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Benzenesulfonamidoquinolino-β-cyclodextrin as a Cell-Impermeable Fluorescent Sensor for Zn^{2+}

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Abstract: A water-soluble benzenesulfonamidoquinolino-β-cyclodextrin has been successfully synthesized in 30% yield by incorporating a *N*-(8-quinolyl)-*p*-aminobenzenesulfonamide (HQAS) group to β-cyclodextrin through a flexible linker. This compound exhibits a good fluorescence response in the presence of Zn^{2+} in water but gives poor fluorescence responses with other metal ions commonly present in a physiological environment under similar conditions. Fluorescence microscopic and two-dimensional NMR experiments showed that benzenesulfonamidoquinolino-β-cyclodextrin could bind to the loose bilayer membranes. As a result, benzenesulfonamidoquinolino-β-cyclodextrin was found to act as an efficient cell-impermeable Zn^{2+} probe, showing a specific fluorescent sensing ability to Zn^{2+}-containing damaged cells whilst exhibiting no response in the presence of healthy cells.

Keywords: cell · cyclodextrins · fluorescent probes · NMR spectroscopy · zinc

Introduction

Zinc, following iron, is the second most abundant transition metal ion present in the body and plays an important role in various biological processes.[1–2] Zinc deficiency or imbalanced zinc distribution within the body, organ, or cell will lead to a broad range of pathologies, such as neuropathic, immune, endocrine, and gastro-enterological systems.[3] For example, the accumulation of intracellular Zn^{2+} will induce neuronal injury and lead to neuronal death.[4–5] Therefore, the detection of Zn^{2+} in live cells has attracted a lot of attention. It is widely regarded that fluorescent probes, which allow the visualization of zinc ions in living cells by fluorescence microscopy, are one of the best tools for tracking Zn^{2+} in cells and organisms.[6–11] Generally, there are two forms of Zn^{2+} probes, namely, cell-permeable and cell-impermeable probes. The former can pass through the cell membrane and have been widely used to detect intracellular Zn^{2+} in living cells, and the latter is designed to be excluded from the contiguous membrane of healthy cells, which have been successfully used in monitoring cell exocytosis, measuring cell viability, indicating cell apoptosis, and so forth.[12–17]

Among various fluorescent probes, 6-methoxy-(8-*p*-toluenesulfonamido)quinoline (TSQ) and its derivatives are the first class of fluorescent probes to be developed for Zn^{2+}, which exhibits a high selectivity for Zn^{2+} as compared to other metal ions.[18–22] On the other hand, possessing a hydrophobic cavity and numerous hydroxyl groups, cyclodextrins (CDs), cyclic oligosaccharides with 6–8 D-glucose units linked by α-1,4-glucose bonds, are widely used as drug carriers and solubilizers. In a preliminary work, we covalently linked *N*-(8-quinolyl)-*p*-aminobenzenesulfonamide (HQAS), an analogue of TSQ, to β-CD to obtain HQAS-β-CD as a water-soluble fluorescent sensor for Zn^{2+}.[23] Herein, we have prepared another HQAS-modified β-CD **1** (Schemes 1

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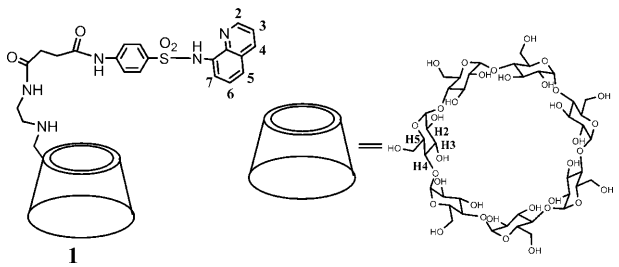

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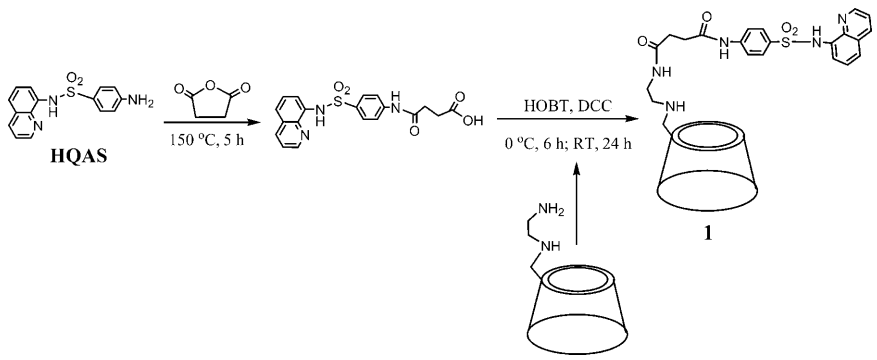
Scheme 1. HQAS-modified β-CD.



Scheme 2. The synthesis method of **1**.

and 2) as an improved cell-impermeable fluorescent probe for Zn^{2+} by the elongation of the spacer between the HQAS group and the β-CD cavity. In this probe, the HQAS group of **1** was cell-permeable and could strongly bind Zn^{2+}. The β-CD cavity of **1** was hydrophobic and could form a self-included complex with a quinoline ring, which restricted the passage of **1** through a healthy cell membrane. In the case of damaged cells, the junction gap in the cell membrane became loose, which allowed the β-CD cavity of **1** to include competitively exposed molecules of the cell membrane and thus anchor in the loose bilayer of the cell. The long flexible spacer allows the HQAS group to insert into the hydrophobic domains of the loose bilayer of the cell. As a result of these factors, **1** was expected to act as an efficient cell-impermeable Zn^{2+} probe. This hypothesis was further validated by fluorescence spectroscopic and fluorescence microscopic investigations, which showed that **1** could selectively sense the damaged cells but gave no response in the presence of healthy cells.

Results and Discussion

Fluorescence Properties and Optical Responses to Zn^{2+}

The target product **1** was synthesized in 30% yield through a typical amide condensation reaction. Arising from the good solubilization property of the β-CD unit, **1** exhibited better water solubility and its solubility limit in water could reach ca. 2 mM. Because the TSQ framework was still retained, **1** exhibited high fluorescence in the presence of Zn^{2+}. The fluorescence titration curves of **1** with Zn^{2+} (Figure 1) show that when excited at 360 nm, the emission intensity of **1** continuously increases upon the stepwise addition of Zn^{2+}, which demonstrates the possibility of **1** as a Zn^{2+} sensor. The coordination stoichiometry of **1** with Zn^{2+} was investigated by UV/Vis spectrometric titration experiments (see Figure S1 in the Supporting Information), and the plot of $\Delta A_{\mathbf{1}/Zn}{}^{2+}$ ($\Delta A_{\mathbf{1}/Zn}{}^{2+} = \Delta A_{\mathbf{1}/Zn}{}^{2+} - A_{\mathbf{1}}$, where $A_{\mathbf{1}}$ is defined as the absorption intensity of **1** at 362 nm) vs $[Zn^{2+}]/[\mathbf{1}]$ shows an inflexion point at a molar ratio of 0.5, which corresponds to a 2:1 coordination stoichiometry between **1** and Zn^{2+}. Therefore, we could deduce the possible structure of the $Zn{\cdot}\mathbf{1}_2$ complex as shown in Scheme 3. On the other hand, the 2-aminoethylamino group of **1** would be partly protonated even in a neutral environment,[27] which is unfavorable towards its coordination with Zn^{2+}. In addition, the ^1H NMR results showed that the quinoline ring proton (H7) of **1** exhibits appreciable shifts ($\Delta\delta$ 0.05 ppm), but the other protons of the substituent of **1** gave no shifts, in the presence of Zn^{2+}. Therefore, we deduced that the structure of **1**/

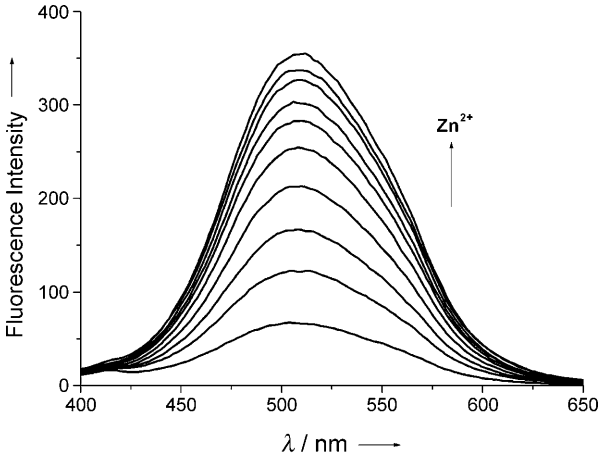
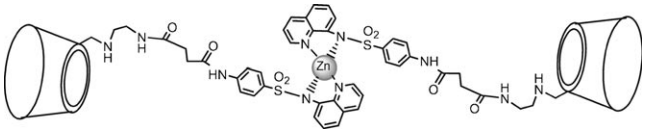


Figure 1. Emission spectra (excitation at 360 nm) of **1** ($[\mathbf{1}] = 20$ μM) with the addition of Zn^{2+} ($[Zn^{2+}] = 0$, 2, 4, 6, 8, 10, 12, 14, 16, 18 μM) in buffer solution (pH 7.2) at 25 °C.



Scheme 3. Possible structure of $Zn{\cdot}\mathbf{1}_2$.

Zn^{2+} complex might be similar to that of the HQAS-β-CD/Zn^{2+} complex as reported previously.[23] That is, two N atoms from each of the two HQAS units of **1** participate in the four-coordinated environment of Zn^{2+}. The binding ability of **1** to Zn^{2+} was quantitatively determined by a competitive binding method[28] using the fluorescence titration experiment (see Supporting Information). The apparent stability constant ($\log K$, $K = [Zn\mathbf{1}_2]/[Zn^{2+}][\mathbf{1}]^2$) of the $Zn{\cdot}\mathbf{1}_2$ complex was observed to be equal to 12.6, which was a little higher than the reported value for the HQAS-β-CD/Zn^{2+} complex ($\log K = 12.4$)[23] but less than that of the Zinquin acid/Zn^{2+} complex ($\log K = 13.7$).[28] This result unambiguously demonstrates the strong binding ability of **1** to Zn^{2+}. The similar coordination mode may also lead to similar fluorescence enhancement mechanism of **1** as that of HQAS-β-

CD. In this mechanism, two nitrogen atoms of HQAS could form an intramolecular hydrogen bond with hydrogen atoms in the absence of Zn^{2+}, which resulted in a photo-induced electron transfer, and the de-excitation of the resulting tautomer occurs mainly through a non-radiative pathway. As a result of this process, **1** emits only weak fluorescence. Once **1** coordinated to Zn^{2+}, the electron transfer process was unfavorable, and an extended π-electron conjugation system, which involves an internal charge transfer (ICT) process from ligand donor to Zn^{2+} acceptor, was formed synchronously.[6,29] Owing to the formation of extended π-electron conjugation system, the **1**/Zn^{2+} system exhibits an intense fluorescence.

The sensing selectivity of **1** to Zn^{2+} was also investigated through a comparative study of the fluorescence responses of **1** to different metal ions. Herein, the effect of other metal ions was tested by monitoring the emission intensities of **1**–metal systems at 506 nm. The results showed (Figure 2) that

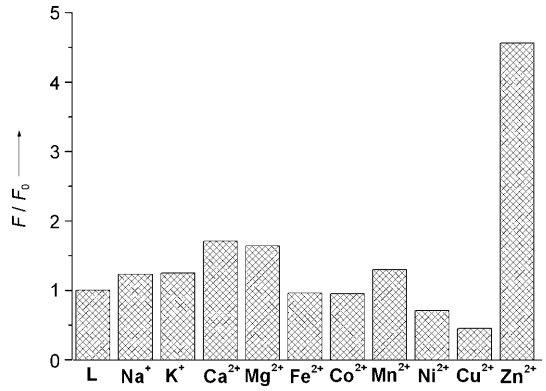


Figure 2. Relative fluorescence intensities of **1** ([**1**] = 20 μM) in the presence of various cations ([Na^+] = [K^+] = [Ca^{2+}] = [Mg^{2+}] = 5 mM, [Zn^{2+}] = [Cu^{2+}] = [Ni^{2+}] = [Co^{2+}] = [Fe^{2+}] = [Mn^{2+}] = 10 μM. These data were measured at pH 7.2 (λ_{ex} = 360 nm, λ_{em} = 506 nm)

the addition of a large excess (250 equiv) of alkaline metals or alkaline earth metal ions (Ca^{2+}, Mg^{2+}, Na^+, and K^+), which always exist at a high concentration in living cells, only slightly change the fluorescence intensity of **1**. Similar phenomena were also observed with the addition of an equivalent amount of transition metal ions including Fe^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+}. These results jointly demonstrated the good fluorescent sensing selectivity of **1** to Zn^{2+}. Although Cu^{2+} quenched the fluorescence of **1**, its interference in the fluorescence response may be masked with a copper binding protein such as bovine serum albumin.

Conformation of 1. Two-dimensional NMR spectroscopy has recently become an important method for the investigation of not only the interaction between mono-modified CDs and guest molecules, but also the self-included mode between the CD cavity and its substituting groups. While guest molecules or substituent groups are included into the β-CD cavity, the NOE correlations between the protons of the guest molecules (or substituent groups) and the interior protons of the β-CD cavity (H3 and H5) will be measured.[30] To study the original conformation of **1** in aqueous solution, the ROESY experiment was performed at 25 °C in D_2O. As illustrated in Figure 3, the NOE correlations between the H3 protons of the quinoline ring and the H5 pro-

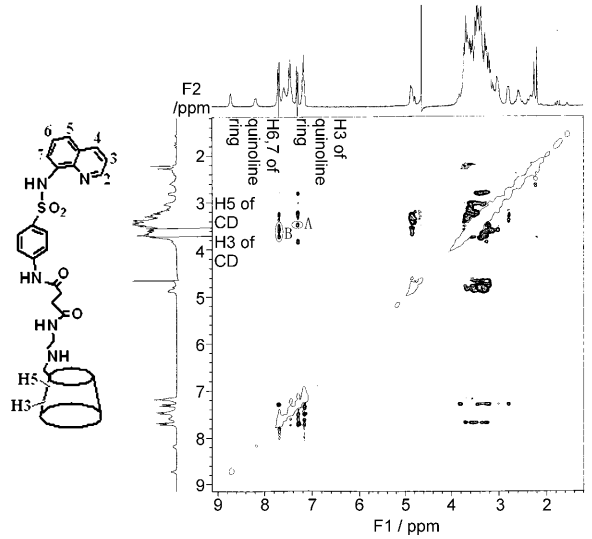


Figure 3. ROESY spectrum of **1** (2 mM) in D_2O at 25 °C.

tons of β-CD (peak A), as well as the NOE correlations between the H6/H7 protons of quinoline ring and the H3/H5 protons of β-CD (peak B), were clearly observed. These NOE correlations indicated that the quinoline ring of **1** was self-included in the β-CD cavity. On the other hand, no NOE correlations between the protons of the quinoline ring and the interior protons of β-CD could be found in the two-dimensional NMR spectrum of the Zn·$\mathbf{1}_2$ complex, which indicated that the self-included substituent group of **1** had been excluded from the β-CD cavity after coordination with Zn^{2+}. Moreover, a molecular modeling study was also performed to support the self-included conformation of **1**. The results (see Supporting Information) showed that the quinoline ring was located in the interior of the β-CD cavity with the H3/H6/H7 protons of the quinoline ring located near the H3/H5 protons of the β-CD cavity, which consequently confirmed the estimated self-included conformation of **1**.

Interaction of 1 with liposome. It is necessary for cell-impermeable probes to possess a suitable affinity for the biological membrane, in order to prevent the rapid disappearance of fluorescence from stained domains through diffusion. Generally, double-chained phospholipids can assemble into a bilayer conformation, wherein the hydrophilic polar head groups orient towards the aqueous environment and the hydrophobic tail regions orient towards the center where they are shielded from the aqueous environment. Spherical lipid bilayers surrounded by an aqueous phase are called liposomes. Biomembranes are composed mainly of phospholipid, cholesterol, and protein. So the liposomes of lecithin and cholesterol have widely been used as biological membrane models to aid in understanding membrane

chemistry. Herein, the interactions of **1** with lecithin liposomes were also investigated by two-dimensional NMR spectroscopy. As seen from the ROESY spectrum of **1** (2 mM) in a mixture of lecithin, cholesterol, and sodium deoxycholate (Figure 4), besides the NOE correlations that

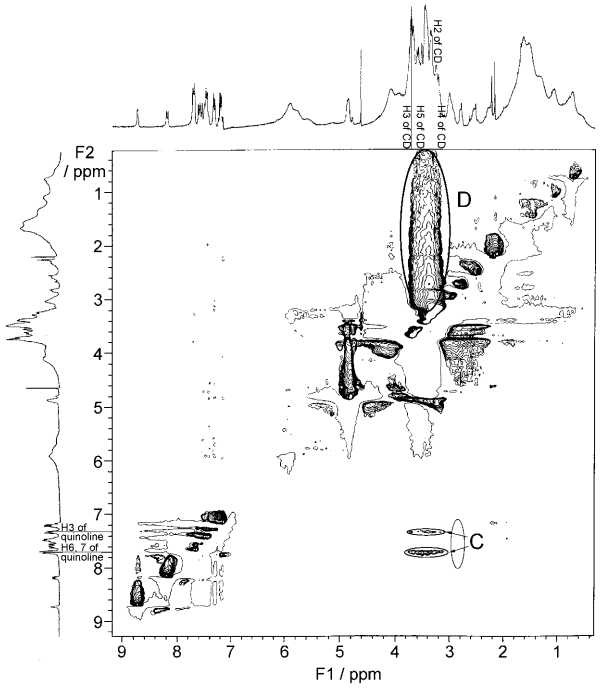


Figure 4. ROESY spectrum of **1** (2 mM) in the presence of liposome at 25 °C.

originate from the self-inclusion of **1** (peaks C), the protons whose δ value ranged from 0 to 2 (including cholesterol protons, sodium deoxycholate protons, and the methylene protons in the alkyl chain of lecithin) presented strong and complicated NOE correlations (peaks D) with the H3/H5 protons of β-CD. Our previous studies demonstrated that the H3/H5 protons of β-CD exhibited NOE correlations with the protons of sodium deoxycholate in a micelle system,[31] but their intensity was far weaker than that shown in Figure 4. Therefore, we deduced that the NOE correlations between cholesterol protons or lecithin protons in liposome and the H3/H5 protons of β-CD would also exist. Since the destroyed liposome had a structure similar to the damaged cell membranes, these results indicated that the β-CD cavities of **1** had the capability of including cholesterol or lecithin of the damaged cell membranes.

Fluorescence microscopy. Yeast cells have many features analogous to mammalian cells. When stained with Zinquin, the intracellular labile zinc pool of yeast cells appeared to localize to small punctuated cytoplasm vesicles, similar to the zincosomes described in mammalian cells.[32] Therefore, yeast cells were used as the substrate to investigate the Zn^{2+}-response ability of **1** in cells. In our experiments, the pre-cultured yeast cells were transferred to a solution of **1**, and fluorescence microscopic images were recorded after ca. 30 min. At the initial stage, only some damaged cells (including splinter and dead cells) exhibited weak fluorescence at cell division domains (Figure 5a and b) but most of the yeast cells did not exhibit any appreciable fluorescence. Interestingly, with time, more and more cells began to emit green fluorescence. This phenomenon may point to a unique fluorescent response mechanism of **1** with damaged cells. Possessing a compact and integrated cell membrane, the healthy cells had the ability to exclude cell-impenetrable molecule.[15] The self-included conformation of **1** also restricted the interaction of **1** with cell membranes. Therefore, **1** gave no response with healthy cells. However, with damaged cells, the junction gap in the cell membrane increased, which allowed the β-CD cavity of **1** to include competitively exposed molecules of the cell membrane and anchor in the loose bilayer of the cell. Subsequently, the free HQAS group could settle in the cell membrane and combine with the intracellular Zn^{2+} to give green fluorescence. On the other hand, when the cells were transferred from the incubated solution to the sample solution for a long time, the cells gradually died. Therefore, the damaged yeast cells that had lost their membrane integrity would allow **1** to fluoresce by catching Zn^{2+}. Interestingly, when we increased Zn^{2+} concentration by adding extra Zn^{2+} solution to the **1**-treated yeast cells, the damaged cells exhibited bright green fluorescence owing to the significant fluorescence enhancement of the **1**–Zn^{2+} system as described above, but the healthy cells still gave no fluorescence signal (Figure 5c and 5d). In another experiment, the incubated solution of yeast cells was heated to boil for 30 min to kill the cells. Then these dead cells were separated from the solution by centrifugation and transferred to the solution containing Zn^{2+} and **1**. The fluorescence microscopic images were recorded under the same conditions. The results showed that all of the cells (containing the budding cells and the candida cells) presented strong fluorescence (Figure 5e and 5f). These observations jointly

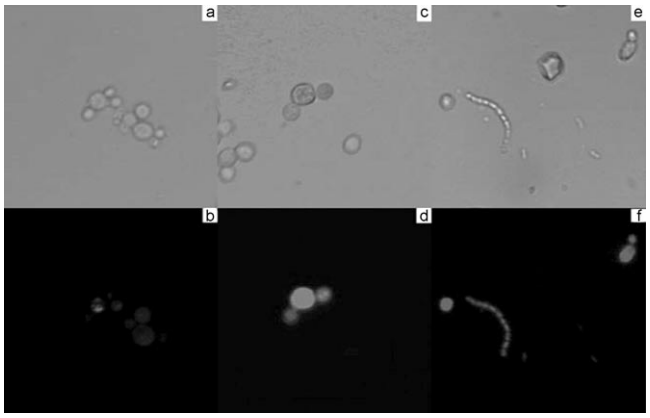


Figure 5. a) Optical and b) fluorescence microscopic images of yeast cells in the presence of **1** (50 μM), c) optical and d) fluorescence microscopic images of yeast cells in the presence of **1** (50 μM) and $ZnSO_4$ (50 μM), e) optical and f) fluorescence microscopic images of killed yeast cells in the presence of **1** (50 μM) and $ZnSO_4$ (50 μM).

validated the specific fluorescent sensing of **1** to the Zn^{2+}-containing damaged cells. It was noteworthy that, because **1** could not adhere to a smooth and integrated surface, the glucose crystals in Figure 5e could not be stained.

This supposition was also proved by the experiments with stained splinter cells, which showed that **1** stained only the division domains of the splinter cells but not other regions (see Supporting Information). A possible reason for this observation may be that the junction gap in the splinter cell membrane was loose, which allowed **1** to include cholesterols of the cell membrane. Moreover, the HQAS group of **1** was also inclined to insert into the hydrophobic regions of the bilayers.[33] These reasons jointly enabled **1** to anchor at the loose bilayer of the damaged cell membrane. In the case of the compact and integrated cell membrane of living cells, **1** could not include any molecules or anchor on the cell surface.

Conclusion

In conclusion, we successfully prepared a water soluble and cell-impenetrable zinc probe, which could selectively sense the Zn^{2+}-containing damaged cells. This property will enable its potential application in the measurement of cell viability or detection of cell apoptosis involving Zn^{2+}.

Materials and Methods

General

All chemicals were of reagent grade and used as received unless otherwise specified. *N*-(8-quinolyl)-*p*-aminobenzenesulfonamide (HQAS)[24] and mono[6-(2-aminoeythylamino)-6-deoxy]-β-CD[25] were prepared according to reported methods. Fluorescence spectra were measured in a conventional rectangular quartz cell (10×10×45 mm) at 25°C on a JASCO FP750 spectrometer equipped with a constant-temperature water bath. Tris-HCl buffer solution (pH 7.2) was used as the solvent in all spectral measurements. NMR experiments were recorded on a Bruker 300 instrument.

Synthesis

HQAS-succinic acid: To succinic anhydride (3.0 g, 30 mmol) dissolved in 50 mL of dry toluene, was added HQAS (3.0 g, 10 mmol). The mixture was stirred at 150°C for 5 h. After cooling to room temperature, the precipitate was collected by filtration and then washed with hot water. After drying in vacuo, a pale yellow product (3.4 g) was obtained in 85% yield. ^{1}H NMR ([D$_6$]DMSO, 300 MHz, TMS): δ=12.13 (s, 1H, -COOH), 10.29 (s, 1H, Quinoline-NH-), 9.79 (s, 1H, Phene-NH-), 8.86 (m, 1H, H^4 of Quinoline), 8.33 (d, J=8.1 Hz, 1H, H^2 of Quinoline), 7.25–7.95 (m, 8H, Quinoline and Phene), 2.50 ppm (s, 4H, -CH$_2$-CH$_2$- overlapped with the peaks of DMSO); MS (ESI): *m*/*z* (%) calcd: 398.09 [M−H]$^-$, 797.18 [2M−H]$^-$; found: 398.50, 797.30; elemental analysis: calcd (%) for $C_{19}H_{17}N_3O_5S$: C 57.13, H 4.29, N 10.52; found: C 56.95, H 4.07, N 10.65.

1: To a solution of DCC (440 mg, 2.1 mmol) and HOBT (150 mg, 1.1 mmol) in 5 mL of dry DMF was added to a solution of HQAS-succinic acid (400 mg, 1 mmol) in DMF (8 mL). The reaction mixture was stirred at 0°C for 1 h. A solution of mono[6-(2-aminoeythylamino)-6-deoxy]-β-CD (1.178 g, 1 mmol) in DMF (5 mL) was added to the reaction mixture, and the mixture was stirred at 0°C for 6 h and then stirred at room temperature for 24 h. Insoluble materials were removed by filtration, and the filtrate was poured into 200 mL of acetone. The precipitate was collected by filtration, washed with ethanol, and subsequently purified twice on a Sephadex C-25 column with 1 M ammonia as eluent. After drying in vacuo, a pale yellow product (460 mg) was obtained in 30% yield. ^{1}H NMR (D$_2$O, 300 MHz, TMS): δ=2.25-2.92 (m, 6H), 2.94-4.12 (m, 44H), 4.92 (s, 7H), 7.25 (m, 2H), 7.39 (s, 1H), 7.55 (d, J=7.8 Hz, 2H), 7.66 (s, 1H), 7.79 (d, J=7.8 Hz, 2H), 8.27 (s, 1H), 8.80 ppm (s, 1H); MS (ESI): *m*/*z* (%) calcd: 1558.51 [M+H]$^+$; found: 1558.63; elemental analysis: calcd (%) for $C_{63}H_{91}N_5O_{38}S\cdot 6H_2O$: C 47.69, H 6.54, N 4.41; found: C 48.05, H 6.45, N 4.69.

Yeast Cell Culture and Cell Staining

Yeast (*saccharomyces cerevisiae*) was obtained from the Agronomy & Forestry Department of Huanghuai University and used as a cell model. Prior to use, it was dispersed on a YPD plate (1.0% yeast extract, 4% peptone, 2.0% glucose, 0.2% $(NH_4)_2SO_4$) and cultured for 2 d at 35°C. For yeast staining, some colonies were selected and added to a solution of **1** (50 μM). Subsequently, the cells were washed twice with distilled water to remove uncombined **1** by centrifugation. A suitable volume of pre-cultured cells was diluted with or without the addition of $ZnSO_4$ (50 μM). Cells for experiments were dropped on glass slides and covered with a cover glass. All imaging experiments were performed on an YZ-2 fluorescence microscope (Beijing Keyi Electro-optic Plant) equipped with a 100W/DC mercury lamp for UV excitation and a SPC-382B color CCD camera for photo collection. The total magnification was 400×.

Liposome Preparation

Lecithin liposome was prepared by a method similar to that described by Wertz et al.[26] The mixture of egg phosphatidylcholine (60 wt.%), cholesterol (20%), and sodium deoxycholate (20%), which increased the solubility of lecithin and keep the solution clear, were dissolved in chloroform/methanol (2:1). The mixture was placed in a culture tube and the solvent was removed with a stream of nitrogen and then dried under high vacuum at room temperature. The solid matter was dissolved in D$_2$O to provide a final lecithin concentration of ca. 2 g dm^{-3}. The suspensions were sonicated at 60°C for ca. 15 min until the solution became clear.

Acknowledgements

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