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A Nile Red/BODIPY-based bimodal probe sensitive to changes in the micropolarity and microviscosity of the endoplasmic reticulum†

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We herein report a fluorescent bimodal probe (1) capable of determining ER viscosity and polarity changes using FLIM and fluorescence ratiometry, respectively; during ER stress caused by tunicamycin, the viscosity was increased from *ca.* 129.5 to 182.0 cP and the polarity of the ER (dielectric constant, ε) enhanced from 18.5 to 21.1.

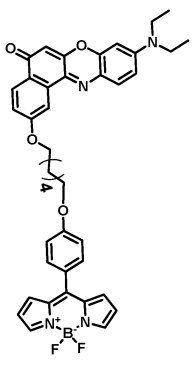
Viscosity and polarity, two significant environmental factors, markedly influence the behaviour of the surrounding targets[1] in various chemical and biological processes, including intracellular diffusions (of nutrients, metabolites, signalling proteins, *etc.*) and changes in the configurations of proteins and cell membranes.[2,3] Endoplasmic reticulum (ER) is a cellular organelle involved in the synthesis of secretory and membrane proteins and their post-translational modification, including glycosylation. The accumulation of large amounts of unfolded or misfolded proteins in the ER activates the ER stress response,[4] which has been implicated in various human diseases such as diabetes and Alzheimer's disease.[4,5] The accumulation of partially unfolded or glycosylated proteins in the ER would certainly alter the viscosity and polarity of the ER.[6] Therefore, it is reasonable to anticipate alteration of viscosity and polarity during ER stress, and it is of interest to monitor the corresponding changes.

With the emergence of biosensing technology, various fluorescent chemosensors have been introduced to measure biological viscosity[7] and polarity.[8] For viscosity measurements, "molecular rotors" have been developed for use with ratiometric[9] or fluorescence lifetime imaging techniques.[10] A bimodal molecular rotor that could be used for dual fluorescence ratiometry and fluorescence lifetime imaging was developed by Peng and coworkers.[11] Subsequently, our group reported a molecular rotor based on through-bond energy transfer, containing a coumarin unit and a BODIPY moiety, to measure mitochondrial viscosity in live cells.[12]

On the other hand, for polarity measurements in biological systems, fluorophores with an intramolecular charge transfer (ICT) (*e.g.*, Nile Red) have been widely adopted.[13] However, to understand cell-based biological events such as ER stress, it would be desirable to have a bimodal fluorescent probe that can be used to measure viscosity and polarity simultaneously and that can also be localised in the cell. To our knowledge, such a probe has not been reported yet.

In this context, we synthesized a new bimodal fluorescent probe (**1**) composed of BODIPY and Nile Red linked with a $-(CH_2)_6-$ spacer (Scheme 1). The BODIPY moiety, designed as a fluorescent molecular rotor, can be used to quantify local cellular viscosity by fluorescence lifetime imaging microscopy (FLIM), and Nile Red is a polarity-sensitive fluorophore.[14] We report herein that the newly synthesized probe localizes in the ER and can be used to quantify distinct changes in both local viscosity and local polarity of ER-stressed HeLa cells.

The probe (**1**) was prepared by conjugation of Nile Red and BODIPY with a $-(CH_2)_6-$ group. Initially, *m*-*N*,*N*-diethylaminophenol was subjected to nitrosation to provide intermediate **4**,



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Scheme 1 Chemical structure of the target compound (**1**).

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† Electronic supplementary information (ESI) available: Limited experimental and spectral data, cell imaging figures, and ^{1}H and ^{13}C NMR. See DOI: 10.1039/c4cc04915b

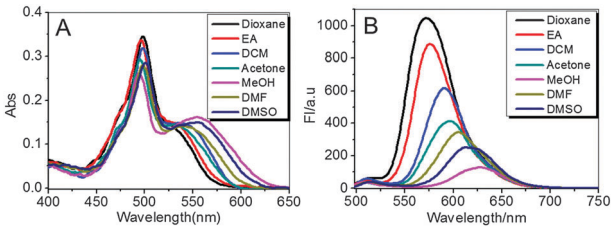

Fig. 1 Absorption (A) and emission (B) spectra of **1** (5.0 μM) in various nonviscous solvents, excited at 488 nm.

which was subsequently reacted with naphthalene-1,6-diol to afford Nile Red (**3**); this was followed by attachment to the $-(CH_2)_6-$ spacer to produce **2**. Reaction of **2** with 4-hydroxylphenyl BODIPY (**6**) afforded **1** in moderate yield. Detailed procedures and physical properties of compounds are provided in the ESI.†

To characterize the spectral properties of the probe, we first studied its solvent dependency. As shown in Fig. 1, the probe exhibits two absorption and two emission bands mainly contributed by its BODIPY and Nile Red moieties. The peaks centred at 490 and 516 nm correspond to the maximum absorption and emission wavelengths of BODIPY, which is almost nonfluorescent in nonviscous solvents because of the free rotation of BODIPY.[12] The maximum absorption and emission wavelengths of Nile Red shifted in solvents of varying polarities (λ_{abs}, 525–575 nm and λ_{em}, 565–635 nm for 1,4-dioxane to MeOH), and the fluorescence intensity gradually decreased with increasing solvent polarity, owing to the ICT process that enables this dye to respond to environmental polarity.

To prove that the probe can be used to determine the local viscosity and polarity of certain media, we investigated fluorescence responses of the sensor towards arbitrary changes in viscosity or polarity. First, fluorescence spectra and the fluorescence lifetime of the probe were measured in solvents of different viscosities. As shown in Fig. 2 and Fig. S1, ESI† the fluorescence

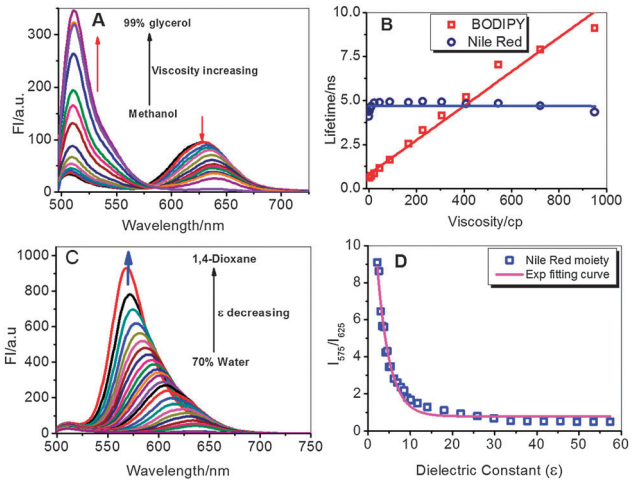

Fig. 2 Fluorescence spectra and fluorescence lifetime of **1** (1.0 μM) in solvents of varying viscosity (A) and polarity (C); fluorescence lifetime of the probe (BODIPY moiety at 516 nm and Nile Red moiety at 635 nm) was measured using a time-correlated single photon counting (TCSPC) system, excited at 375 nm (B); and the fluorescence intensity ratios for Nile Red (I_{575}/I_{625}) were determined in solvents of varying polarity, using an excitation wavelength of 488 nm (D).

intensity of BODIPY was enhanced *ca.* 10-fold with increasing solvent viscosity from 0.6 (methanol) to 950 cP (99% glycerol) at room temperature (25 °C) (Fig. 2A). For fluorescence lifetime measurements, upon extrapolation of the fluorescence decay of BODIPY, we noticed that two factors (BODIPY, τ_1 and Nile Red, τ_2) contributed to the emission at 516 nm. The fluorescence lifetime of BODIPY (τ_1) markedly increased from *ca.* 0.6 ns in MeOH to 10 ns in 99% glycerol because free rotation around the C–C bond between BODIPY and the phenyl unit was gradually suppressed by increasing solvent viscosity. The linear relationship ($R^2 = 0.98$) of τ_1 *vs.* viscosity (Fig. 2B) could be used to quantify the local viscosity. For Nile Red (Fig. 2A), the maximum emission wavelength red shifted and the fluorescence was gradually quenched as the proportion of glycerol in a methanol–glycerol mixture increased. This is probably because the solvent polarity changed (dielectric constants at 25 °C: methanol, $\varepsilon = 32.6$; glycerol, $\varepsilon = 45.8$) or because solubility in a viscous medium is considerably reduced. Furthermore, we found that the fluorescence lifetime of Nile Red (*ca.* 5 ns, mono-exponential decay) in the probe rarely changed even with markedly increased solvent viscosity (Fig. S1, ESI†).

Second, we investigated the changes in fluorescence spectra and the fluorescence lifetime of the probe in nonviscous solvents with different polarities. Very sharp changes in the fluorescence of Nile Red in **1** were observed in solvents of different polarities and low viscosity (Fig. 1B; *e.g.*, 1,4-dioxane, $\varepsilon = 2.25$, $\eta = 1.3$ cP; MeOH, $\varepsilon = 32.6$, $\eta = 0.6$ cP at 25 °C). As the proportion of the polar solvent (water, $\varepsilon = 78.3$ at 25 °C) in the binary mixture (1,4-dioxane–water) increased from 0% to 70%, corresponding to an increase in solvent mixture polarity,[15] the maximum emission wavelength of Nile Red shifted from 565 to 635 nm because of emission from the ICT excited state, and its fluorescence intensity markedly decreased (Fig. 2C). From a plot of the fluorescence intensity ratio I_{575}/I_{625} *vs.* dielectric constant (fitting with an exponential relationship with $R^2 = 0.98$) (Fig. 2D), we could estimate the local polarity of certain media and even live cells.[13] As shown in Fig. 2D, the I_{575}/I_{625} ratio decreases drastically, from *ca.* 6.0 to 0.5, with increasing solvent polarity when the dielectric constant is below 20; this change becomes more gradual when the solvent dielectric constant is over 20, indicating that Nile Red is very sensitive to nonpolar environments. The corresponding fluorescence lifetime (mono-exponential decay) is shortened from *ca.* 9 to 5 ns with increasing solvent polarity. Since the BODIPY moiety in **1** rarely responds to polarity (Fig. S1, ESI†), it exhibits very low fluorescence intensity and an invariable fluorescence lifetime. Therefore, **1** is a dual-function probe that can be used to quantify the local viscosity and polarity of certain media through changes in the fluorescence of BODIPY and Nile Red, respectively.

Before **1** could be used as a bimodal probe to measure viscosity and polarity in the ER, the intracellular localisation of the probe had to be determined. In the preparation for colocalisation experiments, we studied the fluorescence intensity of the probe in HeLa cells incubated for different periods of time. We found that the fluorescence of the probe (5.0 μM) inside HeLa cells was saturated upon incubation for 25 min (Fig. S2 and S3, ESI†). Then the probe and different fluorescent markers specific for intracellular organelles (mitochondria, ER, and lysosomes)

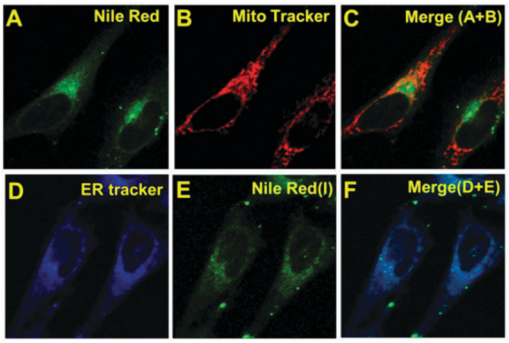

Fig. 3 Colocalisation experiment of **1** (5.0 μM) with organelle-specific markers in HeLa cells; (A, E), fluorescence imaging of Nile Red (collected at 560–590 nm), excited at 488 nm; (B), MitoTracker, excited at 514 nm; (C), merged image of A and B; (D), ER-Tracker, excited at 405 nm; (F), merged image of D and E.

were incubated with HeLa cells for 30 min. The results of colocalisation experiments using a confocal laser microscope with the probe and the organelle-specific dyes are shown in Fig. 3 and Fig. S4, ESI.† The merged colours in panels C and F represent the colocalisation of **1** with MitoTracker and ER-Tracker, respectively. Panel F shows the most overlap between the probe and the organelle-specific dye, indicating that the probe is located mainly in the ER.

To validate the use of **1** for ER polarity imaging, confocal laser fluorescence images of HeLa cells were acquired with or without tunicamycin treatment. And the tunicamycin is known to inhibit glycosylation during protein or glycolipid synthesis, causing the accumulation of proteins or lipids in the ER (ER stress).[16] Confocal laser fluorescence images for BODIPY emissions (515 ± 15 nm), Nile Red I (575 ± 15 nm), and Nile Red II (635 ± 15 nm) are shown in Fig. 4; Fig. S5–S7, ESI.† The fluorescent ratiometric images (Fig. 4C and D) of the probe in HeLa cells were obtained by dividing one emission channel of Nile Red by the other ($I_{(575\pm15)}/I_{(635\pm15)}$) (B/C and F/G, Fig. S5, ESI†).[13]

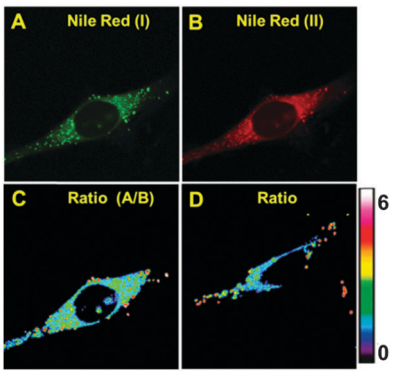

Fig. 4 Fluorescence imaging of 1 (5.0 μM) in HeLa cells; (A), fluorescence of the Nile Red moiety (I) (collected at 560–590 nm); (B), fluorescence of Nile Red (II) (collected at 625–650 nm); (C), ratiometric image of Nile Red obtained from panels A and B untreated by tunicamycin; (D), ratiometric image of Nile Red in HeLa cells treated by tunicamycin for 24 hours; excited at 488 nm, and fluorescence images were obtained using a Leica TCS SP2 confocal laser microscope.

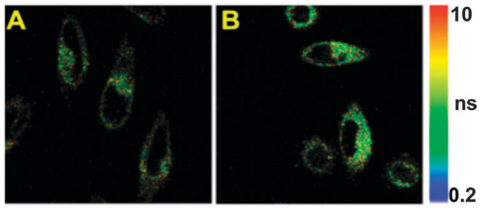

Fig. 5 Fluorescence lifetime imaging (FLIM) of the BODIPY moiety in **1** (5.0 μM) in HeLa cells; (A) FLIM in HeLa cells untreated by tunicamycin; (B) FLIM in HeLa cells treated with tunicamycin (10 μg mL^{-1}) for 24 hours before incubation with **1**; excited at 375 nm and emission was detected at 500–530 nm using an inverted-type scanning confocal microscope (MicroTime 200, PicoQuant, Germany) with a 60× objective.

When the probe was initially incubated with HeLa cells, the average Nile Red ratio was measured to be *ca.* 1.09, corresponding to an ε value of *ca.* 18.5. Upon treatment of HeLa cells with tunicamycin (10 μg mL^{-1}) for 24 h, the average ratio decreased to *ca.* 0.86 (ε = 21.1), indicating a detectable increase in the average polarity of the ER (detection sensitivity is *ca.* 0.6). This increased polarity of the ER could be due to the substantial accumulation of polar proteins in the ER, resulting in ER stress.[6]

FLIM was used to obtain further insight into ER viscosity changes during ER stress in HeLa cells. The probe displayed two exponential decays in HeLa cells before and after treatment with tunicamycin (Fig. S8 and S9, ESI†), which are attributable to the BODIPY and Nile Red moieties. As shown in Fig. 2B and Fig. S1, ESI† the BODIPY moiety was sensitive to viscosity with a viscosity sensitivity of *ca.* 2.3 cP (determined by sensitivity measurements). Therefore, we measured the fluorescence lifetime of BODIPY to calculate the average local viscosity in the ER. As shown in Fig. 5A and Fig. S8, ESI† the average fluorescence lifetime of **1** in untreated HeLa cells was *ca.* 2.07 ± 0.74 ns, corresponding to a viscosity of *ca.* 129.5 ± 7.2 cP. After the cells had been treated with tunicamycin (10 μg mL^{-1}) for 24 h and incubated with **1** for 20 min at 37 °C, the fluorescence lifetime increased to *ca.* 2.58 ± 1.1 ns (η = 182 ± 29.8 cP), confirming that the viscosity of the ER was marginally increased because of accumulation of nascent proteins in the ER.

In conclusion, we have synthesized a novel, microenvironment-sensitive, bimodal fluorescent probe (**1**) for the simultaneous determination of local polarity and viscosity of the ER in live cells. The probe is composed of Nile Red and BODIPY linked with a $-(CH_2)_6-$ spacer, showing low cytotoxicity (Fig. S10, ESI†). Using this probe, the local polarity and viscosity of media could be determined using the ratio of fluorescence intensities at 575 and 625 nm (I_{575}/I_{625}) and the fluorescence lifetime at 516 nm, respectively. We also found that the probe was mainly located in the ER in live cells. Upon treatment of HeLa cells with tunicamycin to cause ER stress, the polarity (ε) of the cells increased from *ca.* 18.5 to 21.1, based on the ratiometric imaging of Nile Red ($I_{(575\pm15)}/I_{(635\pm15)}$). Furthermore, the fluorescence lifetime of BODIPY, as determined by FLIM, increased from *ca.* 2.07 ± 0.74 to 2.58 ± 1.1 ns upon tunicamycin treatment, which corresponded to an increase in the viscosity of the ER from *ca.* 129.5 ± 7.2 to 182 ± 29.8 cP. Therefore, the newly designed bimodal probe

reported herein is a promising tool for the diagnosis of ER-stress-related disease.

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