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pH-Controlled Nanoparticles Formation and Tracking of Lysosomal Zinc Ions in Cancer Cells by Fluorescent Carbazole–Bipyridine Conjugates

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Fluorescent probes for simultaneous detection of multiple organelle specific analytes in cancer cells are essential for bioimaging applications. During abnormalities in cells, among other important parameters (metal ions, reactive oxygen species (ROS), enzymes, etc.) pH and Zn$^{2+}$ are also altered in cells. Herein, we report the formation of nanoparticles of the fluorescent molecules based on carbazole-bipyridine conjugates (CBL1-3) and their use as nanoprobes to simultaneously detect Zn$^{2+}$ and pH variations in lysosome. Upon increasing the pH from 4–6, these probes form nanoparticles with increased size and enhanced fluorescence at 510 nm. Among CBL1-3, nanoparticles of CBL2 upon Zn$^{2+}$ binding, exhibit pH responsive intensity change only at lysosomal pH window at 610 nm and become silent above pH 7. Fluorescent imaging experiments on cancer cells revealed that the CBL2 nanoprobe is capable of localizing at lysosomes and facilitates the detection of endogenous Zn$^{2+}$ and pH variations. Furthermore, the lysosomal Zn$^{2+}$ variation with external stimuli induced programmed cell death was visualized using the nanoprobe.

Introduction

Self-assembled nanoprobes of small molecules are equally powerful when compared to single molecular probes in terms of the response towards target analytes.[4a–f] Recently, self-assembled gold nanoparticles, small molecular aggregates and polymeric nanoparticles have been extensively applied for sensors design.[2a–n] Although molecular assemblies have been used for certain analytes sensing, they have not been much explored for sensing of bioanalytes in cellular system. Especially, external stimuli (pH) induced molecular self-assembly that can influence the optical properties may become more attractive to develop novel fluorescent nanoprobes for metal ion sensing and imaging in specific organelle in cellular system. Non-invasive fluorescent imaging is unequivocally the most versatile and widely applied method in biomedical research due to low detection limit and selective recognition with high resolution.[4a–e] A great deal of effort has been attempted to demonstrate various fluorescent probes for sensing and imaging of essential metal ions, small molecules and macromolecules including various proteins.[4a–d] The interaction of probes with the analytes alters the energy gaps that changes the fluorescence wavelength and intensity which allow the ratiometric detection.

Zn$^{2+}$ is the second most abundant transition metal ion which regulates immune function, signal transduction, neurotransmission, regulation of metalloenzymes etc.[5a–d] Since the disruption of Zn$^{2+}$ homeostasis has been associated with several neurological disorders such as inflammation in Alzheimer and Parkinson’s diseases and amyotrophic lateral sclerosis,[5a–e] the development of non-invasive technique for real-time imaging of endogenous Zn$^{2+}$ is very essential. Especially, the detection of Zn$^{2+}$ in specific subcellular organelle has become a challenging target since it helps to evaluate subcellular functions and cell apoptosis-associated lysosome membrane permeabilization (LMP) in physiologies. These lysosomes act as the foremost site for macromolecular degradation.[5a–e] However, it contains various acid hydrolases to enrich highly acidic pH where protons act as a competing analyte with zinc ions. Thus, the precise localization of the probe to the lysosome and selective recognition of the Zn$^{2+}$ which are associated to apoptosis is the prime concern. Simultaneous, monitoring of pH variations and Zn$^{2+}$ concentration is the major bottleneck to develop a robust diagnostic probe. To date, many attempts have been tried to overcome this issue. So far, very few probes including two-photon probes and FRET based probes have been reported to target lysosomal Zn$^{2+}$.[4a–e] However, none of them endeavour for simultaneous imaging of Zn$^{2+}$ with pH inside lysosome and associated apoptosis. Here we report for the first time, a photostable, self-assembly driven fluorescent nanoprobe for monitoring pH and Zn$^{2+}$ in lysosome in oxidative stress and apoptotic conditions.

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Results and Discussion
Synthesis and characterization.

The direct complexation strategy is used to develop fluorescent probes for $\text{Zn}^{2+}$ by co-ordination with electronegative atoms such as N, present in the fluorophore. When weakly basic aliphatic amines such as morpholine and tertiaryamine are attached to small probes, they can specifically be used to target lysosomes.\(^{3a-6}\) Considering the above aspects, we have designed fluorophores containing a carbazole, linked with a morpholine ($pK_a \sim 7.2$) which is conjugated to $2,2'$-bipyridine ($pK_a \sim 4.2$) moiety. The morpholine moiety facilitates targeting of lysosome whereas the $2,2'$-bipyridine moiety helps binding of $\text{Zn}^{2+}$. As pH varies in aqueous media, either deprotonation or protonation occur at the N-atom at different sites of the molecule that leads to the aggregation to form nanoparticles of different sizes. For the purpose of a comparative study, a similar probe $\text{CBG}$ with a glycol chain which is a non-targeting ligand was prepared. The new molecules were characterized by $^1\text{H}$ NMR, $^{13}\text{C}$ NMR, HRMS and elemental analyses (Scheme S1-S3, Table S1 and S2).

Photophysical data and aggregation properties.

The absorption spectrum of $\text{CBL2}$ in acetonitrile ($c = 10 \mu M$) showed a maximum at 360 nm (Figure 1a). However, in phosphate buffer at pH 7, the absorption spectrum is significantly red shifted with broadening, indicating the possible aggregation of the molecule.

Detailed studies on the pH dependent aggregation are performed with $\text{CBL2}$ in acetonitrile–water mixture at different pH (4, 5 and 6). The emission maximum at 510 nm corresponding to the aggregates disappeared and the monomeric emission of $\text{CBL2}$ at 490 nm appeared (Figure S3) with increasing the acetonitrile percentage and shows maximum intensity in acetonitrile. Formation of the aggregates is further confirmed by the temperature dependent changes in the emission spectra (Figure S4). Similar results were found for others. To evaluate the stability of these aggregated nanoparticles, we measured absorbance and emission properties in buffer solution at different pH for 24 h. Interestingly, there is no significant change of absorbance or emission that refers these nanoparticles are stable in physiological conditions (Figure S5).

Organic small molecular probes have been utilized for sensing and imaging for the development of several biosensors. Among them, a class of low aqueous soluble organic dyes are aggregated to form self-assemble nanoprobes. However, the aggregation behaviors are not carefully studied in many of the recent reports.\(^{2h-1}\) In this work, we studied the pH dependent aggregation behavior of $\text{CBL2}$ for the simultaneous detection of pH and zinc ion concentration. The aggregated molecules formed nanoparticles that are confirmed by transmission electron microscopy (TEM) analysis (Figure 1b). At pH 4, particles were formed around 50 nm in size whereas at pH 6, the size of spherical particles was significantly increased as it can be seen in Figure 1b. DLS study confirmed the formation of nanoparticles at pH 4 and 6 (Figure 2a and b) as illustrated with the size distribution histograms (Figure S6). The size of the particles was gradually increased with increase in pH of the media (Figure S6 and Table S4). The stability of aggregates was found to increase with increase in pH (Figure S8). Interestingly, the particles exhibited reversible size variation with change in pH.

![Scheme 1. Molecular structure of CBL derivatives and CBG.](image_url)
pH as illustrated with pH 4 and 6 without the loss of fluorescence intensity for at least 10 cycles (Figure S7). Notably, prepared aqueous solution was transparent at the experimental conditions and no precipitation was observed for at least one week. This phenomenon suggests that these molecular assemblies are useful in monitoring the pH variations once incubated with lysosome since lysosomal pH alters during pathologies.

At low pH values (pH < 4) due to the protonation at the bipyridyl nitrogen and morpholine nitrogen, molecules are hydrophilic to form non-emissive particles. Above pH 4.2, protonation specifically occurs at the morpholine site, leading to an amphiphilic behaviour to the fluorophore. The amphiphilic character of the molecule facilitate spherical assembly in which the hydrophilic morpholine units are aligned towards the outer surface and 2,2'-bipyridine unit stay in the inner part.

On the contrary, above pH 7, molecules are hydrophobic in nature which facilitates π– stacking, leading to large spherical assemblies. In the case of CBL, random aggregates were formed at different pH (Table S4). Interestingly, the molecular assemblies of CBL probes show better photostability when compared to the monomer and lysotracker green, and no change of intensities were observed even after 30 min of light irradiation and hence are suitable for bioimaging of long time cellular dynamics in lysosome (Figure S8).

To understand the absorbance behavior and HOMO-LUMO energy at different pH, DFT calculations of the molecule, CBL2 in the ground states were carried out using M06X/6-311G(d,p) level as implemented in Gaussian09. HOMO-LUMO energy level of CBL2 (neutral), CBL2(H)+ (protonation only at morpholine nitrogen), CBL2(HH)++ (protonation both at bipyridine and morpholine nitrogen) were found to be nearly unchanged while the LUMO energy level of CBL2(HH)++ showed significant stabilization compared to the CBL2 (Figure S9). The calculated absorption spectra are in good agreement with the experiments (Figure S10). A minimal blue shift of absorbance wavelength was observed for CBL2(H)+, at pH 6 due to protonation at morpholine nitrogen only, whereas a significant red shift of absorbance wavelength for CBL2(HH)++, at pH 4 due to protonation both at bipyridine and morpholine nitrogen.

**pH-Dependent emission changes.**

The absorption maxima of CBL derivatives at pH 2 and 3 occurred at 395 nm and are almost non-emissive. While increasing the pH from 4 to 6 at lysosomal pH window, the emission intensities at 530 nm were increased by 10 folds with a hypsochromic shift of 20 nm (Figure 3a). Below pH 4, the
morpholine and 2,2'-bipyridine are protonated causing the quenching of the fluorescence intensity. Above pH 4, the protonation may gradually be localized at the morpholine unit. Thus, hypsochromic shift occurs due to weaker excited state charge transfer above pH 7. However, in the case of CBG, the emission intensities increased slowly from pH 4 to 8 at 510 nm as there is no morpholine unit (Figure S11). This observation suggests that CBG is not suitable for lysosomal analysis. The pH dependent quantum yield values are tabulated in the supporting information (Table S5).

pH-Dependent Zn$^{2+}$ binding.

It is important to note that CBL2 exhibits maximum fluorescence response at the lysosomal pH window of 4–6 and hence ideal for probing lysosomal pH variation. Upon addition of Zn(ClO$_4$)$_2$, the emission maximum at 530 nm is shifted to 610 nm with 80 nm red shift ($\phi_s = 0.12$). Especially at pH 5, the ratio (530 nm / 610 nm) was gradually decreased with increased in Zn$^{2+}$ concentration (Fig S14). Similar phenomena were observed at pH 4 and 6. The red shift is due to the internal charge transfer from the donor to the acceptor. While increasing the pH 4 to 6, the emission intensity was decreased on addition of Zn(ClO$_4$)$_2$ ($\phi_s = 0.06$). This observation suggests that after binding to Zn$^{2+}$ the molecular aggregates are even stronger and non-radiative (Figure 2c and d, and S14 and 15).

At lower pH (pH 4–6), since the particle size is smaller, the surface area available for interaction with Zn$^{2+}$ is more, resulting in faster response. However, in basic pH since the molecule forms strong and larger aggregates, the surface area available for interaction with Zn$^{2+}$ is less and have weaker response by Zn$^{2+}$ (Figure S14). Similar phenomenon was observed with other CBL derivatives also. In addition, 1.7 eq., 2.8 eq. and 5.4 eq. of Zn$^{2+}$ were required to saturate at pH 4, 5 and 6 respectively suggesting that there may be steric hindrance at higher pH. Results of the detailed investigation of these aggregates with respect to pH are tabulated in Table S4 and Figure S15. We have also tested Zn$^{2+}$ sensing in MES buffer at different pH and the phenomena are similar like PBS buffer. Thus we have continued rest of the experiments in PBS buffer for mimicking the physiology. In addition, we also measured the dissociation constant of nanoparticles with Zn$^{2+}$ at different pH (pH 4 : 1.03 ± 0.4 x 10$^{-5}$ M; pH 5 : 1.92 ± 0.3 x 10$^{-5}$ M; pH 6 : 3.78 ± 0.4 x 10$^{-5}$ M) and the detection limits of Zn$^{2+}$ ranged from 89 nM, 154 nM, and 369 nM at pH 4, 5 and 6 respectively (Figure S16 and S17). The HOMO energy level is nearly unchanged while the LUMO level of the Zn$^{2+}$ complexes shows significant stabilization compared to the neutral form (Figure S18).

Selectivity.

The selective binding towards Zn$^{2+}$ when compared to other cations was investigated by conducting detailed metal ion titration experiments in the presence of a variety of metal cations at different pH (4 to 6). Biologically relevant and abundant cations such as Na$^+$, K$^+$, Mg$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Co$^{2+}$ and toxic metal ions such as Pb$^{2+}$ and Hg$^{2+}$ showed negligible responses (Figure S19). In presence of highly abundant antioxidant, glutathione (GSH; 1 mM), probe CBL-2 preserve its sensing ability of Zn$^{2+}$. However, Cd$^{2+}$, a toxic but least abundant cation in cellular component showed a partial fluorescent enhancement, however may not interfere since Cd$^{2+}$ is not a biologically relevant cation. Obviously the maximum emission intensity at 610 nm in presence of Zn$^{2+}$ is much higher when compared to other ions and hence CBL2 nanoprobe is suitable for Zn$^{2+}$ sensing in physiological conditions. Other derivatives also showed the same behaviour towards different metal ions but, no distinct emission change in lysosomal pH window. Hence, CBL2 is selected as the best fluorescent nanoprobe that can be used for the targeted imaging of lysosomal Zn$^{2+}$ and pH simultaneously.

Co-localization with Lysotracker red.

Before examining the use of CBL2 for evaluating the Zn$^{2+}$ in lysosome, cytotoxicity of the nanoprobe was evaluated on human cervical cancer (HeLa), and fibroblast-like murine pre-adipocyte (3T3 L1) cell lines by MTT assay (Figure S20). No measurable cytotoxicity was observed even at 10 mM probe concentration. Subsequently, cells were co-stained with CBL2 along with lysotracker red, mitotracker red and a nucleus staining dye, Hoechst 3334, to check its specificity in cellular organelles. As shown in Figure 4, CBL2 display reasonably good

![Figure 4. Fluorescence images for intracellular localization of CBL2 (c = 10 μM, DMEM culture media) in HeLa cells and imaged after counter stained with (a) Lysotracker-Red, (b) Mito-Tracker or (c) Hoechst 33342. The co-localization was calculated using Pearson’s correlation coefficient (r) and Manders overlap coefficients (R). Scale bar corresponds to 50 μm.](image-url)
with glycol moiety, no such localization at lysosome was observed (Figure S21).

**Effect of pH regulators.**

Since chloroquine, a lysomotropic agent and Baflomycin A1, a selective inhibitor of the vacuolar-type H\(^+\) ATPase can cause increase of lysosomal pH\(^{[11a–c]}\) and dexamethasone which decreases the cytoplasmic pH and initiate apoptosis,\(^{[12]}\) we examined these external pH regulators for monitoring the Zn\(^{2+}\) in cancer cells. As expected, when pH regulators along with CBL2 nanoprobe were incubated to study intracellular pH, the fluorescence intensity was decreased for chloroquine and Baflomycin A1, whereas intensity was increased for dexamethasome (Figure S22) at yellow channel. It is noted that CBL2 probe is simultaneously responded to intracellular Zn\(^{2+}\) along with pH regulators.

**Zn\(^{2+}\) sensing in oxidative stress.**

Imaging of Zn\(^{2+}\) in lysosome was performed by incubating CBL2 in HeLa cells. It was confirmed that the emission intensity of the Zn\(^{2+}\)-bound nanoprobe was gradually decreased when the pH was changed from 4 to 6 in TRITC channel (Figure 5c and S23). At the same time, the emission intensities were increased at FITC channel (Figure 5d and S23).

As mentioned earlier, at low pH, the molecules formed weak aggregates and readily interacted with Zn\(^{2+}\), whereas at higher pH, the aggregates became strong and the reaction with Zn\(^{2+}\) were weak. Thus at pH 7, we observed intense green emission. Upon treatment with the cell-permeable metal ion chelator N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN),\(^{[3a–b]}\) the emission intensities were reduced drastically, suggesting the decrease of Zn\(^{2+}\) level in the cellular lysosome (Figure S23). Furthermore, lysosomal Zn\(^{2+}\) under oxidative stress was monitored by addition of H\(_2\)O\(_2\) with CBL2 probe (Figure 5a, S5, and S24). During oxidative stress, a rapid influx of hydrogen peroxide takes place which causes the oxidation of cysteine residues in the Zn-bound metallothioneins and subsequently releases the Zn\(^{2+}\). Thus, an administration of hydrogen peroxide causes to release Zn\(^{2+}\) within the cells and rapidly accumulated near the lysosome area. CBL2 nanoprobe are capable of detecting this endogenous Zn\(^{2+}\) in cancer cells (Figure 5a, S5 and S24). Hydrogen peroxide was administrated at low concentration (50 μM) to elicit any cell membrane damage. This observation clearly suggests that CBL2 probe efficiently monitor the intracellular Zn\(^{2+}\) release under oxidative stress.

**Zn\(^{2+}\) sensing in apoptotic conditions.**

In endothelial cells, Zn\(^{2+}\) are occasionally associated to the protection of cells against apoptosis,\(^{[17a]}\) Kolenko et. al., reported that in human peripheral blood T lymphocytes (PBL), Zn\(^{2+}\) depletion may induce cell death due to DNA fragmentation in nuclei.\(^{[17b]}\) However, the effect of Zn\(^{2+}\) to cancer cell proliferation\(^{[18a–b]}\) and the apoptosis are not clearly studied yet. Here we studied the efficiency of CBL2 probe to monitor the Zn\(^{2+}\) in apoptosis conditions. Thus, a cell permeable zinc chelator, TPEN that induces apoptosis and alters the Zn\(^{2+}\) concentration significantly during apoptosis. Upon treatment with the cell-permeable metal ion chelator N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN), the emission intensities were reduced drastically, suggesting the decrease of Zn\(^{2+}\) level in the cellular lysosome (Figure S23). Furthermore, lysosomal Zn\(^{2+}\) under oxidative stress was monitored by addition of H\(_2\)O\(_2\) with CBL2 probe (Figure 5a, S5, and S24). During oxidative stress, a rapid influx of hydrogen peroxide takes place which causes the oxidation of cysteine residues in the Zn-bound metallothioneins and subsequently releases the Zn\(^{2+}\). Thus, an administration of hydrogen peroxide causes to release Zn\(^{2+}\) within the cells and rapidly accumulated near the lysosome area. CBL2 nanoprobe are capable of detecting this endogenous Zn\(^{2+}\) in cancer cells (Figure 5a, S5 and S24). Hydrogen peroxide was administrated at low concentration (50 μM) to elicit any cell membrane damage. This observation clearly suggests that CBL2 probe efficiently monitor the intracellular Zn\(^{2+}\) release under oxidative stress.

**Conclusions**

In conclusion, we have successfully designed fluorescent nanoprobes based on the self-assembled fluorescent molecules, CBL1-3. These molecules display pH dependent formation of fluorescent nanoparticles, exhibiting enhanced emission on increasing the pH. They simultaneously respond towards pH and Zn\(^{2+}\) in lysosome and is silent above or below pH 6–4. The
protonation of nitrogen atoms at the donor and the acceptor sites regulate the hydrophilicity and the self-assembly properties. Interestingly, the CBL2 nanoprobe display better photostability when compared to the corresponding monomer as well as commercial markers. Among CBL1-3, CBL2 based fluorescent nanoprobe is promising for the imaging, of Zn$^{2+}$ and pH variations within lysosomes in cancer cells. In addition, this probe is effectively capable to monitor the alteration of Zn$^{2+}$ under the complex conditions of apoptosis where most reactive oxygen species or free radicals are involved. Thus, CBL2 nanoprobe turns out to be a potential tool for gaining insights on zinc ion dynamics in cancer cells.

**Supporting Information Summary**

Materials and method for the synthesis of lysosome targeting carbazole-bipyridine conjugates and their characterization data including $^1$H NMR, $^{13}$C NMR, Elemental analysis, UV-Vis absorbance and emission spectroscopy with variable pH and Zn$^{2+}$, cell culture and cellular imaging including colocalization study, external stimuli effects such as H$_2$O$_2$ effect of pH regulators, live dead assay and associated apoptosis.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords:** cell imaging · lysosome · nanoprobe · pH sensing · zinc ion


