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Unravelling the correlation between metal induced aggregation and cellular uptake/subcellular localization of Znsalen: an overlooked rule for design of luminescent metal probes†

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Unravelling the unique effects of metal coordination on biological behaviours is of importance to design metal based therapeutic and diagnostic agents. In this work, we chose luminescent Znsalen (ZnL_1) as a case study to demonstrate that metal induced aggregation arising from the intermolecular $Zn\cdots O$ interaction influences its cellular uptake and subcellular localization. Comparative studies with the free bases (L_1 and L_2) show that ZnL_1 undergoes cellular uptake through caveolae-mediated endocytosis and internalizes in endosomal/lysosomal compartments, in contrast to the localization of L_1 and L_2 in the mitochondria. Further studies of photophysical properties, TEM imaging and DLS analysis suggest that ZnL_1 tends to form large sized fibrous structures in aqueous media. To investigate the relationship between ZnL_1 aggregation and the biological behaviour, we used pyridine to tune the "aggregation-to-deaggregation" transition and found that, in the presence of pyridine, ZnL_1 could localize in the mitochondria and internalize into cells through the passive diffusion pathway. Such distinctive biological behaviours resulting from the different Znsalen species clearly point out the importance of metal induced aggregation or metal speciation analysis in designing metal complexes as biological probes.

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Introduction

Luminescent metal complexes have emerged as an important class of imaging agents, because of their photophysical properties such as high luminescence, good photostability, large Stokes shifts and long life-times. 1-10 Moreover, metal coordination gives luminescent metal complexes greater flexibility and diversity in structures, compared to organic fluorophores.^{6,7,11-13} Thus, deciphering the structural rules governing their biological behaviours is of importance to explore the features of luminescent metal complexes in molecular imaging. Despite the tremendous progress which has been made in understanding the relationships between structural information arising from coordination, such as lipophilicity, oxidation state, charge state and coordination mode, and biological behaviours, much less attention has been given to the physical state or association of luminescent metal complexes in aqueous media.3,7,9,14 For metal complexes, the intermolecular metal-ligand or metal-metal interactions reinforce the aggregation process, which may influence the mobility

Znsalens were selected because Zn²⁺ ions possess high Lewis acidity within a planar geometry that allows for extra coordination of a Lewis base ligand or coordinating solvent, or in their absence, self-assembly through an intermolecular Zn···O axial coordination to the phenolic group of another unit.15-22 Such speciation of Znsalens or salophens is accompanied by changes in morphology and "switched off/on" fluorescence corresponding to the "aggregation-to-deaggregation" transition. 15,21,23-25 This forms a chemical basis to design optical sensors to detect anions, biological alkaloids, and even metal cations. 23,26-30 Extending this prominent feature to living cell imaging, Znsalens could be used as low cytotoxicity agents, and display high fluorescence intensity in specific organelles, in which the environment is more hydrophobic than in cytosol or cell culture media.31-34 Since Znsalen tends to aggregate in aqueous media, it is feasible to investigate whether and how Znsalen aggregation affects its biological behaviours such as cellular uptake and subcellular location. This

of the metal complex, and therefore constrain the pathways of exposure to the cell and affect the subcellular distribution. Thus, the chemical and physical form of a metal complex in aqueous solution might be an important factor in designing luminescent imaging agents. To address this issue, we herein chose the Znsalen complex (salen = N,N'-bis(salicylidene)ethylenediamine) as a case study to demonstrate that metal induced aggregation arising from intermolecular Zn···O interaction indeed influences its cellular uptake and subcellular localization.

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would be important to further design luminescent Znsalen complexes as bioprobes, 35,36 combined with the insights gained from the coordination chemistry of Znsalens.

To demonstrate the effect of Zn coordination on biological behaviours, we performed comparative studies of cell imaging experiments between a water-soluble Znsalen, ZnL1, which is conjugated with the mitochondria-targeting triphenylphosphonium cation (TPP), and its free base congeners L₁ and L₂ (Scheme 1a).37-42 Distinctive subcellular distribution between ZnL₁ (lysosomal/endosomal compartments) and the free bases L₁ and L₂ (mitochondria) indicated the significant biological effect arising from Zn coordination. Using L1 and L2 as controls, we excluded the effects of factors such as lipophilicity and charge state on biological behaviours. Then, we hypothesized that the intermolecular Zn···O interaction driving ZnL₁ aggregation plays an important role in its distinctive biological behaviours, according to the photophysical properties and morphology of **ZnL**₁ and **L**₁ in aqueous media. To confirm this hypothesis, we used pyridine as an extra ligand to dissociate the aggregates by breaking the intermolecular Zn···O interaction, and found that ZnL1 undergoes the passive diffusion pathway and mainly localizes in mitochondria (Scheme 1b). Thus, these results point out the importance of metal induced aggregation in determining biological behaviours, which is potentially useful to the design of luminescent metal probes with organelle specificity.

Scheme 1 (a) Synthetic routes and chemical structures of ZnL_1 , L_1 and L_2 . (b) Aggregation and deaggregation forms of ZnL_1 in aqueous media lead to different cellular uptake pathways and subcellular localization.

Results and discussion

ZnL₁ and L₁ were synthesized according to Scheme 1a. Nsubstituted salicylaldehyde was obtained from 3-methoxyl-Nmethylaniline and propargyl bromide, followed by Vilsmeier-Haack formylation. TPP was introduced using the copper-catalyzed "click" reaction. ZnL1 was synthesized through a "one pot" reaction of salicylaldehyde, diimine and zinc acetate, while L₁ was obtained in the absence of zinc acetate. To increase the lipophilicity of L₁, we prepared L₂ as a control using salicylaldehyde with a methyl-group-protected phenol moiety (ESI†). The detailed synthetic procedure and characterization using ¹H NMR, ¹³C NMR, ESI-MS, UV-vis and IR are given in the ESI.†

As shown in Fig. 1a, the absorption spectra of $\mathbf{ZnL_1}$, $\mathbf{L_1}$ and $\mathbf{L_2}$ in DMSO solution $(2 \times 10^{-5} \text{ M})$ show two major absorption bands between 350-450 and 500-600 nm. The free bases L_1 and L₂ display broad absorption bands from 360 to 470 nm, whereas ZnL₁ shows a sharp band centered at 385 nm with a low-energy shoulder at 435 nm. The low-energy bands are likely due to an internal charge transfer (ICT) transition. ZnL1, L1 and L2 exhibit red emission ($\lambda_{\text{max}} = 623$, 631 and 624 nm for $\mathbf{ZnL_1}$, $\mathbf{L_1}$ and $\mathbf{L_2}$ in DMSO, respectively) with fluorescence quantum yields of 0.26, 0.29 and 0.21 (Table S1†). The ¹H NMR spectra of ZnL₁, L₁ and L₂ in d⁶-DMSO display sharp signals with the expected multiplicities for their molecular structures (Fig. S15-S17†). Following Di Bella's procedure,21 we used diffusion-ordered spectroscopy (DOSY) NMR to estimate the molecular weight of $\mathbf{ZnL_1}$ in d^6 -DMSO (Table S4 and Fig. S18†). The obtained molecular weight is ca. 1260, close to the calculated molecular weight. These results clearly suggest that monomeric ZnL₁ is the main species in the coordinating solvent DMSO. In addition, the lipophilicities of ZnL₁, L₁ and L₂ were measured using the logarithm of their octanol/water partition coefficients (log Ps 0.12, -0.77, 0.09) according to Leo's method.⁴³ This suggests that Zn coordination increases the lipophilicity of L1, by an amount comparable to the methoxylation of the phenolic group of L_1 (Table S2†).

Zn coordination affects cellular uptake and subcellular distribution

To demonstrate the effect of Zn coordination on biological behaviours, we investigated the intracellular distribution of

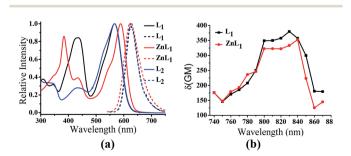


Fig. 1 (a) Normalized UV-vis and fluorescence spectra of ZnL₁, L₁ and L_2 in DMSO. $\lambda_{ex} = 380$ nm. (b) Two photon induced absorption crosssection of ZnL₁ and L₁ in DMSO using Rhodamine B as a reference.

distribution.

ZnL₁, L₁ and L₂ in HeLa cells using confocal laser scanning microscopy (CLSM). FYVE-EGFP, EHD1-EGFP, LysoTracker® Green DND-26 and MitoTracker Green served as markers for the early endosome, late endosome, lysosome and mitochondria organelles, respectively. As shown in Fig. 2a and S1-3,† ZnL₁ showed good cell permeability and was mainly distributed in the lysosomal/endosomal compartments. It exhibited a colocalization level of approximately 0.56 with LysoTracker® Green DND-26. Interestingly, L1 and L2 particularly stained mitochondria with Pearson's correlation coefficients of 0.92 and 0.83 (Fig. 2b and c). The similar subcellular distributions of L₁ and L2 clearly suggested that the lipophilicity of the salen ligands is not the main factor that affects their subcellular

To explore the effect of Zn coordination on the cellular uptake pathway, the temperature-dependent cellular uptake of ZnL₁ or L₁ in HeLa cells was carried out at 4 or 37 °C, and analysed using flow cytometry. As shown in Fig. 3a and b, the cellular internalization of ZnL1 at 4 °C showed a significantly lower intracellular level compared to that of the cells incubated in parallel at 37 $^{\circ}$ C, whereas L₁ was less affected by temperature. This indicates that the cellular internalization of ZnL₁ is an active process despite the low intracellular luminescence in cells incubated at 4 °C. In contrast, passive diffusion might be the dominant mechanism for the free base L1, although a background level of active transport also occurs.

To demonstrate the cellular internalization of ZnL₁, we then used flow cytometry, applying the endocytosis inhibitors chlorpromazine (inhibitor of clathrin-mediated endocytosis),44-46 genistein (inhibitor of caveolae-mediated endocytosis),44,47,48 or cytochalasin D (inhibitor of macropinocytosis). 49-53 As Fig. 3c and d show, the treatment with 100 µg mL⁻¹ genistein resulted in a drastic decrease of 80% in the cellular uptake of ZnL₁, suggesting that ZnL1 is mainly internalized via caveolae-mediated

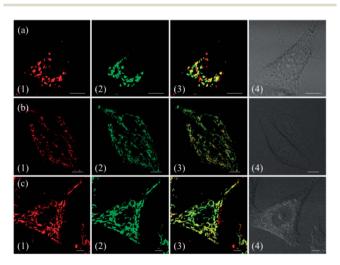


Fig. 2 Co-localization analysis of (a) ZnL₁ with lysosome tracker and (b and c) L₁ or L₂ with mitochondria tracker. (1) Fluorescence images of ZnL₁, L₁ and L₂, ex: 543 nm; (2) fluorescence images of commercial trackers LysoTracker® Green DND-26 in (a) or MitoTracker Green in (b and c), ex: 488 nm; (3) merged images of (1) and (2); (4) differential interference contrast channel. Scale bar: 10 μm.

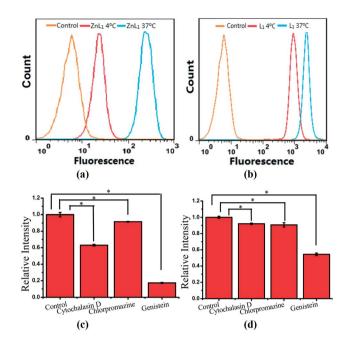


Fig. 3 Cellular uptake of ZnL_1 and L_1 , analysed using flow cytometry. Internalization of ZnL₁ (a) or L₁ (b) was investigated at 4 or 37 °C. HeLa cells were treated with cytochalasin D (5 μ g mL⁻¹), chlorpromazine (10 $\mu g \ mL^{-1}$) or genistein (100 μM) for 30 min and then incubated with inhibitor and ZnL_1 or L_1 (2 μ M) for 1 h. Cells treated with ZnL_1 or L_1 only were used as controls. Mean relative intracellular fluorescence intensities of the intracellular uptake of ZnL_1 (c) and L_1 (d) are shown as histograms (n = 3, *P < 0.001)

endocytosis, which plays a general role in the internalization of large sized particles into cells. 52,54,55 In addition, treatment with cytochalasin D also led to a 40% decrease in cellular uptake, suggesting that a macropinocytosis-mediated pathway might also be partly involved (Fig. 3c). In contrast, for L₁, treatment with genistein only resulted in a 45% decrease in the cellular uptake, and no obvious effect was observed when treated with cytochalasin D or chlorpromazine, which indicates that L₁ partly undergoes caveolae-mediated endocytosis and may mainly cross the cell membrane by passive transport, and then internalize in the mitochondria (Fig. 3d). Furthermore, to exclude the possibility that zinc coordination diminishes the mitochondrial permeation capability, we examined the ability of ZnL₁ and L₁ to stain isolated mitochondria. Following Schagger's procedure,56 we isolated mitochondrial fractions and confirmed the activity using JC-1, shown in Fig. S4.† After the incubation of ZnL1 and L1 with active mitochondria for 0.5 h, the fluorescence of the resuspended mitochondria was detected (Fig. S5†), indicating the mitochondrial permeation capability of ZnL1 and L1. To further verify this, we also used giant unilamellar phospholipid vesicles (GUVs) as mitochondria models following Pielak's method (Fig. S6†).⁵⁷ As shown in Fig. 4, both **ZnL**₁ and **L**₁ permeated the mitochondria mimic membranes and localized in the GUV matrix after 20 min. These results suggested that Zn coordination does not affect the mitochondria permeation capability, and the different subcellular distributions of ZnL1 and L1 might be due to their cellular uptake pathways.

Photophysical properties and morphology in aqueous media

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Since cellular uptake of ZnL₁ is related to caveolae-mediated endocytosis and macropinocytosis, we envisioned that the behaviour of ZnL₁ in aqueous media might influence the cellular uptake and subcellular distribution. To understand this behaviour, we carried out UV-vis absorption and emission spectroscopy of ZnL₁ and L₁ (Fig. 5b and c, S7 and S8†) in aqueous media. With increasing water content in the DMSO solution, ZnL₁ and L₁ displayed blue shifts of 15 nm and 26 nm in the low-energy absorption spectrum, and the emission was quenched gradually, with blue shifts of 6 and 11 nm, respectively. The lack of a clear isosbestic point in the absorption spectra and the quenched fluorescence for ZnL1 and L1 suggested the formation of aggregates in aqueous media. 19,58-60 Interestingly, plotting the emission intensities of ZnL₁ and L₁ versus the water contents (%) showed a logistic regression with P values of 2.71 and 1.04 (Fig. 5d), respectively. The larger P value for ZnL₁ indicated a cooperative effect on the formation of ZnL₁ aggregates in aqueous media.

The ¹H NMR spectra of **ZnL**₁ and **L**₁ (concentration: 1 mM) in the mixtures of D₂O and d⁶-DMSO solvents (Fig. S11†) show that the signals of the protons of the salen skeleton became broadened and their intensity decreased progressively with increasing D₂O content, which is probably due to restricted motion of the hydrophobic salen moiety, whereas the signals of the triphenylphosphonium group did not significantly change. In addition, diffusion-ordered spectroscopy (DOSY) NMR for ZnL₁ in d⁶-DMSO solution containing 30% D₂O showed an average molecular mass of 1602 (Table S4 and Fig. S19†), indicating partial aggregation of ZnL1 under these conditions. However, for the broad and weak signals, we cannot obtain reliable DOSY data at higher concentrations of D2O, which are predicted to form larger aggregates. Nevertheless, optical spectroscopy data and NMR spectroscopic studies clearly indicate the "deaggregation to aggregation" transition of ZnL₁ when the solvent was switched from DMSO to water.

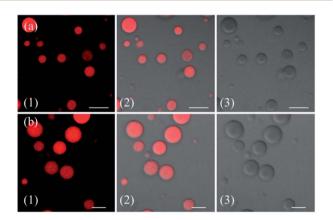


Fig. 4 Mitochondria permeability investigation of ZnL_1 and L_1 with GUVs as mitochondria membrane mimics. Confocal images of (a) ZnL_1 and (b) L_1 confined in GUVs. (1) Fluorescence images of ZnL_1 and ZnL_2 merged images of (1) and (3); and (3) differential interference contrast channel. Scale bar: 10 ZnL_2

To further examine the morphology of ZnL₁ or L₁ in aqueous media, dynamic light scattering (DLS) experiments were performed. As shown in Fig. 5e, L1 (1 µM) has a smaller hydrodynamic diameter (Dr, ca. 60 nm) and narrower distribution than $\mathbf{ZnL_1}$ (D_r , ca. 720 nm). No change of the $\mathbf{ZnL_1}$ or $\mathbf{L_1}$ aggregates were observed in cell culture media (Fig. S9†), indicating that the ingredients in the cell culture media have little effect on the aggregates. Then, the morphologies of ZnL₁ and L₁ aggregates were studied by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) by drop-casting the aqueous solutions of ZnL₁ or L₁ onto Cu-C (200) substrates. As shown in Fig. 5f and g, the TEM and SEM images clearly show the formation of a fibrous nanostructure (with dimensions of 43.5 ± 2.6 nm and more than 1 μ m in length) for ZnL_1 , while L_1 shows a distinct globular morphology with dimensions ranging from 20 nm to 70 nm, which are consistent with the DLS results. To exclude the effect of lipophilicity, we used L_2 as a control, and L_2 exhibited a similar particle size and distribution to those of L₁ in both aqueous solution and cell culture medium (Fig. S9 and 10†). Given the similar structures of ZnL₁, L₁ and L₂, we ascribed the different morphologies in aqueous media to the intermolecular Zn···O interaction in ZnL1 aggregates (Scheme 1b).

Although the aggregation of Znsalen complexes through the intermolecular Zn···O interaction in non-coordinating solvents is a usual feature, such studies in water have much less been reported. There are several examples of water soluble Znsalens that were found to be monomeric species in aqueous solutions,27,28,61 and thus the effect of water on the aggregation of ZnL₁ in water needs to be further discussed. Generally, water is a Lewis base and a coordinating solvent, and can compete with the intermolecular Zn···O interaction, and hence may prevent aggregation. However, another facet of water as solvent, in which the hydrophobic effect of Znsalen promotes such aggregation, cannot be neglected. Obviously, the role of water is complicated and highly dependent on the different binding affinities of the H₂O-Zn and the intermolecular phenolic O-Zn interaction, the structure and lipophilicity of Znsalens, the properties of the conjugates and the solvent effect. In this work, the formation of ZnL₁ aggregates in water, confirmed by UV-vis absorption, fluorescence and NMR spectroscopic studies, together with morphological studies clearly indicates that the intermolecular Zn···O interaction is more prominent than the coordination of water. On the other hand, since lipophilic triphenylphosphonium cations were introduced, ZnL₁ is to some extent hydrophobic (log P 0.12). The flexible spacer (C4 chain and triazole linker) promotes the aggregation of the Znsalen moiety and the triphenylphosphonium cations as head groups located in the interfaces, which is in agreement with the changes of the proton signals in the ¹H-NMR spectra. Thus, the hydrophobic effect is another important factor that determines ZnL₁ aggregation in water.

Stability of ZnL₁ aggregates in aqueous media

To understand the contribution of the intermolecular $Zn\cdots O$ interaction to ZnL_1 aggregation, we carried out competitive

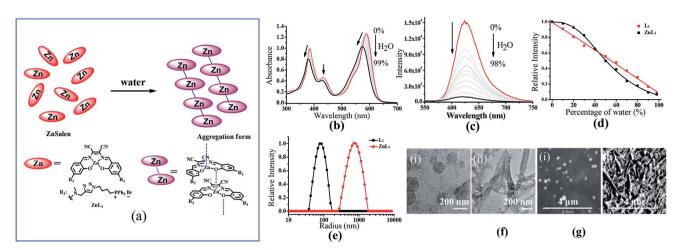


Fig. 5 (a) The change of speciation of ZnL_1 in water solution. (b) UV-vis and (c) fluorescence spectra ($\lambda_{ex} = 380$ nm) of ZnL_1 (20 μ M) in mixed solutions of H₂O/DMSO. The content of H₂O was varied from 0% to 99%. (d) Fluorescence changes of ZnL₁ and L₁ with different water percentage. (e) DLS analysis of ZnL₁ and L₁ in water containing 0.1% DMSO. (f) TEM images of self-assembled (i) L₁ and (ii) ZnL₁ (scale bar: 200 nm). (g) SEM pictures of two assemblies of (i) L_1 and (ii) ZnL_1 (scale bar: 4 μ m).

pyridine binding experiments. According to previous studies,15,17-19 the competitive binding of Lewis bases such as pyridine would dissociate the aggregates if the intermolecular Zn···O interaction was the main driving force for the selfassembly process in non-coordinating solvents. As shown in Fig. 6b, when pyridine was added to an aqueous solution of ZnL₁, the bands at around 380 and 550 nm were red shifted to 387 and 590 nm, indicating the dissociation of the ZnL₁ aggregates. As expected, the fluorescence intensity increases along with the increasing amount of pyridine (Fig. 6c). Fluorescence lifetimes were recorded before and after pyridine addition. In water, the luminescence decays are fitted as doubleexponential decays with lifetimes of 2.0 (76%) and 5.2 (24%) ns. After pyridine titration, the fluorescence lifetime is 4.0 ns fitted as a single-exponential decay, slightly shorter than that in DMSO (5.2 ns). The complete deaggregation of ZnL₁ requires up

to 2×10^5 equiv. pyridine (Fig. S12†), which is comparable to the extremely stable bimetallic Znsalen complex reported by Kleij and coworkers.20 Monitoring the pyridine titration using ¹H NMR spectra demonstrated the sharpened proton signals of ZnL₁ with downfield shifts (Fig. S13†), also supporting the deaggregation of ZnL₁ by pyridine. Moreover, the dissociation of ZnL₁ aggregates is evident from DLS results and TEM imaging. Fig. 6d shows the decrease in hydrodynamic radius from 720 nm to 27 nm with increasing pyridine from 0 to 300 thousand equiv. TEM imaging confirmed this trend and showed that the size of aggregates was reduced from 1250 \pm 117 to 31 \pm 2.3 nm (Fig. 6e).

In addition to pyridine, we attempted to test the effect of anions on ZnL₁ aggregation in aqueous media. Previous studies demonstrated that anions such as phosphate23,27,61 and Br⁻²⁵ are able to coordinate Zn and dissociate Znsalen

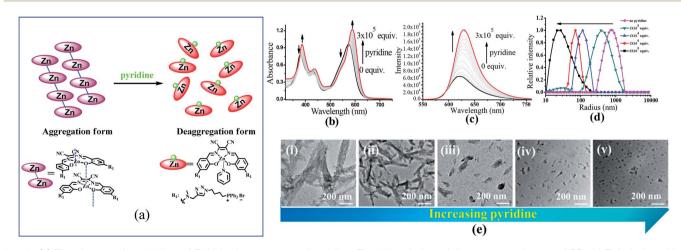


Fig. 6 (a) The change of speciation of ZnL₁ in the presence of pyridine. The dissociation of the aggregated state of 20 μM ZnL₁ induced by competitive pyridine binding monitored using (b) UV-vis and (c) fluorescence spectra. (d) DLS analysis of ZnL_1 (1 μ M) in water with the addition of 0-300 thousand equiv. pyridine. (e) TEM images of self-assembled ZnL₁ in the presence of (i-v) 0, 1×10^3 , 1×10^4 , 1×10^5 , 3×10^5 equiv. pyridine. Scale bar is 200 nm.

equation:

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aggregates, however, with much smaller binding constants than pyridine. 61,62 In this work, we chose PO₄ ³⁻ (PBS buffer), Br⁻ ("Bu₄NBr) and HBSS buffer (containing various inorganic anions), which are relevant to biological studies. As shown in Fig. 7a-c, with increasing the concentration of PBS, ⁿBu₄NBr and HBSS from 0 to 50 mM, the UV-vis spectra showed increasing bandwidths and blue-shifted with decreasing intensity at 500-600 nm, indicating increased aggregate formation. To understand this, we proposed that the high concentration of anions promoting the ZnL₁ aggregation is due to a salt effect arising from PBS, "Bu₄NBr and HBSS. To test this, we measured the UV-vis spectra of ZnL1 in the presence of NaCl (0-49 mM). As shown in Fig. 7d, similar spectroscopic changes were observed. Thus, we concluded that the aggregation of ZnL₁ might be affected by the ionic strength of these salts. This also could be used to explain why pyridine is very effective to disassociate ZnL₁ for the high binding constant to Zn ion and negligible ionic strength of pyridine. Although the "aggregation-to-deaggregation" process for Znsalen/salophens has been extensively studied,15,18-21 few studies on the stability of such aggregates in aqueous media have been performed. This is important to verify whether the aggregates are stable enough to affect the biological behaviours. Following Kleij's method, 18,20

$$(Znsalen)_n + nPy \Rightarrow nZnsalen(Py)$$

 $K = K_{Py}^n / K_{agg}, K_{agg} = K_{n/n+1}^{n-1}$ (1)

In this equation, $K_{\rm py}$ represents the coordination constant of pyridine to the Zn center of **ZnL**₁. To evaluate $K_{\rm py}$, we carried out pyridine titration on the solution of **ZnL**₁ in dichloromethane (DCM) (Fig. S14†). The coordination constant was calculated to be 1.4×10^4 M⁻¹, which is lower than those (5.3 and 5.9×10^5)

we also estimated the stability constants of the ZnL1 aggregates

in aqueous media. The relationship of the overall aggregation

constant K_{agg} , stepwise aggregation constant $K_{n/n+1}$, and the

aggregation number n can be described using the following

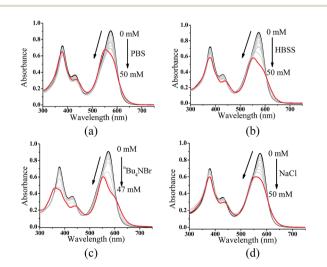


Fig. 7 UV-vis spectra of 20 μ M ZnL₁ in the presence of (a) 0–50 mM PBS, (b) HBSS, (c) n Bu₄NBr, or (d) NaCl.

reported by Kleij,^{18,26} and the value of 4.7×10^6 reported by Di Bella.⁶² The lower coordination constant was probably due to the interference of the large triphenylphosphonium groups. As in the pyridine titration in aqueous solution, the increase at 590 nm was monitored to calculate the total reaction constant K. $K_{\rm agg}$ and $K_{n/n+1}$ depend on the aggregation number n. Table S3† lists all the calculated values of $K_{\rm agg}$ and $K_{n/n+1}$ with increasing aggregation numbers. Each addition of monomer to a previously formed oligomer has a high association constant, in the order of about $10^{10} \, {\rm M}^{-1}$, which is about 2 orders higher than for the dimerization of Znsalens in organic solvents.¹⁸ This clearly demonstrates that the solvent effect reinforces the intermolecular Zn···O interaction and enhances the stability of the **ZnL**₁ aggregates.

Effect of the "aggregation/deaggregation" process on the biological behaviours

To confirm the hypothesis that ZnL₁ aggregation affected its biological behaviours, we performed cell imaging experiments using **ZnL**₁ in the presence or absence of pyridine. According to the above results, ZnL₁ aggregates can be dissociated by the addition of pyridine, which would exhibit different cellular uptake pathways and subcellular distributions from the sole use of ZnL1. The intracellular luminescence and subcellular distribution of ZnL1 were investigated in HeLa cells using confocal laser scanning microscopy (CLSM). As shown in Fig. 8a, in the absence of pyridine, ZnL₁ exhibited punctate red fluorescence in the lysosomal/endosomal compartments, which were mainly co-localized with LysoTracker® Green DND-26 (Pearson's correlation coefficient 0.56). In contrast, in the presence of pyridine (about 6000 equiv.), ZnL₁ was mainly localized in the mitochondria and co-localized with commercial Mito-Tracker Green (Pearson's correlation coefficient 0.60) (Fig. 8b). These results clearly demonstrate that the "aggregation/deaggregation" transition influences the subcellular distribution.

To understand whether the deaggregation of $\mathbf{ZnL_1}$ in aqueous solution influences the internalization mechanism, we examined the temperature-dependent cellular uptake using

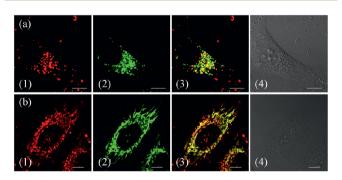


Fig. 8 Co-localization analysis of (a) ZnL_1 in the absence of pyridine with lysosomes and (b) ZnL_1 in the presence of pyridine with mitochondria: (1) fluorescence images of ZnL_1 , ex = 543 nm; (2) fluorescence of commercial trackers LysoTracker® Green DND-26 in (a) or MitoTracker Green in (b), ex = 488 nm; (3) merged picture of (1) and (2); (4) differential interference contrast channel. Scale bar: 10 μ m.

flow cytometry. As shown in Fig. 9a, a significantly lower intracellular fluorescence intensity was observed for ZnL₁ in the presence of pyridine at 4 °C than that at 37 °C, indicating that the ZnL₁ species were internalized by cells via a temperaturedependent process. Then, we employed the endocytosis inhibitors chlorpromazine (inhibitor of clathrin-mediated endocytosis), 44-46 genistein (inhibitor of caveolae-mediated endocytosis),44,47,48 and cytochalasin D (inhibitor of macropinocytosis)49-53 as described above, to investigate whether ZnL1 internalizes into the cell through endocytosis. As shown in Fig. 9b, in the presence of pyridine, the treatments with genistein, cytochalasin D or chlorpromazine only resulted in a moderate decrease of intracellular fluorescence (27%, 17% and 14%, respectively) and thus cellular uptake of ZnL₁ in the presence of pyridine is almost not correlated to endocytosis, which is markedly different from the endocytosis pathway of ZnL₁ in the aggregated state.

Given that the TPP conjugate possesses a positive charge, the internalization of $\mathbf{ZnL_1}$ with pyridine may be facilitated by organic cation transporters (OCT) or driven by the plasma membrane potential (-50 to -70 mV, negative inside). When using tetrabutylammonium bromide as the OCT inhibitor, 67 HeLa cells showed no obvious intracellular fluorescence change (Fig. 9c and d). To investigate the effect of the plasma

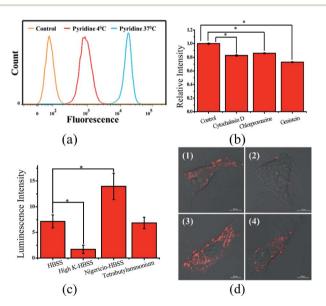


Fig. 9 Cellular uptake of ZnL₁ analysed by flow cytometry and CLSM. (a) Effect of incubation temperature on the internalization of ZnL_1 in the presence of pyridine was investigated at 4 or 37 °C. (b) Effect of endocytosis on the internalization of ZnL₁ in the presence of pyridine: HeLa cells were treated with cytochalasin D (5 μg mL⁻¹), chlorpromazine (10 μ g mL⁻¹) or genistein (100 μ M) for 30 min and then incubated with inhibitor and ZnL_1 (2 μM) for 1 h. Cells treated with ZnL_1 only were used as controls. (c) and (d) Effect of the plasma membrane potential and organic cation transporter inhibitor on the internalization of ZnL₁: HeLa cells treated with ZnL_1 in the presence of pyridine buffered in (1) HBSS, (2) high K⁺-HBSS, (3) 5 μM nigericin-HBSS (cells were treated with 5 μM nigericin first for 30 min) or (4) 1 mM tetrabutylammonium bromide-HBSS (cells were treated with 1 mM tetrabutylammonium bromide first for 20 min). Mean relative intracellular fluorescence intensities of intracellular uptake are shown in histograms (n = 3, *P < 0.001). Scale bar: 10 µm.

membrane potential, we examined the cellular uptake of ZnL₁ in the presence of pyridine under the conditions of high potassium buffer (K+-HBSS, 170 mM K+, depolarization)65,67,68 or nigericin-HBSS (10 μM, hyperpolarization).65,69 As shown in Fig. 9c and d, the K⁺-HBSS-treated cells displayed a remarkable decrease in intracellular fluorescence (80%), while the nigericin-treated cells showed an intracellular fluorescence increase of nearly 2 fold. This clearly demonstrated that ZnL₁ in the presence of pyridine is internalized mostly by membrane potential dependent passive diffusion. Since we have demonstrated that PBS, HBSS, and Br cannot lead to ZnL1 deaggregation and couldn't disturb pyridine coordination, combined with its photophysical properties and morphology in aqueous media, we hypothesized that the distinctive internalization pathways may be due to the different morphologies between the "aggregation/deaggregation" transition of ZnL1, because the caveolae-mediated or macropinocytosis-mediated endocytosis is related to the uptake of large sized particles into cells. 44,66,68

Conclusions

Taking our results together, we demonstrated that the intermolecular Zn···O interaction between Znsalen played an important role in determining its cellular uptake pathway and subcellular distribution. Through comparative studies between ZnL_1 and the free bases L_1 and L_2 , Zn coordination was found to lead to a distinctive cellular uptake pathway and subcellular distribution. More importantly, the photophysical and morphology studies on ZnL₁ and the free bases in aqueous media suggest that the different aggregation states arising from the intermolecular Zn···O interaction play a critical role in influencing the biological behaviours. This hypothesis was confirmed by cell imaging experiments using ZnL1 in the presence or absence of pyridine, which clearly demonstrated the effect of tuning the "aggregation/deaggregation" transition of **ZnL**₁ in aqueous media on the cellular uptake pathway and subcellular distribution. These results point to a new factor, "metal induced aggregation", which effectively influences cellular uptake and subcellular distribution, and which should not be overlooked in designing luminescent metal complexes as biological probes.

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Notes and references

- 1 K. L. Haas and K. J. Franz, Chem. Rev., 2009, 109, 4921-4960.
- 2 Q. Zhao, C. Huang and F. Li, Chem. Soc. Rev., 2011, 40, 2508–2524.
- 3 E. Baggaley, J. A. Weinstein and J. Williams, *Coord. Chem. Rev.*, 2012, **256**, 1762–1785.
- 4 M. Schäferling, Angew. Chem., Int. Ed., 2012, 51, 3532-3554.

5 D.-L. Ma, V. P.-Y. Ma, D. S.-H. Chan, K.-H. Leung, H.-Z. He and C.-H. Leung, *Coord. Chem. Rev.*, 2012, 256, 3087–3113.

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- 6 D. L. Ma, H. Z. He, K. H. Leung, D. S. H. Chan and C. H. Leung, *Angew. Chem., Int. Ed.*, 2013, 52, 7666–7682.
- 7 K. K.-W. Lo, A. W.-T. Choi and W. H.-T. Law, *Dalton Trans.*, 2012, 41, 6021–6047.
- 8 V. Fernández-Moreira, F. L. Thorp-Greenwood and M. P. Coogan, *Chem. Commun.*, 2010, 46, 186–202.
- 9 F. L. Thorp-Greenwood, R. G. Balasingham and M. P. Coogan, *J. Organomet. Chem.*, 2012, **714**, 12–21.
- 10 D.-L. Ma, D. S.-H. Chan and C.-H. Leung, Acc. Chem. Res., 2014, 47, 3614–3631.
- 11 Z. Guo and P. J. Sadler, Angew. Chem., Int. Ed., 1999, 38, 1512-1531.
- 12 E. Meggers, Chem. Commun., 2009, 1001-1010.
- 13 M. Frezza, S. Hindo, D. Chen, A. Davenport, S. Schmitt, D. Tomco and Q. P. Dou, *Curr. Pharm. Des.*, 2010, 16, 1813–1825.
- 14 R. P. Haugland, *The handbook: a guide to fluorescent probes and labeling technologies*, Molecular probes, 2005.
- 15 C. T. L. Ma and M. J. MacLachlan, Angew. Chem., Int. Ed., 2005, 44, 4178–4182.
- 16 J. K. H. Hui, Z. Yu and M. J. MacLachlan, Angew. Chem., Int. Ed., 2007, 46, 7980-7983.
- 17 A. W. Kleij, Dalton Trans., 2009, 4635-4639.
- 18 M. M. Belmonte, S. J. Wezenberg, R. M. Haak, D. Anselmo, E. C. Escudero-Adan, J. Benet-Buchholz and A. W. Kleij, *Dalton Trans.*, 2010, 39, 4541–4550.
- 19 G. Consiglio, S. Failla, P. Finocchiaro, I. P. Oliveri, R. Purrello and S. Di Bella, *Inorg. Chem.*, 2010, 49, 5134–5142.
- G. Salassa, M. J. J. Coenen, S. J. Wezenberg,
 B. L. M. Hendriksen, S. Speller, J. A. A. W. Elemans and
 A. W. Kleij, *J. Am. Chem. Soc.*, 2012, 134, 7186–7192.
- 21 G. Consiglio, S. Failla, P. Finocchiaro, I. P. Oliveri and S. Di Bella, *Inorg. Chem.*, 2012, **51**, 8409–8418.
- 22 I. P. Oliveri, S. Failla, G. Malandrino and S. Di Bella, J. Phys. Chem. C, 2013, 117, 15335–15341.
- 23 M. Cano, L. Rodríguez, J. C. Lima, F. Pina, A. Dalla Cort, C. Pasquini and L. Schiaffino, *Inorg. Chem.*, 2009, 48, 6229–6235.
- 24 I. P. Oliveri and S. Di Bella, *J. Phys. Chem. A*, 2011, **115**, 14325–14330.
- 25 I. P. Oliveri, G. Malandrino and S. Di Bella, *Dalton Trans.*, 2014, 43, 10208–10214.
- 26 E. C. Escudero-Adán, J. Benet-Buchholz and A. W. Kleij, *Inorg. Chem.*, 2008, 47, 4256–4263.
- 27 S. Khatua, S. H. Choi, J. Lee, K. Kim, Y. Do and D. G. Churchill, *Inorg. Chem.*, 2009, 48, 2993–2999.
- 28 A. D. Cort, P. De Bernardin and L. Schiaffino, *Chirality*, 2009, 21, 104–109.
- 29 S. J. Wezenberg, D. Anselmo, E. C. Escudero-Adán, J. Benet-Buchholz and A. W. Kleij, *Eur. J. Inorg. Chem.*, 2010, 29, 4611–4616
- 30 Y.-B. Cai, J. Zhan, Y. Hai and J.-L. Zhang, *Chem.-Eur. J.*, 2012, **18**, 4242–4249.
- 31 J. Jing, J. Tang, D. Xie, Y.-B. Cai, J.-J. Chen and J.-L. Zhang, *Sci. Sin.: Chim.*, 2014, 44, 191–203.

- 32 Y. Hai, J.-J. Chen, P. Zhao, H. Lv, Y. Yu, P. Xu and J.-L. Zhang, *Chem. Commun.*, 2011, 47, 2435–2437.
- 33 J. Jing, J.-J. Chen, Y. Hai, J. Zhan, P. Xu and J.-L. Zhang, *Chem. Sci.*, 2012, 3, 3315–3320.
- 34 D. Xie, J. Jing, Y.-B. Cai, J. Tang, J.-J. Chen and J.-L. Zhang, *Chem. Sci.*, 2014, 5, 2318–2327.
- 35 J.-J. Chen, J. Jing, H. Chang, Y. Rong, Y. Hai, J. Tang, J.-L. Zhang and P. Xu, *Autophagy*, 2013, **9**, 894–904.
- 36 J. Jing and J.-L. Zhang, Chem. Sci., 2013, 4, 2947-2952.
- 37 R. A. J. Smith, C. M. Porteous, A. M. Gane and M. P. Murphy, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 5407–5412.
- 38 A. T. Hoye, J. E. Davoren, P. Wipf, M. P. Fink and V. E. Kagan, *Acc. Chem. Res.*, 2008, **41**, 87–97.
- 39 B. C. Dickinson and C. J. Chang, J. Am. Chem. Soc., 2008, 130, 9638–9639.
- 40 S. Marrache and S. Dhar, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 9445–9450.
- 41 S. K. Bae, C. H. Heo, D. J. Choi, D. Sen, E.-H. Joe, B. R. Cho and H. M. Kim, *J. Am. Chem. Soc.*, 2013, 135, 9915–9923.
- 42 R. K. Pathak, S. Marrache, D. A. Harn and S. Dhar, *ACS Chem. Biol.*, 2014, **9**, 1178–1187.
- 43 A. Leo, C. Hansch and P. Y. C. Jow, *J. Med. Chem.*, 1976, **19**, 611–615.
- 44 J. Rejman, V. Oberle, I. Zuhorn and D. Hoekstra, *Biochem. J.*, 2004, 377, 159–169.
- 45 J. P. Richard, K. Melikov, H. Brooks, P. Prevot, B. Lebleu and L. V. Chernomordik, *J. Biol. Chem.*, 2005, **280**, 15300–15306.
- 46 E. Blanchard, S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski and Y. Rouillé, *J. Virol.*, 2006, 80, 6964–6972.
- 47 I. R. Nabi and P. U. Le, J. Cell Biol., 2003, 161, 673-677.
- 48 J. Rejman, A. Bragonzi and M. Conese, *Mol. Ther.*, 2005, **12**, 468–474.
- 49 J. S. Wadia, R. V. Stan and S. F. Dowdy, *Nat. Med.*, 2004, **10**, 310–315.
- 50 I. M. Kaplan, J. S. Wadia and S. F. Dowdy, *J. Controlled Release*, 2005, **102**, 247–253.
- 51 C. A. Puckett, R. J. Ernst and J. K. Barton, *Dalton Trans.*, 2010, **39**, 1159–1170.
- 52 S.-F. Peng, M. T. Tseng, Y.-C. Hoc, M.-C. Wei, Z.-X. Liao and H.-W. Sung, *Biomaterials*, 2011, 32, 239–248.
- 53 F. Madani, S. Lindberg, Ü. Langel, S. Futaki and A. Gräslund, *J. Biophys.*, 2011, **2011**, 1–10.
- 54 R. G. W. Anderson, Annu. Rev. Biochem., 1998, 67, 199-225.
- 55 S. Trabulo, A. L. Cardoso, M. Mano and M. C. P. De Lima, *Pharmaceuticals*, 2010, **3**, 961–993.
- 56 K. Minezaki, M. Suleiman and R. Chapman, J. Physiol., 1994, 476, 459–471.
- 57 I. G. Zigoneanu, Y. J. Yang, A. S. Krois, M. E. Haque and G. J. Pielak, *Biochim. Biophys. Acta, Biomembr.*, 2012, **1818**, 512–519.
- 58 D. M. Cohen and E. Fisher, J. Chem. Soc., 1962, 3044-3052.
- 59 J. Brynestad and G. P. Smith, *J. Phys. Chem.*, 1968, 72, 296–300.
- 60 G. Consiglio, S. Failla, P. Finocchiaro, I. P. Oliveri and S. D. Bella, *Dalton Trans.*, 2012, 41, 387–395.

61 O. Jurček, M. Cametti, M. Pontini, E. Kolehmainen and K. Rissanen, *Org. Biomol. Chem.*, 2013, 11, 4585–4590.

- 62 I. P. Oliveri, G. Maccarrone and S. Di Bella, *J. Org. Chem.*, 2011, 76, 8879–8884.
- 63 S. Davis, M. Weiss, J. Wong, T. J. Lampidis and L. B. Chen, *J. Biol. Chem.*, 1985, **260**, 13844–13850.
- 64 D. Piwnica-Worms, J. F. Kronauge and M. L. Chiu, *Circulation*, 1990, **82**, 1826–1838.
- 65 M. L. Chiu, J. F. Kronauge and D. Piwnica-Worms, *J. Nucl. Med.*, 1990, **31**, 1646–1653.
- 66 A. C. Komor and J. K. Barton, *Chem. Commun.*, 2013, **49**, 3617–3630.
- 67 C. A. Puckett and J. K. Barton, *Biochemistry*, 2008, 47, 11711–11716.
- 68 J. Wang, J. D. Byrne, M. E. Napier and J. M. DeSimone, *Small*, 2011, 7, 1919–1931.
- 69 E. E. Bellocchio, R. J. Reimer, R. T. Fremeau and R. H. Edwards, *Science*, 2000, **289**, 957–960.