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Luminescent zinc salen complexes as single and two-photon fluorescence subcellular imaging probes†

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A novel class of ZnSalens (ZnL_{1-10}) with lipophilic and cationic conjugates as optical probes in single and two-photon fluorescence microscopy images of living cells were prepared, which exhibited chemo- and photostability, low cytotoxicity and high subcellular selectivity.

There is a wide interest in developing novel molecular imaging techniques for living cells towards subcellular organization and structures, even of molecular events during biological processes.[1] As a non-invasive optical imaging technique, fluorescent microscopy using fluorescent stains plays a vital role for its convenience, high spatial resolution and good sensitivity. However, many of the currently available set of fluorescent dyes suffer from low water solubility, high toxicity and photobleaching as well as the small Stokes shift, which can lead to an unwanted background signal due to autofluorescence of endogenous fluorophores.[2] Moreover, for further application in tissue imaging, the short excitation wavelength (<650 nm) seriously decreases the penetration depth.[3,4] To circumvent these problems, developing an ideal optical probe not only with high luminescence, low cytotoxicity, high stability and large Stokes shift but also suitable for two-photon fluorescence spectroscopy which can be excited by NIR or longer wavelengths to increase penetration depth and reduce cell damage would be of importance.

To address these issues, recent progress for luminescent d^6 metal complexes such as Ir, Ru, Re[5] and d^8 metal complexes Pt[6–9] had been made to demonstrate their advantages such as stability, large Stokes shift, long lifetime, *etc.* However, compared with the overwhelming status of organic dyes in cell imaging, luminescent metal complexes, except for a few examples such as SYPRO Ruby dye, have attracted much less attention due to the potential toxicity of heavy metals, possible active oxygen species involved, and lack of tailor-made specificity in intracellular localization. To extend the scope of luminescent transition metal probes, we turned our attention to the d^{10} metal Zn(II), which is a bioavailable metal that widely exists in metalloenzymes and has a closed-shell electronic configuration anticipated to produce fluorescence characteristics dependent on the ligand. On the other hand, through Zn(II) metalation, the flexibility of the ligand might be reduced to enhance the ligands' chemo- and photochemical stability. However, to our knowledge, although luminescent Zn complexes are of increasing interest in photochemistry, organic optoelectronics, and luminescence sensors,[10,11] their application as optical probes in biological studies remains extremely sparse.[12,13] In this work, we report the luminescent ZnSalens (**ZnL_{1-10}**) (**L** = 2,3-bis[(4-dialkylamino-2-hydroxybenzylidene) amino] but-2-enedinitrile) and demonstrate their use as single and two-photon fluorophores to perform non-invasive organelle labeling *in vitro*.

ZnSalens (salen = N,N′-ethylenebis(salicylimine)) or ZnSalophens (salophen = N,N′-phenylenebis(salicylimine) are important kinds of fluorescent zinc complexes. When salen ligands were functionalized by an electron-donor (D)/electron-acceptor (A) pair (cyanine part) with intramolecular charge transfer (ICT), the associated complexes may have nonlinear optical (NLO) properties. In this work, we chose an electron withdrawing moiety such as a cyano group (A) on diamines and dialkylamino substituents (D) at the conjugated position as the "luminescent part". To achieve tailor-made specificity in intracellular localization, we anchored ZnSalens to two different kinds of carriers based on the lipophilic and cationic properties to enhance their affinity to particular organelles. Following a previously reported procedure,[10] ZnSalen complexes **ZnL_{1-10}** were obtained in yield of 18–63% by condensation of *cis* diaminomaleonitrile and 2-hydroxy-4-(dialkylamino) benzaldehyde in the presence of $Zn(OAc)_2{\cdot}2H_2O$ in ethanol. The syntheses and characterization of these compounds are given in the electronic supporting information† (Scheme 1).

The electronic absorption spectra of complexes **ZnL_{1-10}** in DMSO at 298 K are listed in Fig. S1a† and show intense absorption features (ε on the order of 10^4 M^{-1} cm^{-1}) in the UV region (*ca.* 300–400 nm) and in the visible region (*ca.* 500–620 nm). Emission spectra with an emission maximum at 618–635 nm of **ZnL_{1-10}** are shown in Fig. S1b.† Fluorescence quantum yields Φ of 0.34–0.69 for **ZnL_{1-10}** in DMSO solution are summarized in Table S1.† In aqueous media ($DMSO/H_2O$, v/v = 5:95), the

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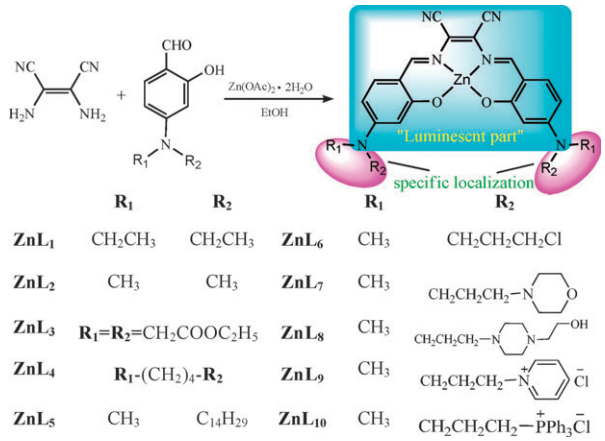

	R_1	R_2		R_1	R_2
ZnL_1	CH_2CH_3	CH_2CH_3	ZnL_6	CH_3	$CH_2CH_2CH_2Cl$
ZnL_2	CH_3	CH_3	ZnL_7	CH_3	$CH_2CH_2CH_2$–N O
ZnL_3	R_1=R_2=$CH_2COOC_2H_5$		ZnL_8	CH_3	$CH_2CH_2CH_2$–N N–OH
ZnL_4	R_1-$(CH_2)_4$-R_2		ZnL_9	CH_3	$CH_2CH_2CH_2$–$\overset{+}{N}$ Cl^-
ZnL_5	CH_3	$C_{14}H_{29}$	ZnL_{10}	CH_3	$CH_2CH_2CH_2$–$\overset{+}{P}Ph_3Cl^-$

Scheme 1 Synthesis of Zn(II) Salens ($\mathbf{ZnL_{1–10}}$).

photophysical properties of $\mathbf{ZnL_{1–10}}$ were studied. With hydrophobic conjugates, $\mathbf{ZnL_{1–6}}$ showed poor solubility and the large blue-shift absorptions (46–77 nm) in the visible region and Φ significantly decreased (<0.01, Table S2†), consistent with the existence of aggregate species.[14] Complexes $\mathbf{ZnL_{7–8,10}}$ with cationic or hydrophilic conjugates bearing good solubility exhibited absorptions with *ca.* 20 nm blue shift in aqueous media compared to that in DMSO and fluorescence quantum yields decreased (Φ of $\mathbf{ZnL_{7–8,10}}$: 0.03–0.44) which may be due to water quenched fluorescence of ZnSalen.

Upon two-photon excitation by a mode-locked femtosecond Ti:sapphire laser at 840 nm, the recorded two-photon induced is virtually identical to the one-photon induced emission spectrum (Fig. 1a). A two-photon process was confirmed by a power dependence experiment: a log–log linear relationship between the emission intensity and incident power with a slope of 1.9 (Fig. S7†). Furthermore, with reference to Rhodamine B,[15,16] the two-photon cross-section of $\mathbf{ZnL_4}$ was estimated to be 196 GM in DMSO at 840 nm (Fig. 1b). Thus, compared with the recent reported sulfer-terminal Zn(II) complex,[12] complexes $\mathbf{ZnL_{1–8,10}}$ showed higher fluorescence quantum yields (0.51–0.69) and some of them exhibited larger two-photon absorption (2PA) cross-sections.

To demonstrate the use of $\mathbf{ZnL_{1–10}}$ as dye stains for cell imaging, the chemo- and photostability as well as the cytotoxicity or cell viability were assessed first. The stability of ZnSalens in aqueous solution was exemplified by time dependent UV-vis spectra of $\mathbf{ZnL_8}$ in water and a very small change was observed after 72 h, suggesting ZnSalens possess reasonable stability (Fig. S6†). Moreover, the pH dependent absorption and emission spectra of $\mathbf{ZnL_{3,4,7}}$ in the solution of the mixed buffers (NaOAc, Mes, Mops and Tris) and DMSO (0.5%, V_{DMSO}/V_{buffer}) were measured and shown in Fig. S3–5,† which indicated that (1) these ZnSalen complexes were stable over the pH ranges (2–12), and (2) the UV/vis spectra changes were reversible when we continued to change pHs in the same solution.

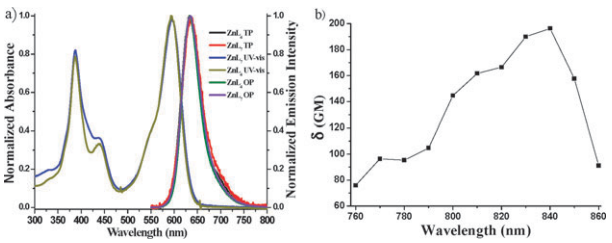

Fig. 1 (a) Normalized absorption (yellow line for $\mathbf{ZnL_4}$, blue line for $\mathbf{ZnL_7}$), steady state one-photon excitation fluorescence emission (olive line for $\mathbf{ZnL_4}$, violet line for $\mathbf{ZnL_7}$, λ_{exc} = 593 nm) and two-photon excitation fluorescence emission (black line for $\mathbf{ZnL_4}$, red line for $\mathbf{ZnL_7}$, λ_{exc} = 840 nm) spectra of $\mathbf{ZnL_4}$ and $\mathbf{ZnL_7}$ in DMSO. (b) Two-photon induced excitation spectrum of $\mathbf{ZnL_4}$ in DMSO (1×10^{-5} M).

The photostability of $\mathbf{ZnL_4}$ was examined in comparison with commercially available ER Tracker Green in living HeLa cells (Fig. 2a and b). As shown in Fig. 2a and b, the green color of the commercial ER Tracker Green decayed with the increase of irradiation time, and after being irradiated for 350 s the dye was almost completely photoleached (the fluorescence intensity decreased 92%). However, the fluorescence intensity of $\mathbf{ZnL_4}$ decreased 36% after the same irradiation time, strongly suggesting that $\mathbf{ZnL_4}$ showed resistance to photobleaching compared with commercial ER marker.

Cell viability assays in HeLa cells were conducted *via* the MTT assay. Fig. 2c displays the cell viability data for HeLa cells after treatment with several concentrations of $\mathbf{ZnL_4}$ and $\mathbf{ZnL_7}$ for 24 h (the untreated cells were used as a control). The data indicated that HeLa cells showed nearly ~100% viability following 24 h of treatment over a concentration range from 2 μM to 20 μM, which suggests that both $\mathbf{ZnL_4}$ and $\mathbf{ZnL_7}$ are virtually nontoxic over a period of 24 h.

To further explore the application of $\mathbf{ZnL_{1–10}}$ in living cell imaging, we carried out the colocalization study with several commercial available lysosome, mitochondria and ER trackers and transfected cells with EGFP encoded plasmids for endosomes and Golgi apparatus to determine the tailor-made specificity in intracellular localization. The emission spectra of $\mathbf{ZnL_{4,7}}$ in living cells indicated that these dyes in cells presented in

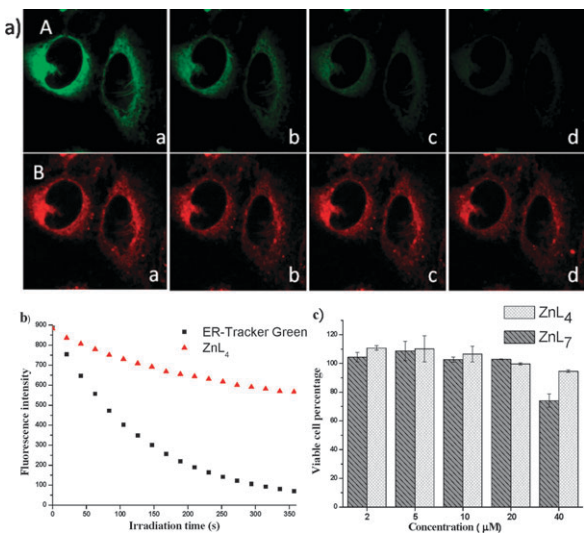

Fig. 2 Photostability comparison of $\mathbf{ZnL_4}$ and ER-Tracker Green as ER makers in HeLa cells. (a) Confocal Microscopy Image. Row A and Row B show one-photon confocal microscopy images of HeLa cells co-stained with (Row A) ER-Tracker Green and (Row B) $\mathbf{ZnL_4}$. The images were taken at (a) 0, (b) 84, (c) 168, and (d) 315 s under successive irradiation. All the images are obtained under the same condition. (b) Fluorescence intensity curves of $\mathbf{ZnL_4}$ and ER Tracker Green. (c) Viability of HeLa cells with $\mathbf{ZnL_4}$ and $\mathbf{ZnL_7}$.

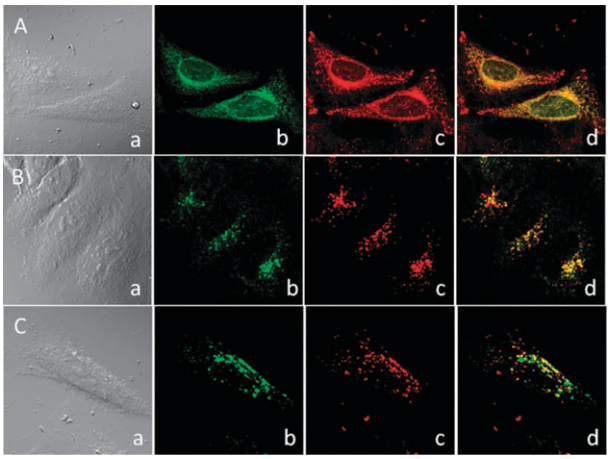

Fig. 3 One-photon fluorescence colocalization images of HeLa cells incubated with probes (2 μM, 0.5 h) and commercial dyes or GFP. (a) Differential interference contrast (DIC) image, (b) image of commercial marker, (c) image of ZnSalen, (d) merged images of (b) and (c). Row A: colocalization study of $\mathbf{ZnL_4}$ and ER Tracker Green (Pearson's coefficient: 0.74). Row B: colocalization study of $\mathbf{ZnL_7}$ and LysoTracker Green DND-26 (Pearson's coefficient: 0.70). Row C: colocalization study of $\mathbf{ZnL_9}$ and FYVE-EGFP (Pearson's coefficient: 0.66).

hydrophobic environments (Fig. S9†). The confocal microscopy images showed that, except $\mathbf{ZnL_5}$, the other Zn complexes could be taken up efficiently by HeLa cells and the optimum concentrations are 2 μM. Through comparison with colocalization of subcellular markers, we found that $\mathbf{ZnL_{3–10}}$ localized in organelles such as lysosome, endosome and ER whereas no staining of mitochondria, Golgi apparatus and cell membrane was observed. As shown in Fig. 3, we selected fluorescent images of HeLa cells incubated with $\mathbf{ZnL_{4,7,9}}$ (2 μM, 0.5 h) and commercially available lysosome, endosome and ER markers. The other images for colocalization study of other ZnSalen complexes were given in ESI†. In particular, $\mathbf{ZnL_4}$ with cyclic alkyl conjugate at salicyladehyde were specifically colocalized with an endoplasmic reticulum marker (Fig. 3A) and $\mathbf{ZnL_{3,6}}$ with linear alkyl conjugate exhibited similar subcelluar labeling (Fig. S12 and Fig. S14, repectively†). $\mathbf{ZnL_7}$ tailed by mopholino mainly exhibited lysosomal specificity while partly colocalized with early endosome marker (Fig. 3B). $\mathbf{ZnL_8}$ tailed by mopholino derivative showed similar organelle localization (Fig. S16†). With cationic conjugates such as pyridinum and triphenyl phosphonium (TPP) ions which were supposed to accumulate in mitochondria,[17] $\mathbf{ZnL_{9–10}}$ displayed punctate luminescence in the cytoplasm and only colocalizated with FYVE-EGFP (Fig. 3C and Fig. S19,† respectively), an early endosome marker and the reason need to be elucidated. These results demonstrate the efficiency of these conjugates to tailor luminescent ZnSalen in specific organelles.

To demonstrate the application of ZnSalen complexes in two-photon fluorescence microscopy (2PFM) imaging in living cells, we used $\mathbf{ZnL_4}$ and $\mathbf{ZnL_7}$ as probes to stain HeLa cells. The 2PFM image and the merged image confirm that the molecules of $\mathbf{ZnL_4}$ and $\mathbf{ZnL_7}$ are presented within the cells (Fig. 4, Fig. S21†) and showed similar images to that using

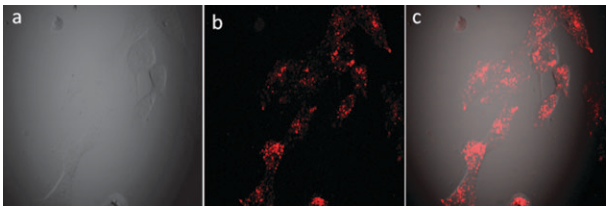

Fig. 4 (a) Bright-field image of the HeLa cells stained with $\mathbf{ZnL_7}$. (b) Two-photon microscopy image of the same cells with excitation at 840 nm. (c) Merged image of (a) and (b).

single-photon fluorescence microscopy (1PFM) for HeLa cells. It is also worthy to note that $\mathbf{ZnL_4}$ could be photobleached under two-photon excitation (Fig. S23–24†), which might be due to the intrinsic higher power 2PFM laser used (*ca.* 30 times higher than 1PFM laser).

In conclusion, we present a novel class of ZnSalen complexes $\mathbf{ZnL_{1–10}}$ with lipophilic and cationic properties conjugates. These luminescent complexes exhibited chemo- and photostability, low cytotoxicity to living cells, and subcellular selectivity, which are essential to be ideal optical probes in living cell imaging. More importantly, the high 2PA cross section of these compounds makes them potentially applicable in 2PFM imaging.

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