JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Activatable Rotor for Quantifying Lysosomal Viscosity in Living Cells

Lu Wang,[†] Yi Xiao,*[,†] Wenming Tian,[‡] and Liezheng Deng[‡]

[†]State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116024, China

[‡]State Key Laboratory of Molecular Reaction Dynamics, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

Supporting Information

ABSTRACT: We have developed **Lyso-V**, the first fluorescent probe of lysosomal viscosity. Because of its lysosome-actived fluorescence characteristics, **Lyso-V** has proved to be an ideal lysosomal tracer with high spatial and temporal resolution under laser confocal microscopy. More importantly, **Lyso-V** shows its practical applicability in real-time quantification of lysosomal viscosity changes in live cells through fluorescence lifetime imaging microscopy.

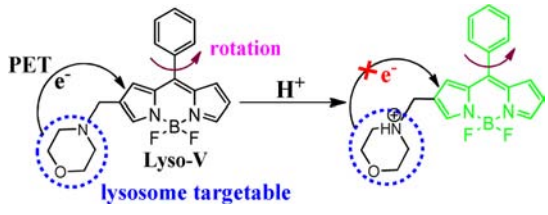
Detection of intracellular viscosity remains one of the challenges in chemical biology, although for a long time viscosity has been well-recognized as an essential microenvironmental parameter in a variety of biological systems. Viscosity contributes to biological functions by affecting the mobility of substances.[1] Also, its abnormal changes are closely linked with disorders and diseases (e.g., diabetes, infarction, and hypertension).[2] However, conventional viscometers suitable for macroscopic fluids cannot be applied in live cells, necessitating the development of new tools.[3] One predictable obstacle is the fact that the intracellular microenvironment, which is composed of many different organelles (e.g., mitochondria and lysosomes), is highly inhomogeneous. Not surprisingly, the local viscosity differs considerably from one region to another. For the sake of better indicating cells' local changes, sensing the intracellular viscosity (and other environmental parameters, e.g., pH and polarity, and important chemical species, e.g., Ca^{2+} and nitric oxide) should to the greatest possible extent be performed within distinct organelles rather than in an unknown intracellular area.[4]

Lysosomal viscosity reflects the status and function of this kind of organelle, whose locations, morphologies, and components are always changing. Normally, as the cell's recycling centers, lysosomes receive senescent cellular debris or needless macromolecules and process them into small molecules that the cell can utilize.[5] However, in cases of lysosomal dysfunction, especially lysosomal storage diseases[6] caused by the deficiency of single lysosomal enzymes, macromolecular substances do not decompose but accumulate within lysosomes. Since the lysosomal viscosity fluctuates along with changes in the amounts and densities of such macromolecules, real-time monitoring of its dynamic alteration would be very meaningful for not only fundamental cell biology but also diagnosis of lysosome-related disorders.[7] Unfortunately, detecting lysosomal viscosity in living cells remains a "blank space", without any attempt reported in the literature.

To our knowledge, fluorescent probes, which are powerful tools in cell biology, can hardly meet the requirements for monitoring lysosomal viscosity. Recently, several research teams, including the groups of Haidekker,[8] Suhling,[9] and Peng,[10] have developed a number of viscosity-sensitive probes based on different kinds of molecular rotors that respond to the increased viscosity by enhancement of the fluorescence intensity or increases in the fluorescence lifetime. These inspiring works have enriched our knowledge of molecular design ideas for viscosity probes. However, almost all of these probes showed uneven, uncharted, and nonspecific intracellular distributions that might influence the reliability of their viscosity mapping. The only two exceptions were reported by Haidekker[8b] and Suhling,[9] who developed molecular rotors containing long chains with high lipophilicity to bind to membranes or other hydrophobic domains of cellular organelles and to probe the local microviscosity of these regions. To date, none of these fluorescent probes is well-suited for lysosomal applications.

In this work, we developed **Lyso-V**, the first lysosome molecular probe for viscosity, by attaching a morpholine moiety to a typical boron dipyrromethene (BODIPY) molecular rotor (Scheme 1). The rotor unit provides sensitivity toward the viscosity because of the free rotation around the single bond connecting the BODIPY core and the phenyl group.[9a] The rotor can freely rotate, without being bound to proteins [see the Supporting Information (SI)]. BODIPYs themselves are highly lipophilic, giving them a large tendency to accumulate in lipid-rich intracellular regions rather than in lysosomes. Within **Lyso-V**, moderate alkalinity of the morpholine moiety is critical for its specific localization in acidic lysosomes. According to our previous study,[11] morpholine has a pK_a of 5−6, so it can be protonated only in lysosomes (pH 4.5−5.5) but not in cytosol

Scheme 1. Working Principle of Lysosomal Viscosity Probe Lyso-V



Received: November 30, 2012
Published: February 14, 2013

dx.doi.org/10.1021/ja311688g | J. Am. Chem. Soc. 2013, 135, 2903−2906

or in other subcellular organelles. Once **Lyso-V** diffuses into lysosomes and is protonated, it becomes more hydrophilic and is retained in the lysosomes. In addition, introduction of the morpholine unit also endues **Lyso-V** with a lysosome-actived fluorescence characteristic that is very beneficial for lysosomal tracing. This occurs because the electron-rich morpholine unit quenches the BODIPY fluorescence through photoinduced electron transfer (PET).[12] Thus, even though there is a trace amount **Lyso-V** distributed outside the lysosomes, its background fluorescence is negligible. However, in lysosomes, the morpholin moiety is protonated and can no longer serve as an electron donor for PET. Hence, only **Lyso-V** molecules that are localized in lysosomes are fluorescent.

Lyso-V's fluorescence intensity is highly sensitive to both pH and viscosity (Figure 1). On the one hand, at a particular

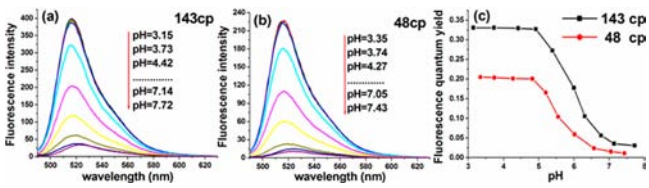


Figure 1. (a, b) Fluorescence spectra of **Lyso-V** at different pH in methanol/water/glycerol mixtures at two viscosities: (a) 143 and (b) 48 cP. (c) Fluorescence quantum yields of **Lyso-V** at different pH.

viscosity (e.g., 143 or 48 cP), **Lyso-V**'s fluorescence band at 517 nm increased as the pH decreased from 6 to 4; for example, at a viscosity of 48 cP, the fluorescence quantum yield (ϕ) at pH 4 was 0.21, which is almost 20 times that at pH 7 (ϕ = 0.011). On the other hand, when the pH was kept the same, the fluorescence intensity decreased with decreasing viscosity. For instance, at pH 4, ϕ was 0.21 at 48 cP, which is only two-thirds of that at 143 cP (ϕ = 0.33). The above discovery revealed that **Lyso-V**'s fluorescence intensity is not suitable for quantifying viscosity because of the interference from pH changes. Because the pH of lysosomes varies from 4 to 6 at different stages, laser confocal imaging (fluorescence intensity imaging) might not be a good choice for accurately determining the lysosomal viscosity. However, it should be noted that for pH > 7, **Lyso-V**'s fluorescence became very weak and negligible at all viscosities. This characteristic, ascribed to PET from the neutral morpholine donor to the excited BODIPY, helps eliminate the background fluorescence outside the lysosomes and thus is very beneficial for the use of **Lyso-V** to trace the locations of lysosomes under laser confocal microscopy.

The fluorescence lifetime of **Lyso-V** exhibits satisfactory specificity and sensitivity toward viscosity changes. First, the fluorescence lifetimes of both **Lyso-V** and its protonated form (obtained by acidifying the medium with a small but sufficient amount of HCl) were measured in a series of viscosity gradient buffers composed of methanol and glycerol in various proportions (Figure 2a,b). Interestingly, the lifetime−viscosity curve of neutral **Lyso-V** coincides with that of its protonated counterpart, as they displayed almost identical lifetimes at every viscosity (Figure 2c). Along with the gradual increase in viscosity from 0.6 to 359.6 cP, the liftetimes became remarkably longer, from 0.30 to 4.62 ns. Second, at the four chosen viscosity levels (0.7, 10, 30, and 69 cP), the fluorescence lifetimes were determined at different pH. At a given viscosity level, the lifetimes at different pH were very similar; according to Sauer and co-workers,[13] this can be ascribed to "static

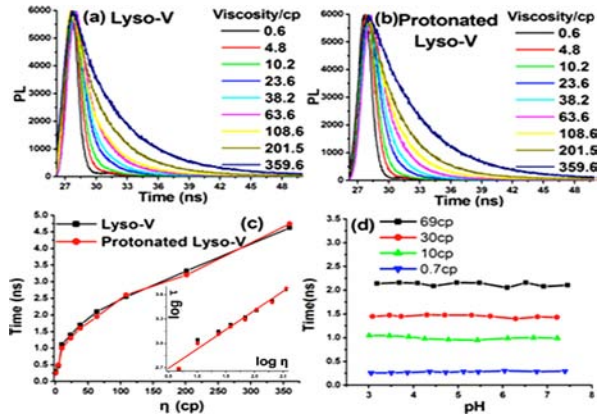


Figure 2. (a, b) Fluorescence lifetime spectra at 517 nm for 2 μM (a) **Lyso-V** and (b) protonated **Lyso-V** [obtained by acidifying methanol/glycerol systems with 4 μL of 37% HCl(aq)] in methanol/glycerol mixtures with varying proportions to adjust the viscosity. (c) Fluorescence lifetime vs viscosity curves for neutral (black) and protonated (red) **Lyso-V**. (d) Fluorescence lifetimes of **Lyso-V** at various pH in water/methanol/glycerol mixtures with varying portions to adjust the viscosity to the four indicated levels and diluted with HCl and NaOH solutions to adjust the pH.

quenching" by the very fast PET process due to the short distance between the fluorophore and the quencher. It was also found that at different viscosities, the fluorescence lifetimes at the same pH were quite different. When we precisely tuned and determined the pH, we had to add large portions of water (see the SI), and the as-obtained water/methanol/glycerol ternary systems would have had considerably different polarities than the binary methanol/glycerol mixtures, as water has a much higher dielectric constant (ε = 78) than methanol (ε = 33) and glycerol (ε = 43). However, it is worth noting that the lifetimes determined in the two viscosity buffer systems were coincident. The lifetimes at 69, 30, 10, and 0.7 cP were around 2.10, 1.43, 1.02, and 0.25 ns, respectively, over a range of pH (Figure 2d); at all four viscosities, we found the identical coordinate positions in the viscosity−lifetime curves for neutral and protonated **Lyso-V** (Figure 2c). These results indicate that **Lyso-V**'s fluorescence lifetime is sensitive only to the viscosity and is independent of changes in pH and polarity. Hence, the viscosity−lifetime curve in Figure 2c can be used as a calibration curve for quantification of the viscosity by fluorescence lifetime imaging microscopy (FLIM).[14]

The cytotoxicity of **Lyso-V** is very low within 24 h of incubation (see the SI). An MTT experiment showed that >90% of MCF-7 cells survived after 12 h (5 μM **Lyso-V** incubation), and after 24 h the cell viability remained at ~80%. For many known lysosomal probes, long incubation can induce an increase in the cell death rate. Compared with those of most commercial lysosomal probes, the cytotoxicity of **Lyso-V** toward MCF-7 cells is very low, which is a favorable property for applications in living cells.

Lyso-V displayed strong localized fluorescence within lysosomes. MCF-7 cells were costained with **Lyso-V** and Neutral Red (**NR**), a commercial lysosome tracker, for 3 min at 37 °C. **Lyso-V** showed bright-green fluorescence in channel 1 (Figure 3b). At the same time, **NR** showed red fluorescence in channel 2 (Figure 3c). The merged image (Figure 3d) indicates that the two channel images overlapped very well, confirming that **Lyso-V** can specifically localize in the lysosomes of living cells. The changes in the intensity profile of linear regions of

dx.doi.org/10.1021/ja311688g | J. Am. Chem. Soc. 2013, 135, 2903−2906

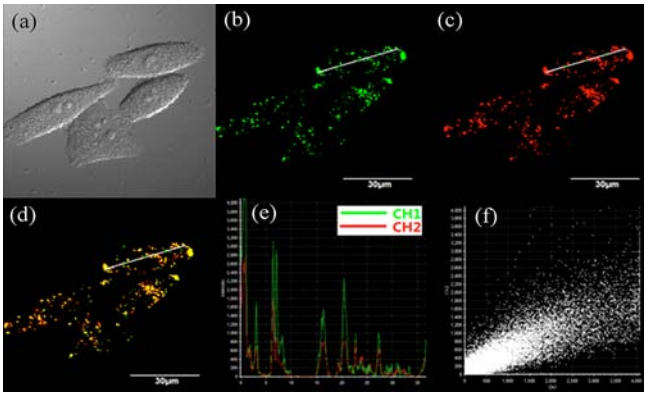


Figure 3. Colocalization imaging of MCF-7 cells stained with 5.0 μM **Lyso-V** and 5.0 μM **NR** for 3 min at 37 °C. (a) Bright-field image. (b) Confocal image from **Lyso-V** on channel 1 (λ_{ex} = 488 nm, λ_{em} = 515−545 nm). (c) Confocal image from **NR** on channel 2 (λ_{ex} = 559 nm, λ_{em} = 585−610 nm). (d) Merged image of channels 1 and 2. (e) Intensity profile of ROIs across MCF-7 cells. (f) Correlation plot of **Lyso-V** and **NR** intensities.

interest (ROIs) (**Lyso-V** and **NR** costaining) tended toward synchronization (Figure 3e). From the intensity correlation plots (Figure 3f), a high Pearson's coefficient and overlap coefficient of 0.915 and 1.616, respectively, were obtained. The above colocalization investigation confirmed that **Lyso-V** can stain lysosomes specifically.

The capability of **Lyso-V** to trace lysosomes with high spatial and temporal resolution was also demonstrated (Figure 4).

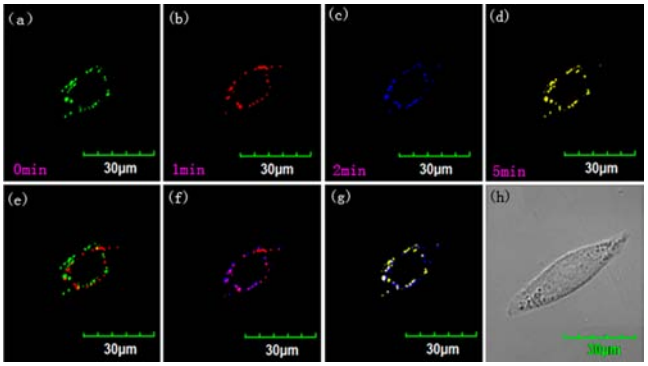


Figure 4. (a−d) Confocal images of an MCF-7 cell stained with 5 μM **Lyso-V** and stimulated using 3 μM chloroquine. Different pseudocolors are used to illustrate the fluorescence images at different stimulation times of 0, 1, 2, and 5 min. (e−g) Merges of images at two different times: (e) 0 and 1 min, (f) 1 and 2 min, and (g) 2 and 5 min. (h) Bright-field image.

MCF-7 cells were stimulated by a low dose (3 μM) of chloroquine, a typical lysosomal toxicant, in order to drive lysosomal migration without inducing any other apparent disturbance in the cells. A time series of confocal microscopy images with the aid of **Lyso-V** was recorded in a short period of 5 min. These pictures at different times (Figure 4a−d) revealed that the lysosomes largely kept their original shapes but their locations changed little by little. Although these short-term shifts were not very large, they could be unambiguously traced in the merges of images at any two continuous times (Figure 4e−g). Such a good image quality of **Lyso-V** for tracing lysosomes should be ascribed to the bright fluorescence within the lysosomes and the negligible background fluorescence outside them.

In combination with FLIM, **Lyso-V** is the first reliable tool for determining the local viscosity within lysosomes. Figure 5b

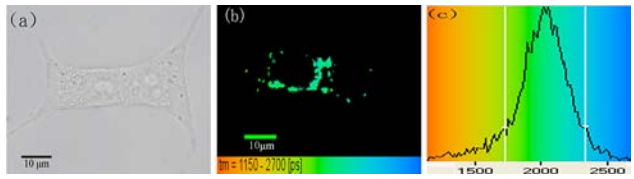


Figure 5. FLIM investigation of an MCF-7 cell stained with 5 μM **Lyso-V**. (a) Bright-field image. (b) Fluorescence lifetime imaging using fluorescence detection at 535 ± 15 nm after pulsed excitation at 405 nm. (c) Histogram of lifetimes.

shows a FLIM image of a single normal MCF-7 cell, in which the fluorescence lifetimes in the lysosomes have been mapped with considerable spatial resolution. The FLIM image also shows a narrow lifetime distribution range between 1.75 and 2.35 ns (Figure 5c). In the lifetime histogram, the maximum probability is located at 2.05 ns. According to our calibration graph in Figure 2c, the lifetimes of **Lyso-V** in lysosomes correspond to the viscosity range 50−90 cP, and the average viscosity is ∼65 cP. To our knowledge, these fundamental data of importance for cellular biology have not been available previously.

Lyso-V has practical applicability in real-time monitoring of dynamical changes in lysosomal viscosity under stimulation by different mechanisms. To confirm this, we adopted two kinds of medicines commonly used in the clinic. First was dexamethasone, an anti-inflammatory and immunosuppressant drug that works as a stabilizer of lysosomal membrane[15a] and an inhibitor of lysosomal enzymatic release.[15b] During a 20 min period of stimulation using a low dose (5 μM) of dexamethasone, continuous FLIM images revealed gradually extended lifetimes within the lysosomes (Figure 6). According to the lifetime histograms listed in the SI, the average lifetime in the total cell increased from 1.99 to 2.24 ns, which means that the average lysosomal viscosity increased from 67 to 85 cP. We also checked the lifetimes of two distinct regions of lysosomes (marked as 1 and 2 in Figure 6a) and found a similar tendency of the lifetime increases (Figure 6h). Obviously, dexamethasone

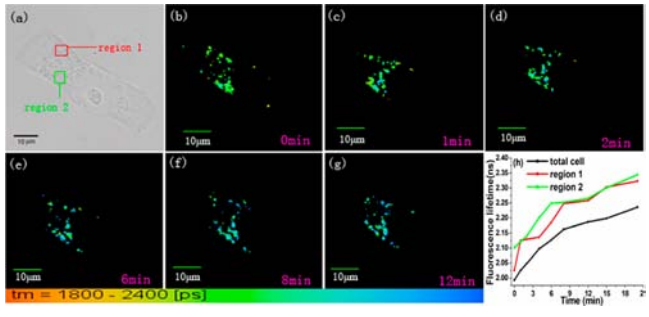


Figure 6. Fluorescence lifetime imaging of a MCF-7 cell stained with 5 μM **Lyso-V** and stimulated for different times (0−20 minutes) using 5 μM dexamethasone, with fluorescence detection at 535 ± 15 nm. (a) Bright-field image. (b−g) Fluorescence lifetime imaging of the cell stimulated for different times usinig 5 μM dexamethasone. (h) Plot of fluorescence lifetimes of **Lyso-V** stimulated for different times using 5 μM dexamethasone.

dx.doi.org/10.1021/ja311688g | *J. Am. Chem. Soc.* 2013, 135, 2903−2906

stimulation results in an increase in lysosomal viscosity. In view of the fact that these cells' lysosomal viscosity before stimulation was only ~67 cP, the increase in magnitude of ~20 cP is remarkable. The other stimulating agent was chloroquine, which is a typical toxicant for lysosomes because its stronger basicity can raise the lysosomal pH.[16] However, even at chloroquine concentrations as high as 20 μM, the FLIM images revealed that the fluorescence lifetimes within the lysosomes changed very slightly (see the SI). The average lifetime after chloroquine stimulation for 20 min was ~2.06 ns, which is almost the same as the original lifetime (2.05 ns). Thus, under our stimulation conditions, chloroquine stimulation did not greatly influence the lysosomal viscosity, in contrast to dexamethasone stimulation. The above experiments demonstrate that application of **Lyso-V** in FLIM to monitor lysosmal viscosity changes will be a valuable method in lysosome-related pharmacology and toxicology studies.

In conclusion, we have developed **Lyso-V**, the first fluorescent probe for lysosomal viscosity, in a straightforward manner by attaching a morpholine moiety to a BODIPY rotor. Because of its lysosome-activated fluorescence characteristics, **Lyso-V** is an ideal lysosomal tracer with high spatial and temporal resolution under laser confocal microscopy. More importantly, **Lyso-V** can be practically applied in real-time quantification of lysosomal viscosity changes in live cells through the FLIM technique. For the first time, lysosomal viscosity data for MCF-7 cells have been obtained and the different influences of two typical lysosome-stimulating drugs on the lysosomal viscosity have been discovered.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, characterization, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

xiaoyi@dlut.edu.cn

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (21174022 and 21173217), the National Basic Research Program of China (2013CB733702), and the Specialized Research Fund for the Doctoral Program of Higher Education (20110041110009). We also thank Prof. Junle Qu at Shenzhen University for helpful discussions on FLIM.

■ REFERENCES

(1) (a) Elcock, A. H. *Curr. Opin. Struct. Biol.* **2010**, *20*, 196. (b) Minton, A. P. *J. Biol. Chem.* **2001**, *276*, 10577.

(2) (a) Luby, P. K. *Int. Rev. Cytol.* **2000**, *192*, 189. (b) Stutts, M. J.; Canessa, C. M.; Olsen, J. C.; Hamrick, M.; Cohn, J. A.; Rossier, B. C.; Boucher, R. C. *Science* **1995**, *268*, 847. (c) Reinhart, W. H. *Biorheology* **2001**, *38*, 203. (d) Uchimuar, I.; Numano, F. *Diabetes Front.* **1997**, *8*, 33. (e) Simon, A.; Gariepy, J.; Chironi, G.; Megnien, J. L.; Levenson, J. J. *Hypertension* **2002**, *20*, 159. (f) Moriarty, P. M.; Gibson, C. A. *Cardiovasc. Rev. Rep.* **2003**, *24*, 321. (g) Bosman, G. J. C. G. M.; Bartholomeus, I. G. P.; Grip, D. W. J. *Gerontology* **1991**, *37*, 95.

(3) (a) Haidekker, M. A.; Theodorakis, E. A. *Org. Biomol. Chem.* **2007**, *5*, 1669. (b) Kaliviotis, E.; Yianneskis, M. *Proc. Inst. Mech. Eng., Part H* **2007**, *221*, 887.

(4) Kuimova, M. K.; Botchway, S. W.; Parker, A. W.; Balaz, M.; Collins, H. A.; Anderson, H. L.; Suhling, K.; Ogilby, P. R. *Nat. Chem* **2009**, *1*, 69.

(5) (a) Dell'Angelica, E. C.; Mullins, C.; Caplan, S.; Bonifacino, J. S. *FASEB J.* **2000**, *14*, 1265. (b) Burleigh, M. C.; Barrett, A. J.; Lazarus, G. S. *Biochem. J.* **1974**, *137*, 387.

(6) (a) Futerman, A. H.; van Meer, G. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 554. (b) Vellodi, A. *Br. J. Haematol.* **2005**, *128*, 413. (c) Neufeld, E. F. *Annu. Rev. Biochem.* **1991**, *60*, 257. (d) Simonsa, K.; Gruenberg, J. *Trends Cell Biol.* **2000**, *10*, 459.

(7) (a) Harkness, J. *Biorheology* **1971**, *8*, 171. (b) Huizing, M.; Helip-Wooley, A.; Westbroek, W.; Gunay-Aygun, M.; Gahl, W. A. *Annu. Rev. Genomics Hum. Genet.* **2008**, *9*, 359.

(8) (a) Haidekker, M. A.; Brady, T. P.; Lichlyter, D.; Theodorakis, E. A. *J. Am. Chem. Soc.* **2006**, *128*, 398. (b) Haidekker, M. A.; Ling, T.; Anglo, M.; Stevens, H. Y.; Frangos, J. A.; Theodorakis, E. A. *Chem. Biol.* **2001**, *8*, 123.

(9) (a) Kuimova, M. K.; Yahioglu, G.; Levitt, J. A.; Suhling, K. *J. Am. Chem. Soc.* **2008**, *130*, 6672. (b) Levitt, J. A.; Kuimova, M. K.; Yahioglu, G.; Chung, P. H.; Suhling, K.; Phillips, D. *J. Phys. Chem. C* **2009**, *113*, 11634.

(10) Peng, X. J.; Yang, Z. G.; Wang, J. Y.; Fan, J. L.; He, Y. X.; Song, F. L.; Wang, B. S.; Sun, S. G.; Qu, J. L.; Qi, J.; Yan, M. *J. Am. Chem. Soc.* **2011**, *133*, 6626.

(11) Yu, H. B.; Xiao, Y.; Jin, L. J. *J. Am. Chem. Soc.* **2012**, *134*, 17486.

(12) de Silva, A. P.; Gunaratne, H. Q. M.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Fademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515.

(13) Doose, S.; Neuweiler, H.; Sauer, M. *ChemPhysChem* **2009**, *10*, 1389.

(14) Berezin, M. Y.; Achilefu, S. *Chem. Rev.* **2010**, *110*, 2641.

(15) (a) Hinz, B.; Hirschelmann, R. *Pharm. Res.* **2000**, *17*, 1489. (b) Ackerman, N. R.; Beebe, J. R. *J. Pharmacol. Exp. Ther.* **1975**, *193*, 603.

(16) Gonzalez-Noriega, A.; Grubb, J. H.; Talkad, V.; Sly, W. S. *J. Cell Biol.* **1980**, *85*, 839–852.