/TOF (MALDI-TOF mass spectrometer, Bruker Daltonics Co.) using sinapinic acid as a matrix and by using 355 nm laser as an ionization source. The FTIR studies were performed using Perkin Elmer FT-IR spectrometer. One mg of lyophilised sample of \textit{lyz-Cu NCs} was grinded with KBr and a pellet is made from this. The spectrum for only lysozyme was also recorded for comparison. In order to carry out field emission gun – transmission electron microscopy (FEG-TEM) or just TEM microscopy studies, samples were spread over carbon coated copper grids having 200 mesh and dried and analyzed by using JEOL JEM-2100F (FEG-TEM) operating at 200 kV. X-ray photoelectron spectroscopy (XPS) was performed using Mg K\textalpha excitation (1253.6 eV) with a VG ESCALAB MKII spectrometer. Calibration for the binding energy was done based on carbon 1s being at 284.6 eV.

\textbf{Results and discussion}

\textbf{Lysozyme coated copper nanoclusters (\textit{lyz-CuNCs})}

The \textit{lyz-CuNCs} were initially prepared from a solution of lysozyme and copper (II) chloride at pH = 12 upon treating with a mild reducing agent, such as, hydrazine and the solution was adjusted to the requisite pH using HCl. The \textit{lyz-CuNCs} were characterized by UV-Vis absorption, fluorescence, CD and XPS spectroscopy, and also by TEM.

\textbf{Emission and absorption studies}

Among the studies carried out at pH = 12, 10, 7, 5 and 3, the one that is studied at the neutral pH, i.e., a mixture of \textit{lyz} and copper salt solution (details given in experimental)
resulted in dark yellow colour after one day of incubation and the fluorescence emission suggested the formation of CuNCs. Photographs of the vials of lyz–CuNCs in aqueous solution at all pH values under visible and UV light are given in Fig. 1. At pH =12 and 10, the solution were transparent and exhibit no green fluorescence when observed under UV light. The green fluorescence was stronger at neutral pH. However, there was only a minimal amount of green fluorescence at pH = 5 and 3 due to the precipitation and/or agglomeration. In the literature, the lysozyme capped CuNCs were synthesized at pH 11-12 and temperature of 40-45 °C, but using two different ratios of protein to the copper salt to result in blue emission ($\lambda_{ex} = 360 \ & \ \lambda_{em} = 450 \ nm$) in one case$^{13}$ and red emission ($\lambda_{ex} = 365 \ & \ \lambda_{em} = 600 \ nm$) in the other.$^{14}$ Therefore, the literature reports of lyz–CuNCs sharply differ from our case where our lyz–CuNCs were synthesized at neutral pH and RT and these show green fluorescence with $\lambda_{em} = 510 \ nm$, and not the blue or the red emission. While the excitation is in UV region (360-365 nm), in the present case the excitation is in the visible region at 490 nm. Thus the lyz–CuNCs reported in this paper exhibited a shift of 60 nm in their fluorescence emission when compared to the blue lyz–CuNCs reported in the literature.$^{13}$ Thus, our green fluorescent lyz–CuNCs compliments the blue$^{13}$ and red$^{14}$ nanoclusters reported in the literature by completing the range of emission of 450 to 600 nm via bridging the gap with its emission centered at 510 nm.
Fig. 1 Photographs of lyz–CuNCs in aqueous solution under visible (left side vial) and UV (right side vial) light in each pair at the indicated pH.

As compared to other pH conditions studied, at pH = 7, the agglomeration is prevented due to the capping of lyz that results in exhibiting significant fluorescence emission intensity at 510 nm (Fig. 2a). Minimal fluorescence emission intensity was noticed for the solution of lyz–CuNCs at pH = 5 and no fluorescence intensity was noticed for that studied at pH = 12, 10 and 3. Solution of lyz–CuNCs was dialyzed for removing unbound copper ions as well as other small molecular components. Even after the dialysis, the solutions exhibited fluorescence emission for the study carried out at pH = 7 (Fig. 2b). The quantum yields of the lyz–CuNCs determined as given in the experimental are 0.014, 0.016, 0.18, 0.037 and 0.0093 at pH values of 3, 5, 7, 10 and 12 respectively, with respect to quinine hemisulfate, thus showing highest quantum yield at pH = 7. The protein lyz alone shows maximum emission at ~350 nm upon excitation at 280 nm at all the pH values studied due to the presence of aromatic amino acids, viz., Trp and Tyr as can be noticed from Fig. 2c. When excited at 490 nm, lyz alone doesn’t show any fluorescence (Fig. 2d) which suggested that the green fluorescence is emitted only by the protein coated CuNPs, viz., lyz–CuNCs, but not simply by the protein.

Fig. 2 (a) pH dependent fluorescence spectra of (λex 490 nm) lyz–CuNCs. (b) Emission spectra of undialyzed (--) and dialyzed lyz–CuNCs (--) at pH = 7. (c and d) pH dependent
fluorescence spectra ($\lambda_{ex} = 280$ and 490 nm respectively) of lyz. Colour code for pH in Fig. a, c & d: 3 (―); 5 (―), 7 (―), 10 (―), 12 (―).

The absorption spectrum of lysozyme alone shows a typical protein absorption spectrum with maximum peak at 280 nm and without any SPR band at all the pH studied (Fig 3a). However, the lyz–CuNCs shows two peaks, one broad peak at ~300 nm and a sharp peak at ~220 nm (Fig 3b). In the absorption spectra at pH = 7, no SPR band was noticed at 550 nm due to smaller size of the copper nanoparticles as measured based on TEM (3-5 nm) and these are referred as nanoclusters (NCs) (Fig. 3b and c). Thus we find a significant increase in the absorbance of the SPR band observed at ~550 nm in case of pH =12 which distinguishes this from the remaining pH values and this supports the nano particle formation as can be noted from the inset of Fig. 3b. Thus, the SPR band almost diminishes as the pH of the solution decreases and turns to acidic (Fig. 3b). The spectrum of lyz alone is totally different from the absorption spectra obtained for lyz–CuNCs and the latter is characteristic of the copper bound lyz (Fig. 3a and 3b).

Fig. 3 pH dependent absorption spectra of (a) lysozyme alone and (b) lyz–CuNPs. The SPR band is shown in inset. The colour code for pH in both (a) and (b) are: 12 (―); 10 (―), 7 (―), 5 (―), 3 (―). (c) Absorption spectra of undialyzed (―) and dialyzed lyz–CuNCs (―) at pH = 7.

CD and XPS studies
While the simple *lyz* is predominantly present in α-helical (222 nm band in CD) structure, the *lyz–CuNC* s exhibit mostly random coil (200 nm band in CD) like structure at all the pH conditions studied (Fig. 4a). Even after the dialysis, at pH = 7, the random coil structure was still seen to be predominant (Fig. 4b). This suggested the partial denaturation of the protein in *lyz–CuNC* s which helps in opening of α-helix as well as in reducing Cu$^{2+}$ to Cu$^{0}$ and the protein, *lyz* is coated onto the *CuNCs*. XPS analysis was performed with *lyz–CuNC* s obtained after the dialysis. The XPS spectra were characteristic of the reduction of Cu$^{2+}$ to Cu$^{0}$ both based on the main peak as well as the satellite peaks. While the Cu$^{2+}$ shows two strong satellites of which one is at 962 eV, the Cu$^{0}$ shows two weak satellites with the main peak being centered around 933 eV. All these features can be clearly seen from the XPS spectra given in Fig. 4c and the spectra corresponds to the Cu$^{0}$ species.

Fig. 4  (a) Circular dichroism spectra of *lyz–CuNP* s at different pH values: 12 (–), 10 (–), 7 (–), 5 (–), 3 (–), and simple *lyz* at pH = 7 (–).  (b) CD spectra of simple *lyz* (–) and dialyzed *lyz–CuNCs* (–) at pH = 7.  (c) The XPS spectrum of copper in *lyz–CuNCs* (dialyzed) at pH = 7.

**Characterization by MALDI and FTIR studies**
The lysozyme, in its native state showed m/z peak around 14300 Da (for z = 1) and 7145 Da (for z = 2). Upon addition of 20 equivalents of Cu$^{2+}$ at pH 10, the peak shifts in the molecular weight corresponding to the presence of higher copper clusters, such as, Cu$_1$, Cu$_2$, Cu$_4$ and Cu$_6$ as noticed from the m/2 peak (Fig. 5a). Under these conditions, the peak corresponding to the protein alone was almost completely diminished and the copper bound ones were noticed as marked in the spectra. Upon addition of hydrazine, the Cu$^{2+}$ ion is reduced to Cu$^0$ and results in lysozyme bound nanocluster which was then dialyzed. As the desorption of the dialysed nanocluster is not abundant, the corresponding MALDI spectra resulted in greater noise. In case of blue fluorescent lyz–CuNCs, 2 to 9 copper bound nanoclusters (Cu$_2$, Cu$_4$ and Cu$_9$) were reported,$^{13}$ while no MALDI studies were reported in case of red lyz–CuNCs.$^{14}$ An FTIR spectrum of simple protein shows amide peak at ~1600 cm$^{-1}$ along with a shoulder at ~1700 cm$^{-1}$. In case of lyz–CuNCs, only the shoulder disappears and the amide band is retained, suggesting the capping of CuNCs by the protein, lysozyme. Thus, the protein acts as a capping as well as stabilizing agent in these CuNCs (Fig. 5b).

![Fig. 5](image)

Fig. 5 (a) MALDI-TOF spectra of simple lyz (—) and the lyz treated with Cu$^{2+}$ in 1:20 ratio at pH 7 (—) and at pH 10 (—). (b) FTIR spectra of simple lyz (—) and lyz–CuNCs (—).
TEM studies

The TEM micrographs were measured for *lyz-CuNCs* at pH=7 both before and after the dialysis. Before the dialysis, the *lyz-CuNCs* exhibited particle sizes in the range 5-20 nm (Fig. 6a, e, f). The protein coating in *lyz–CuNCs* was proven by EDAX, which shows peaks for copper along with N, C, O, and S (Fig. 6b) and particle size distribution is shown in inset. Since, some larger size particle of 10-20 nm was noticed in this sample, TEM was performed again with the dialyzed and centrifuged solution for establishing the size distribution of *lyz–CuNCs*. After the dialysis, the particle size distribution was restricted to 3-5 nm (Fig. 6c, d, g, h). The particles in the size distribution (Fig. 6d) of 3-5 nm of *lyz–CuNCs* were expected to show fluorescence emission and these indeed showed green fluorescence as presented in this paper.

![TEM images and EDAX spectra](image)

**Fig. 6**  *Lyz–CuNCs* at pH = 7. (a) TEM image before dialysis (scale bar is 50 nm). (b) EDAX of the sample before dialysis. The inset bar diagram is a particle size distribution.
plot. (c) TEM image after dialysis (scale bar is 20 nm). (d) Particle size distribution after dialysis. HR-TEM of lyz-CuNCs at pH 7.0: (e, f) before and (g,h) after dialysis. (i, j) are TEM and (k) is HR-TEM images of lyz–CuNCs at pH = 5. The scale bar in (i) is 50 nm and in (j) is 5 nm. (l) EDAX of the lyz–CuNCs at pH = 5. The inset in (l) is the particle size distribution plot wherein the x-axis corresponds to size (nm) and the y-axis corresponds to percent (%) of the nanoparticles.

At acidic pH, the size of lyz–CuNCs was in the range of 5-10 nm but these nanoparticles were precipitated out from the solution and hence weak fluorescence was noticed (Fig. 6i, j and Fig. 7a, b). The micrograph indicates a clear-cut aggregation of the small nanoparticles into clusters of 50-100 nm size. Even the EDAX measurement (Fig. 6l & Fig. 7d) carried out with the samples at pH = 5 and 3 clearly showed the protein coating by exhibiting the peaks for C, N, O, S along with copper. On going from pH = 5 to 3, spherical aggregates are more prevalent. Thus, the TEM clearly supports that 3-5 nm size lyz–CuNCs are formed mainly at pH = 7 and these gives rise to green emission, while the particles observed at all other pH values are of larger in size or aggregates leading to minimal or no fluorescence. At pH = 5 and 3, the HR-TEM shows inter planar distance of 0.21 and 0.19 nm respectively that corresponds to (111) lattice planes of metallic copper, supporting the reduction of Cu$^{2+}$ to Cu$^{0}$ (Fig. 6k & 7c). The presence of Cu$^{0}$ was already shown by XPS. The inter planar distance observed in the present case agrees well with that reported in the literature. At pH = 12 and 10, larger aggregates of the small nanoclusters were noticed clearly showing the agglomeration at these pH values to result in fiber like structures (Fig. 7e, f, g, h).
Fig. 7 (a, b) are TEM and (c) HR-TEM images of the lyz–CuNCs at pH = 3. The scale bar in (a) is 50 nm and in (b) is 5 nm. (d) EDAX of the same. The inset in (d) is the particle size distribution plot wherein the x-axis corresponds to size (nm) and the y-axis corresponds to percent (%) of the nanoparticles. TEM images of the lyz–CuNPs at pH =12 (e, f) and at pH =10 (g, h). The scale bars are 100, 50, 100 and 50 nm respectively in (e), (f), (g) and (h).

**Cell viability of the fluorescent lyz–CuNCs prepared at pH = 7**

The MTT assay was carried out to determine the cell viability of lyz–CuNCs upon treating the NIH3T3 cells with these nanoclusters and incubating these for a period of 24 hrs. The cell viability was studied as a function of the concentration of lyz–CuNCs as shown in (Fig. 8). Up to 20 µg/ml concentration of lyz–CuNCs, the cell viability is ~100%. Even at very high concentration of lyz–CuNCs (i.e., 80 µg/ml), ~90% cell viability was noticed, supporting that these nanoclusters are non-toxic to cells and hence are well suited for cellular imaging work.
Fig. 8. Percent viability of NIH3T3 cells after the treatment with various concentrations of lyz–CuNCs.

**Cell imaging by the fluorescent lyz–CuNCs prepared at pH = 7**

The utility of lyz–CuNCs in cellular imaging was successfully demonstrated using three cell lines, namely, NIH3T3 cells (mouse embryonic fibroblast cell), MCF7 cells (human breast cancer cells) and MDA-MB-231 cells (human estrogen negative breast cancer cells) using fluorescence confocal microscopy. The fluorescence image collected under 490 nm excitation showed bright green fluorescence in cells (Fig. 9). In all the three types of cells, green fluorescence was mostly located in the periphery, though some fluorescence intensity was seen inside the cells particularly in case of MCF7 cells.
Evidence for the cellular uptake of lyz–CuNCs

Initially, the cellular uptake of lyz–CuNCs was studied using the same three cell lines, viz., NIH3T3, MCF7 and MDA-MB-231 using fluorescence confocal microscopy. The localization of lyz–CuNCs was determined in L292 cells by Z-stack experiment and it is noted that lyz–CuNCs were present in the cells predominantly in perinuclear cytoplasm (Fig. 10). The fluorescence in blue lines in YZ and XZ axis clearly indicate that lyz–CuNCs entered the cells and mostly localized in cytoplasm.
Fig. 10 Ortho-view images of z-stack of L292 cells showing lyz–CuNCs in the cells.

Conclusions and comparisons

Lysozyme was chosen as the model protein to stabilize the copper nanoclusters to give, lyz–CuNCs. In the literature, the lyz was used for the synthesis of gold nanoparticles within a single crystal of lysozyme.\textsuperscript{26,27} In our paper, we have shown that the protein, lyz stabilizes the copper nanoparticles due to its high pKa over a wide pH range. Lysozyme is a monomer containing 129 amino acid residues with 4 disulfide bridges and has a molecular weight of 14.3 kDa.\textsuperscript{26} The presence of 14\% basic amino acids, such as, Lys and Arg makes this protein well suited for binding to copper depending upon the pH and this has been clearly demonstrated from the studies reported in this paper by carrying out these at different pH and varying protein to copper mole ratios.
The fluorescence intensity of lyz–CuNCs observed at pH = 7 was high with a quantum yield of 0.18 with respect to quinine hemisulfate. The small size (3-5 nm) of lyz–CuNCs results in the formation of fluorescent nanoclusters. As compared to other pH values studied, at pH = 7, the TEM showed relatively homogeneous distribution of 3-5 nm particles without any significant aggregation of protein resulting in green fluorescence. However, at both lower and higher pH, aggregated species of different types were observed in TEM, supporting that at neutral pH the binding of copper dominates resulting in smaller size nanoclusters to give green fluorescence emission. A schematic representation of the nanoparticle formation at different pH conditions was shown in Fig. 11 a,b and c.

Fig. 11 (a,b,c) are the schematic representation of the formation of lyz–CuNPs and lyz–CuNCs at the indicated pH conditions. Confocal microscopy images: (d) BT-CD63 cells labeled with GFP-CD63 exosomes\textsuperscript{30} and (e) MCF7 cells labeled with lyz–CuNCs at pH 7.

To our knowledge, this is the first example of the formation of protein mediated green fluorescent copper nanoparticles. Blue and red fluorescent CuNCs stabilized with protein, DNA and ascorbic acid were reported in the literature\textsuperscript{12-18} However, in the present study,
the emission is around ~510 nm owing to its green fluorescence. The green fluorescence reported in our paper emerges from the clusters of the size 3-5 nm when the study was carried out at neutral pH.

The report of our green fluorescent lyz–CuNCs compliments the blue ($\lambda_{\text{ex}} = 360 \& \lambda_{\text{em}} = 450$ nm)$^{13}$ and red ($\lambda_{\text{ex}} = 365 \& \lambda_{\text{em}} = 600$ nm)$^{14}$ nanoclusters reported in the literature by completing the range of emission of 450 to 600 nm through bridging the gap with its emission centered at 510 nm. The green fluorescent property of the lyz–CuNCs has been proven to be useful in the cell imaging of normal (NIH3T3 cells) as well as cancerous cells (MCF7 and MDA-MB-231 cells). As reported in the literature, the lyz–CuNCs were used for imaging only the HeLa cell lines.$^{13}$ Our study reports the imaging of one healthy cell line (NIH3T3 cells) and two cancerous cells (MCF7 and MDA-MB-231 cells) along with proving its presence inside the cell by Z-stacking experiment. The experiment of Z-stack showed that lyz–CuNCs goes inside the cell and are localized in the cytoplasm. Thus, our lyz–CuNCs provides broader applicability for cell or tissue imaging. Our attention was attracted towards green fluorescent CuNPs due to its similarity to the green fluorescence protein (GFP) reported in the literature.$^{25}$ Similar to the present lyz–CuNCs, the GFP from A. victoria has its excitation peak at 395 nm and the emission peak is at 509 nm.$^{28}$ GFP is widely used for cell labeling and as a marker for gene expression.$^{29-31}$ The lyz–CuNCs material can also be used in labeling various types of cells similar to that wherein GFP being used. Comparison of these two in case of cancer cell labeling can be viewed from Fig. 11d,e. In our case the excitation wave length is clearly into the visible region unlike those reported in the literature regarding the blue and the red copper nanoclusters wherein the excitation wave length was in the UV region. BT-CD63 cells
labeled with GFP-CD63 exosomes has bright green fluorescence.\textsuperscript{30} Similar green fluorescence was also noticed in our studies of \textit{lyz–CuNC}s with MCF-7 cells. Hence, green fluorescent \textit{lyz–CuNC}s can be used for the cancer diagnosis and for the treatment when appropriately tuned and conjugated with a drug molecule. Therefore, the green fluorescent \textit{lyz–CuNC}s can be an alternate choice for GFP, since the GFP needs tedious procedure to express, purify and conjugate and is also expensive to procure. Hence these lysozyme coated copper nano cluster (\textit{lyz–CuNC}s) material may find real time applications in biology, medical diagnosis and even in therapy when appropriately tuned.

\textbf{Associated content}

Supporting Information

Absorption and fluorescence spectra of lysozyme at different pH are available free of charge via the Internet at \url{https://www.rsc.com/}.

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