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COMMUNICATION

A near-infrared emission fluorescent probe with multi-rotatable moieties for high sensitivity detection of mitochondrial viscosity in inflammatory cell model

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A near-infrared emission viscosity fluorescent probe (TPE-V) which contains multi-rotatable moieties was developed for the first time. TPE-V exhibited a large stokes shift (190 nm) and large enhancement of fluorescence intensity (100-fold) in response to viscosity. Moreover, TPE-V was successfully applied for detection of mitochondrial viscosity in inflammatory cells model.

Viscosity is a crucial paremeter of reaction in solution, which is mainly determined by the diffusion rate of substance. In living system, viscosity strongly influences intracellular signal transport, the diffusion of active metabolites and interactions between biomacromolecules.[1,2] The changes of intracellular viscosity are closely related to the physiological function and a variety of diseases, such as diabetes, Alzheimer disease (AD), hypertension and Parkinson disease (PD). [3-7] It is reported that the viscosity is about 1-2 cP in the cellular cytoplasm of normal cells, while in the diseased cells it is significantly increased, which can reach 140 cP, or even higher. [8-11] Mitochondria are the main compartment of energy production which play significant roles for various vital cellular processes including ATP production and central metabolism.[12] Changes of mitochondrial viscosity may influences mitochondrial network organization and metabolite diffusion rate. Studies have shown that the changes of mitochondrial viscosity could cause the body to be in a state of disease.[13] Therefore, it is significant to develop an effective method for monitoring the viscosity variations in the mitochondrial.

Although some analytical methods have been used to detect viscosity including viscometer and electrochemical analysis, nondestructive strategies appropriated for biological samples measurements in living cells are still unmet. To solve this problem, extensive molecular rotors were developed as microviscosity sensors, [14-17] but most of them have short emission wavelength, which may lead to damage to living biological systems.[18] Furthermore, the molecular rotors are responsive to viscosity variation. However, there is still room for development more sensitive viscosity fluorescent probe. Recently, Tang group developed a series of dyes displaying aggregation-induced emission (AIE) characters. [19-21] Mechanistic studies show that AIE phenomenon is mainly due to restriction of intramolecular motions. According to this characteristic, fluorescence viscosity probe based on AIE fluorophore was reported. [22] Compared with traditional molecular rotors, viscosity-sensitive AIE dyes contain more rotatable parts and is more sensitive to viscosity changes in microenvironment. However, it is still urgent to develop a method for improving the sensitivity of detection viscosity changes with near-infrared emission. Taking advantage of these viscosity-dependent characteristics, we envisaged that connection of the traditional molecular rotors with AIE dyes can result in probes ultrasensitive to environmental viscosity with near-infrared emission, which, however, has not yet been reported.

Herein, we report a unique near-infrared (NIR) emission mitochondrial viscosity fluorescent probe (**TPE-V**) which contains multi-rotatable moieties for the first time. Interestingly, **TPE-V** exhibited ultrasensitive response to viscosity (<15 cP) and the fluorescence intensity at 650 nm increased about 100-fold in 99 vol% glycerol. Moreover, **TPE-V** showed a large Stokes' shift (190 nm) which can avoid self-absorption effect. Importantly, **TPE-V** was successfully targeted in mitochondria and employed for monitoring viscosity variations under characteristic pathological conditions.

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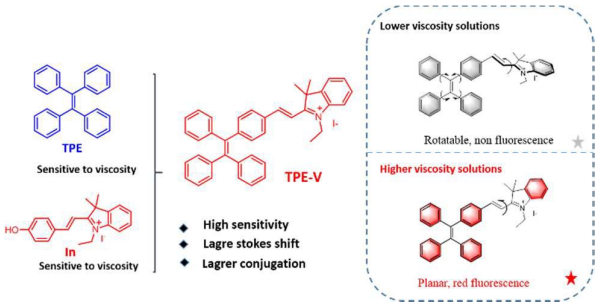

Scheme 1. Design of near-infrared emission fluorescence viscosity probe TPE-V.

TPE contains more rotatable parts and may more sensitive to viscosity changes. The molecular rotors can be freely rotated in low viscosity solutions, while the rotation is suppressed in high viscosity solutions. As shown in Fig. S1, both TPE moiety and an indole salt moiety can response to viscosity. According to these features, we designed a viscosity fluorescence probe **TPE-V** (Scheme 1), which contained a TPE moiety and an indole salt moiety. We anticipated that the probe has no fluorescence under low viscosity solutions due to the free rotation of TPE moiety and an indole salt moiety, while in high viscosity solutions, the rotation is suppressed and the fluorescence intensity would be dramatically enhanced. In addition, the probe can localize in mitochondria due to the indole cation. The synthesis steps and characterization data were shown in supporting information.

To verify whether **TPE-V** is responsive to viscosity as designed, we first investigate the optical property of **TEP-V** in water and viscosity solutions. As shown in Fig. S2a, **TPE-V** exhibited the maximum absorption at 440 nm in water solution, while in 99 % V/V glycerol solution, the maximum absorption shifted to 465 nm, which may be due to the inhibition of rotation in viscosity solution and enlarged the degree of conjugation. As expected, **TPE-V** displayed almost no fluorescence under excited at 460 nm in water solution. However, in 99 % V/V glycerol solution, the fluorescence intensity enhanced about 100-fold at 650 nm (Fig. S2b), which far higher than the reported viscosity fluorescence probes (Table S1). Moreover, the fluorescence intensity of **TPE-V** at 650 nm was enhanced with the increase of viscosity from 1.4 cP to 950 cP (Fig. 1a) and the obvious fluorescence changes could be observed by the naked eye (inset of Fig. 1a). As shown in Fig. 1b, there is a good linear relationship between log I_{650} and log η (R^2=0.99) by fitting the Förster–Hoffmann equation [23] and the value of x (as high as 0.67) presented the possibility for the monitoring the changes of viscosity *in vitro*. Notably, the emission intensity of **TPE-V** was increased about 10-fold in viscosity solutions from 1.4 cP to 15 cP (Fig. S3). These results exhibit that **TPE-V** is highly sensitive to detect of viscosity.

Fluorescence lifetime is another way to validate the sensitivity of the probe for viscosity detection and it is not effected by the concentration, absorption, and emission intensity of the probe. [24] Therefore, we measured the sensitivity of probe in different water-glycerol system by fluorescence lifetime. As shown in Fig. 1c, the fluorescence lifetime increased with the increment of viscosity (1.4 -15 cP) and exhibited a good linear relationship between log τ and log η (R^2= 0.99148, x =0.21618) according to the Förster–Hoffmann equation (Fig. 1d). The observed changes of fluorescence lifetime is accordant with the restricted rotation of TPE and indole salt groups in the system of high viscosity. The non-radiative decay rate decreases with increasing viscosity, and thus the fluorescence lifetime of **TPE-V** is prolonged with the increase of viscosity. All these results indicate that **TPE-V** can serve as an excellent fluorescent probe for monitoring the viscosity changes.

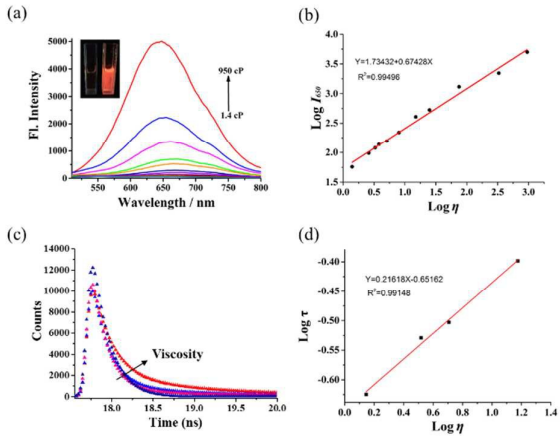

Fig. 1. (a) The fluorescence spectra of 10 μM **TPE-V** in variation ratios of water-glycerol system. λ_{ex} = 460 nm. (b) The linear relationship between log I_{650} and log η, R^2 = 0.99496, x =0.67428. (c) Fluorescence lifetime spectra of **TPE-V** (10 μM) in different water–glycerol system, λ_{ex} = 460 nm, λ_{em} = 650 nm. The viscosities were 1.4 cP-15 cP, respectively. (d) The linear relationship between log τ and log η, R^2= 0.99148, x =0.21618.

Generally polarity is an important influence factor for molecular rotors to detect viscosity, [25] therefore, the fluorescence intensity of **TPE-V** in some solvents with different polarity were conducted. As shown in Fig. S4, the probe **TPE-V** did not show significant fluorescence changes at 650 nm in different polar solutions compared with that in 99 % V/V glycerol. In addition, the quantum yields of **TPE-V** in other low-viscosity solvents are very low (almost about 0.025). However, in 99 % V/V glycerol, the quantum yield is about 0.286 (Table S2). These results demonstrate that environmental polarity brings ignorable interferences to the viscosity detection.

To examine the specificity of **TPE-V**, the fluorescence spectra of **TPE-V** were conducted coexisting with some possible competitive species including 500 μM S^{2-}, HSO_3^-, GSH, Cys, Hcy, (tert-butyl hydroperoxide (TBHP), ditert-butyl peroxide (DTBP), Mg^{2+}, Ca^{2+}, Na^+, Zn^{2+}, K^+, Fe^{2+} and 100 μM H_2O_2, ClO^- and BSA. As shown in Fig. 2, the fluorescence intensity of **TPE-V** had no obvious changes after treated with other bioactive molecules. Only viscosity solution can triggered an obviously fluorescence enhancement at 650 nm. Moreover, the probe exhibited aggregation caused quenching (ACQ) effect in physiological environment (Fig. S5), which avoided the interference from the aggregation-induced emission. These results suggest that the probe **TPE-V** has an excellent specificity to viscosity. In addition, the fluorescence intensity of **TPE-V** under various pH values at 650 nm had no significant

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changes (Fig. S6). These results indicate that **TPE-V** has potential application in complex biological systems.

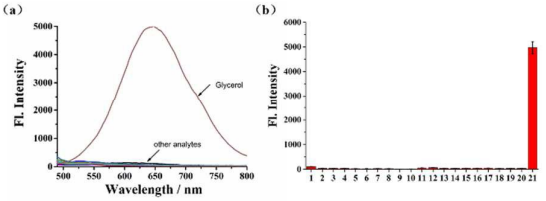

Fig. 2. Selectivity of **TPE-V** to viscosity. (a) Fluorescence spectra change of **TPE-V** in 99% V/V glycerol solution and other representative bioactive molecules in PBS buffer (10 mM, pH=7.4). The probe was treated with these molecules for 30 min. (b) Fluorescence intensity of **TPE-V** at 650 nm after 30 min incubation with representative bioactive molecules in PBS buffer. 1. BSA, 2. $CaCl_2$, 3. $MgCl_2$, 4, $CuSO_4$, 5. Cys, 6.GSH, 7. H_2O_2, 8. HClO, 9. Na_2S, 10. $NaHSO_3$, 11. $FeSO_4$, 12. $ZnCl_2$, 13. KI, 14. Hcy, 15. DTBP, 16. NaOAc, 17. NaF, 18. TBHP, 19. NaCl, 20. Only probe **TPE-V**, 21. **TPE-V** in 99% V/V glycerol solution. λ_{ex} = 460 nm.

Encouraged by the above desirable attributes of **TPE-V**, we further evaluated the capability of **TPE-V** to monitor the viscosity changes in living cells. Initially, the toxicity of **TPE-V** to living cells were examined by MTT assays. [26] As shown in Fig. S7, the survival rate of HeLa and NHA cells were more than 80%, demonstrating that **TPE-V** has almost no toxicity to HeLa and NHA cells and can be used for cell imaging.

Considering the overall positive charge of **TPE-V**, we reasoned that the probe may be accumulated in the mitochondria. Therefore, the intracellular distribution of **TPE-V** was further studied by colocalization experiment (Fig. 3). HeLa cells were cultured with **TPE-V** (10 μM) for 30 min, and then cultured with 500 nM Mito-Tracker Green (MTG, the commercial mitochondrial dye) for another 10 min. The imaging results indicated that the green channel of MTG and red channel of **TPE-V** can overlap well. The Pearson's correlation coefficients were calculated as high as 0.95. Moreover, the variations of intensity profiles in green and red channel exhibited a tendency toward synchrony. These results suggest that **TPE-V** has good membrane permeability and primarily accumulated in mitochondrial.

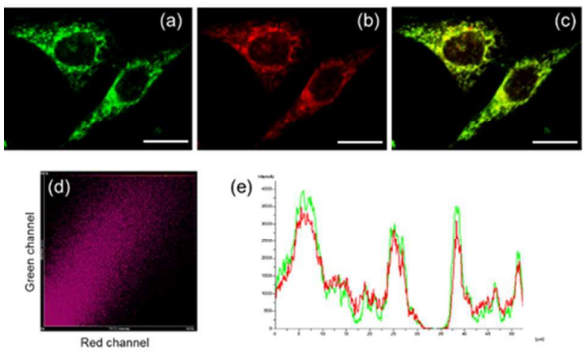

Fig. 3. The colocalization fluorescence imaging of HeLa cells co-treated with (a) Mito-Tracker Green (500 nM) and (b) **TPE-V** (10 μM) under excitation at 488 nm. (c) The merged image of (a) and (b). (d) The intensity scatter plot of green channel and red channel. (e) Intensity profile of two channels. Green channel: λ_{em} = 500–550 nm. Red channel: λ_{em} = 570–620 nm nm. Scale bar: 20 μm.

Dysfunction of mitochondrial may directly lead to several human diseases accompanied by an increase of mitochondrial matrix viscosity. [27] To verify whether **TPE-V** could detect mitochondrial viscosity variations in living cells by fluorescence imaging, monensin (Mo) and nystatin (Ny) were used to change mitochondrial viscosity. [28,29] The fluorescence imaging of **TPE-V** monitoring the mitochondrial viscosity changes are shown in Fig. 4. When HeLa cells were treated with 10 μM **TPE-V**, fluorescence intensity could be observed in red channel and mitochondrial morphology exhibited tubule-like. While the Hela cells were pre-incubated with 10 μM Mo or 10 μM Ny for 40 min, and then cultured with **TPE-V** for another 30 min, an obvious fluorescence enhancement was obtained. Importantly, obvious changes of mitochondria morphology from tubule form to globular-like could be observed, which probably due to the structural changes of mitochondria stimulated by Mo and Ny. Moreover, the similar results were also observed in the imaging experiments in NHA cells (Fig. S6). Differently, under the same experimental conditions, the fluorescence intensity in HeLa cells is obviously stronger than that in NHA, which illustrates that the mitochondrial viscosity in HeLa cells is higher than in NHA cells. These results suggest that **TPE-V** can detect the viscosity changes in mitochondrial of living cells sensitively.

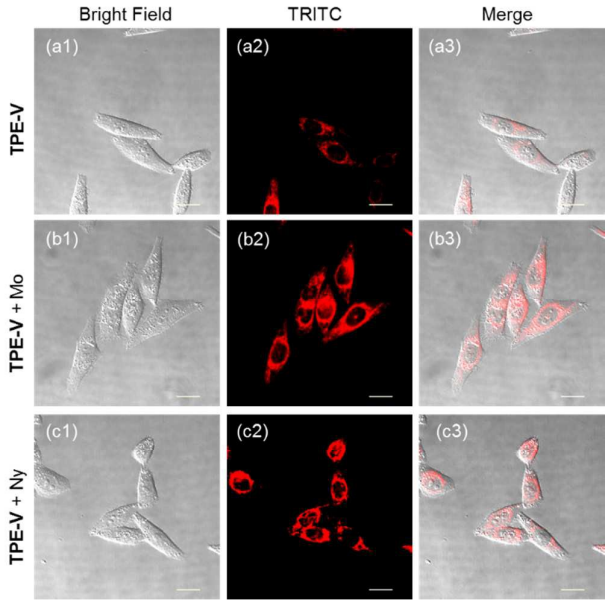

Fig. 4. The fluorescence imaging of HeLa cells. (a1-a3) Images of HeLa cells stained with 10 μM **TPE-V** for 30 min and then treated with 0.4 % trypan blue for anothr 5 min. (b1-b3) HeLa cells exposed to monensin (10 μM), and then incubated with **TPE-V** (10 μM) and trypan blue (0.4 %). (c1-c3) Cells pre-treated with nystatin (10 μM), and then treated with **TPE-V** (10 μM) and trypan blue (0.4 %). (a1-c1) Bright-field images of HeLa cells. (a2-c2) Fluorescence images of HeLa cells in red channel. (a3-c3) the overlay of bright-field and red channel. λ_{ex} = 488 nm, λ_{em} = 570–620 nm. Scale bar: 20 μm.

As reported that inflammation can stimulate the increase of viscosity. [30] Lipopolysaccharide (LPS), a cell wall ingredient of gram-negative bacteria, has been verified to trigger cells to produce inflammation. [31] Therefore, LPS induced cells is a well-established model for inflammation study. [32] To further study the feasibility of **TPE-V** to detect viscosity changes in inflammation cell models, LPS was used to establish inflammatory model in NHA and HeLa cells (Fig. 5). When treated with **TPE-V** (10 μM), NHA cells displayed almost no fluorescence. However, NHA pre-treated with LPS (20 μM), and then incubated with **TPE-V** showed marked fluorescence enhancement. The similar results can also be demonstrated in

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HeLa cells. We further recorded the fluorescence lifetime images of **TPE-V** with or without the LPS stimulations in NHA and HeLa cells (Figure 6). After the LPS treatment, observable much longer fluorescence lifetime were observed in NHA and HeLa cells. In this process, mitochondrial viscosity of NHA cells increased from about 50 to 600 cp and HeLa cells increased from about 70 to 600 cp. These studies reveal that **TPE-V** is suitable for detecting viscosity in cell inflammatory model.

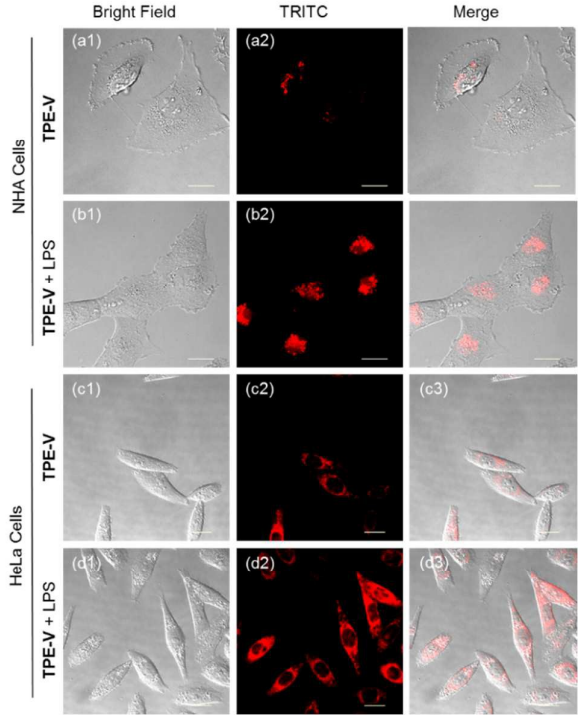

Fig. 5. The fluorescence imaging of NHA and HeLa cells. (a1-a3) Images of NHA cells stained with 10 μM **TPE-V** for 30 min and then treated with 0.4 % trypan blue for 5 min. (b1-b3) NHA cells exposed to LPS (20 μM), and then incubated with **TPE-V** (10 μM) and trypan blue (0.4 %). (c1-c3) Images of HeLa cells stained with 10 μM **TPE-V** for 30 min and then treated with 0.4 % trypan blue for 5 min. (d1-d3) HeLa cells pretreated with LPS, and then treated with **TPE-V** and trypan blue (0.4 %). (a1-d1) Bright-field images of NHA and HeLa cells. (a2-d2) Fluorescence images of NHA and HeLa cells in red channel. (a3-d3) the overlay of bright-field and red channel. λ_{ex} = 488 nm, λ_{em} = 570–620 nm nm. Scale bar: 20 μm.

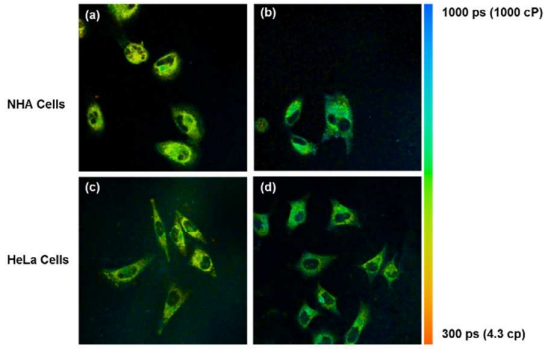

Figure 6. The fluorescence lifetime imaging of **TPE-V** in HeLa and NHA cells. (a) Images of NHA cells stained with 10 μM **TPE-V** for 30 min. (b) NHA cells exposed to 20 μM LPS for 40 min, and then incubated with 10 μM **TPE-V** for another 30 min. (c) Images of HeLa cells stained with 10 μM **TPE-V** for 30 min. (d) HeLa cells pretreated with 20 μM LPS for 40 min, and then treated with 10 μM **TPE-V** for another 30 min.

In conclusion, we have designed a highly sensitive viscosity fluorescent probe with near-infrared emission by using TPE moiety and an indole salt moiety as rotational groups. **TPE-V** exhibited a large "off-on" fluorescence response (about 100-fold enhancement) from water to 99 %V/V glycerol with a large stokes shift (about 190 nm). Moreover, **TPE-V** displayed an excellent sensitivity (<15 cP) and specificity to viscosity. More importantly, **TPE-V** successfully realized the detection of mitochondria viscosity changes and vividly showed the influence of LPS on mitochondria viscosity. We expect that the unique viscosity probe **TPE-V** will be further applied to disease research as a powerful tool. Furthermore, the design strategy may be extended for various functional fluorescent probe with extraordinary optical properties.

Conflicts of interest

The authors declare no competing financial interest.

ACKNOWLEDGMENT

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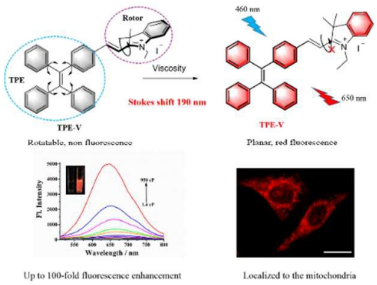

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