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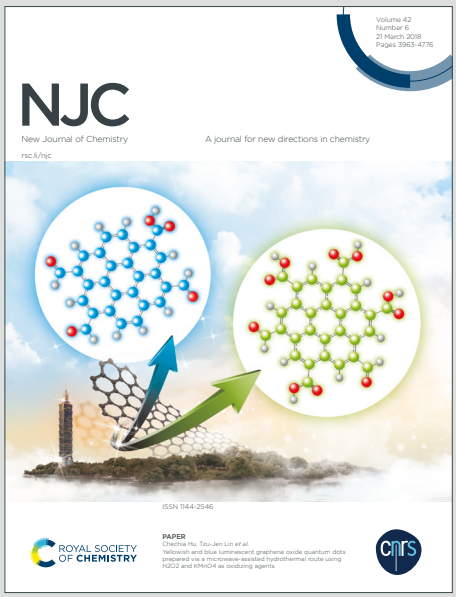

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A novel carbazolyl GFP chromophore analogue: Synthesis strategy and acidic pH-activatable lysosomal probe for tracing endogenous viscosity changes

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Abstract

Intracellular viscosity changes of the internal microenvironment will lead to many diseases, including cancer, inflammation, neurodegenerative diseases. A novel carbazolyl GFP chromophore analogue Lys-CzFP with acidic pH-activatable was designed for tracing lysosomal viscosity changes. The synthesis developed is a new efficient, novel, one-pot procedure. Lys-CzFP, as a fluorescent molecular rotor, the internal carbazole and benzazole moieties of Lys-CzFP are served as rotators, which could rotate around the single C—C bond in the π-conjugated bridge. Lys-CzFP had a long emission wavelength at 560 nm and a large Stokes shift of 78 nm. The fluorescence intensity of Lys-CzFP exhibited a significant enhancement with increasing viscosity. The fluorescence intensity increased by 98-fold within 5 s from 1.0 cP to 1412 cP, with the fluorescence quantum yield (ϕ_F) increasing from 0.003 to 0.253 as well as the fluorescence lifetime increasing from 0.25 ns to 1.12 ns, respectively. Furthermore, interference experiments indicated that Lys-CzFP could respond to viscosity particularly. Besides, Lys-CzFP had excellent biocompatibility, low cytotoxicity, and lysosomal localization. Together, Lys-CzFP was successfully applied for tracing endogenous viscosity changes in living cells.

Keywords: Fluorescent protein; Viscosity probe; Excellent biocompatibility; Lysosome labelling; Living cell imaging

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1 . Introduction

Abnormal changes of intracellular viscosity are closely related to various diseases of humans,[1, 2] such as Alzheimer's disease(AD),[3] diabetes,[4] and amyloid beta-peptide.[5] Traditional mechanical viscometers cannot measure the viscosity changes of cells or organelles in real-time.[6] In contrast, the fluorescence probe plays a vital role in tracing viscosity changes in living cells.[7-10] Therefore, the development of fluorescence platform for tracing viscosity in cells or subcellular levels is crucial.[11-14] Lysosomes play a critical role in maintaining intracellular secretion, digestion organelles, and plasma membrane repair.[15] Viscosity changes in lysosomes can bring about the dysfunction of lysosomes.[16] Tons of evidence have shown that lysosomes are the pathogenesis of diseases such as cancer, storage disorders, neurodegenerative disorders.[17, 18] Therefore, the development of probes that accurately trace viscosity in living cells is in urgent demand.

Green Fluorescent Protein (GFP) has been applied as a bio-optical marker for tracing numerous analytes.[19-22] Liu et al. reported a series of GFP molecule derivatives detected protein conformational collapse and demonstrated that they could fluoresce in protein aggregates.[23] Li et al. reported a viscosity probe of GFP derivative, the probe has two C=C double bonds between phenol and imidazolidinone, which showed a maximum of about 10-fold fluorescence response and successful traced lysosomal viscosity changes in living cells. However, the probe was not sensitive to pH and lacked the characteristic of lysosomal-activated fluorescence. The background fluorescence interference could not be ignored under physiological conditions, and the fluorescence intensity did not change significantly.[24] Besides, there are various classical fluorophores, including Pyrrolic,[25] Carbazole,[26] BODIPY,[27, 28] Cyanine,[29] Naphthalene,[30] and the like (Table S1 and Scheme 1a), [31-36] they also serve as molecular rotor fluorophores applied in viscosity probe field. Whereas the

development of viscosity probe with highly sensitive, fast response, low cytotoxicity, and large stokes shift as well as the targeted organelle-specific release is a prerequisite. Compared to conventional fluorophores, the GFP chromophore is extracted from the organism, which has natural biocompatibility.[37] Its fluorescence is extremely weak in a free state that attributed to the rotation of intra-molecule, which lead to a quick non-radiative transition and deactivation from the fluorescent state, short fluorescence lifetime, and vice versa.[38]

In the exploitation of the viscosity probe, sensitivity, response time, and background luminescent noise signal that might influence the reliability of viscosity tracing. Therefore, a novel GFP chromophore analogue Lys-CzFP with acidic pH-activatable was first designed for tracing lysosomal viscosity in living cells (Scheme 1b). Here, the carbazolyl GFP chromophore analogue was synthesized firstly. Further, indole moiety was introduced via Knoevenagel condensation. Thus, Lys-CzFP has a larger molecular plane and viscosity sensitivity than two-sided phenols. Morpholine moiety was attached to the GFP structure to localize the lysosomes.[39, 40] Besides, the electron-rich morpholine moiety weakened fluorescence of the GFP fluorophore via photoinduced electron transfer (PET). However, after diffused into the lysosomal microenvironment (pH 3.8−6.6), morpholine moiety can be protonated for the moderate alkalinity (pKa 5−6), the morpholine moiety will be converted to be electron-withdrawing. After that, the PET process will be cut off, and the fluorescence released,[41] which endued Lys-CzFP with an acidic pH-activatable lysosome-targeting fluorescence characteristic that eliminated the background fluorescence outside of lysosomes,[42] allowed a low background luminescent noise signal to be obtained. After obtaining the Lys-CzFP, its structure was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS (Fig. S3–S5).

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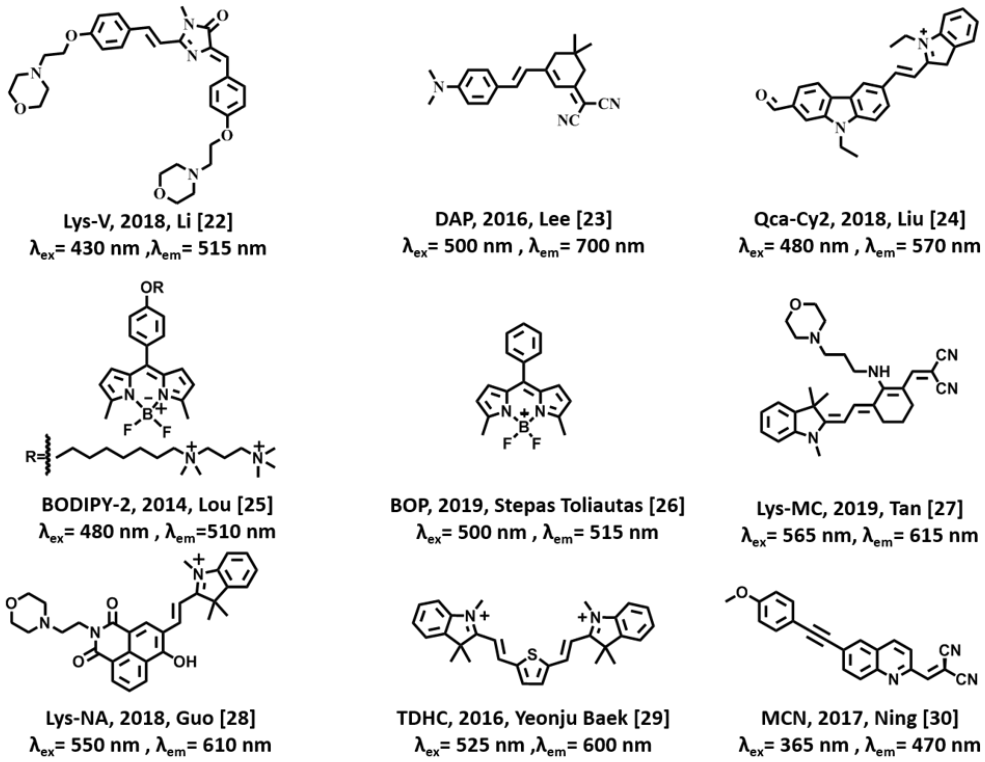
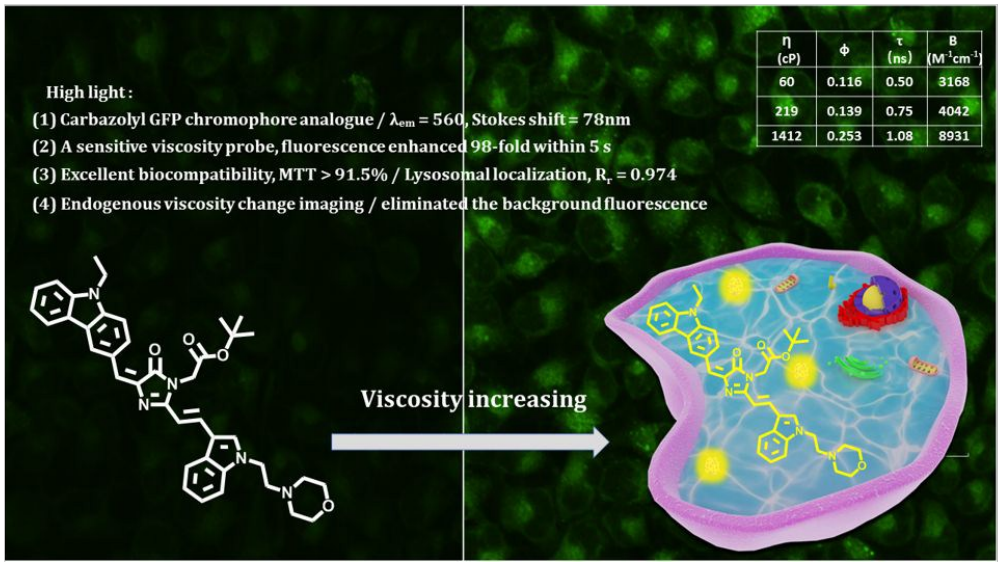
(a) Previous work



(b) This work



Scheme 1 （a）Previous work for tracing viscosity.（b）Carbazolyl GFP chromophore analogue was modulated to serve as a fluorescence probe for tracing lysosomal viscosity changes.

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2. Experimental

2.1 Materials and methods

Reagents. All chemicals are commercially available, and the solvents used in the reactions were the anhydrous solution. The reaction was monitored by TLC plate using Silicycle® glass sheets precoated with silica gel 60 Å with detection by UV-absorption (254 nm or 365 nm). Flash column chromatography was performed using Silica Flash® F60 silica gel in the indicated solvent mixture. Stock solutions of the probe in DMSO and of F^-, HCO_3^-, CO_3^{2-}, I^-, SO_3^{2-}, S^{2-}, Cl^-, HSO_3^-, ClO^-, Ca^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Glucose, H_2O_2, H_2S, Cys, Aln, Ala, Asp, Pro, Tyr, Lys, GSH, Arg, Hcy, Phe, Thr, Gln, Val, Gly in distilled water were prepared. All amino acids and ions were purchased from Macklin Biochemical Co., Ltd, Tokyo Chemical industry Co., Ltd and Sinopharm Chemical Reagent Co., Ltd.

Apparatus. ^1H NMR and ^{13}C NMR spectra were obtained on a Bruker NMR (600 /150 MHz) and (300/75 MHz) spectrometer in the given solvents. Chemical shifts are reported as δ-values in ppm relative to the $CDCl_3$, DMSO-d_6 residual solvent peak or tetramethylsilane (TMS) as an internal standard. Coupling constants are provided in Hz. All given ^{13}C spectra are proton-decoupled. High-resolution mass spectra were recorded with a Waters Q-TOF Premier quadrupole/time-off flight (TOF) mass spectrometer. Fluorescent images were observed using confocal laser scanning microscopy (FLUOVIEW FV3000. OLYMPUS).

General procedure for viscosity determination and spectral measurements

The solvents were acquired by mixing water-glycerol systems in different proportions. Viscosity values of solutions were obtained according to Table S2. The stock solution of Lys-CzFP (2 mM) was prepared in DMSO. The test solutions contained Lys-CzFP (10 μM), water-glycerol systems in different proportions. These solutions were sonicated for 10 min to remove air bubbles. After standing at room temperature for 1 h at 20 °C, then measured in a UV

spectrophotometer and a fluorescence spectrophotometer.

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Fluorescence lifetime measurements[43]

The solutions of Lys-CzFP (10 μM) in different viscosity were prepared in the solvent mixture (water–glycerol). These solutions were sonicated for 10 min to eliminate air bubbles. After standing for 1 h at 20 °C, the solutions were measured in a fluorescence lifetime measuring equipment with the excitation wavelength at 460 nm.

Cell culture.

MTT cytotoxicity assay

Cytotoxicity of the probe Lys-CzFP was carried out by a standard MTT assay. The logarithmic phase of Bel-7402 cells was seeded into a 96-well cell culture plate (1×10^4 cells per well) and incubated under 95% air and 5% CO_2 at 37°C for 24 h. Next, the cells were incubated with different concentrations (0 mM, 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM) of the probe Lys-CzFP for another 24 h. Then, each cytotoxicity experiment was repeated three times by the standard method.

Co-location experiment[44]

Bel-7402 and Hela cells line were used in this work from the American type culture collection (ATCC). Cells were incubated using the standard method and incubated in 95% air and 5% CO_2 at 37°C. Then, the cells were split into a 35 mm diameter confocal dishes, the density of cells should keep around 3×10^5 cells in each dish, then incubated in 1640 medium for 24 h under standard culture conditions. The probe Lys-CzFP (10 μM) and Lyso-Tracker Red (10 μM) were added and incubated for 30 min under standard culture conditions. After that, the imaging sample was washed with PBS three times to remove the free probe. Then, the cells were imaged using confocal laser scanning microscopy. (FLUOVIEW FV3000. OLYMPUS)

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Tracking viscosity changes experiment

Bel-7402 cells seeded in a 35 mm petri dish with a glass cover slide. After culturing overnight, a specific concentration of the Lys-CzFP (10 μM) solution prepared in DMSO was added to the above petri dish. And it was incubated with the cells for 30 minutes under standard culture conditions. Then, dexamethasone (20 μM) was added and incubated for 60 min. Before imaging, the cells were washed three times with PBS solution (pH 7.4) to remove the free probe. Recording real-time living cells images every 20 min under confocal laser scanning microscopy. (FLUOVIEW FV3000. OLYMPUS)

2.2 Synthesis

Synthesis of Compound M_1:

Glycine tert-butyl ester hydrochloride (1500 mg, 8.95 mmol) and NaOH (1500 mg, 8.40 mmol) were added to 2-methylpropan-2-ol (30 mL), which was stirred at room temperature for 1 h. Then, N-ethyl-3-carbazolecarboxaldehyde (1000 mg, 4.48 mmol) was added and stirred overnight. (E)-2-((1-ethoxyethylidene)amino)acetate (1800 mg, 8.97 mmol) was prepared and added. The mixture was stirred at room temperature for 36 h and then the reaction was quenched by water. The mixture was diluted with DCM and washed with water three times. The organic layer was collected and evaporated. The crude product was further purified by silica gel column to get yellow solid. Yield: 91.3%.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.37 (d, J = 8.6 Hz, 1H), 8.17 (d, J = 7.6 Hz, 1H), 7.49 (t, J = 7.6 Hz, 1H), 7.42 (dd, J = 8.3, 2.6 Hz, 2H), 7.37 (s, 1H), 7.28 (t, J = 7.4 Hz, 1H), 4.37 (q, J = 7.3 Hz, 2H), 4.31 (s, 2H), 2.38 (s, 3H), 1.49 (s, 9H), 1.45 (t, J = 7.3 Hz, 3H).

Synthesis of Compound M_2:

N-(2-chloroethyl) morpholine hydrochloride (3000 mg, 16.21 mmol), Indole-3-

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carboxaldehyde (1180 mg, 8.11 mmol) and potassium carbonate (2240 mg, 16.21 mmol) was added to DMF (50 mL). The mixture was stirred at 50°C for 48 h. After reaction, the mixture was extracted with DCM and the organic layer was washed with water three times. The DCM was evaporated and the crude product was further purified by silica gel column to afford yellow oil. Yield: 95.4%.

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.91 (s, 1H), 8.32 (s, 1H), 8.11 (d, J = 7.8 Hz, 1H), 7.64 (d, J = 8.2 Hz, 1H), 7.32 – 7.29 (m, 1H), 7.26 – 7.24 (m, 1H), 4.39 (t, J = 6.3 Hz, 2H), 3.53 (t, J = 6.3 Hz, 4H), 2.70 (t, J = 6.3 Hz, 2H), 2.43 (s, 4H).

Synthesis of Compound Lys-CzFP:

Compound M_1 (110 mg, 0.26 mmol) and compound M_2 (56.71 mg, 0.22 mmol) were dissolved in benzene. Acetic acid (30 μL) and piperidine (50 μL) were added and the mixture was refluxed overnight at 110°C. After reaction, The organic layer was washed with water (150 mL × 3), dried over anhydrous $MgSO_4$ and filtered. The benzene was evaporated and the crude product was further purified by silica gel column to get red solid. Yield: 78.2%.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.08 (s, 1H), 8.59 (d, J = 8.6 Hz, 1H), 8.36 (d, J = 15.6 Hz, 1H), 8.26 (d, J = 7.7 Hz, 1H), 8.14 (s, 1H), 8.07 (d, J = 7.7 Hz, 1H), 7.71 (d, J = 8.8 Hz, 1H), 7.64 (t, J = 7.1 Hz, 2H), 7.51 (t, J = 7.9 Hz, 1H), 7.35 – 7.23 (m, 3H), 7.19 (s, 1H), 6.79 (d, J = 15.6 Hz, 1H), 4.69 (s, 2H), 4.54 – 4.47 (m, 2H), 4.36 (s, 2H), 3.56 (s, 4H), 2.72 (s, 2H), 2.45 (s, 4H), 1.43 (s, 9H), 1.35 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.77, 167.46, 159.01, 140.32, 140.08, 137.44, 136.95, 135.24, 134.11, 130.15, 126.24, 125.96, 125.29, 124.89, 124.78, 122.74, 122.63, 122.32, 121.04, 120.53, 120.41, 119.56, 112.55, 110.94, 109.52, 106.76, 81.96, 66.16, 57.35, 53.23, 43.14, 41.79, 37.19, 27.59, 13.69. HRMS: $[M+H]^+$ Calcd: 658.3388, Obsd: 658.3385.

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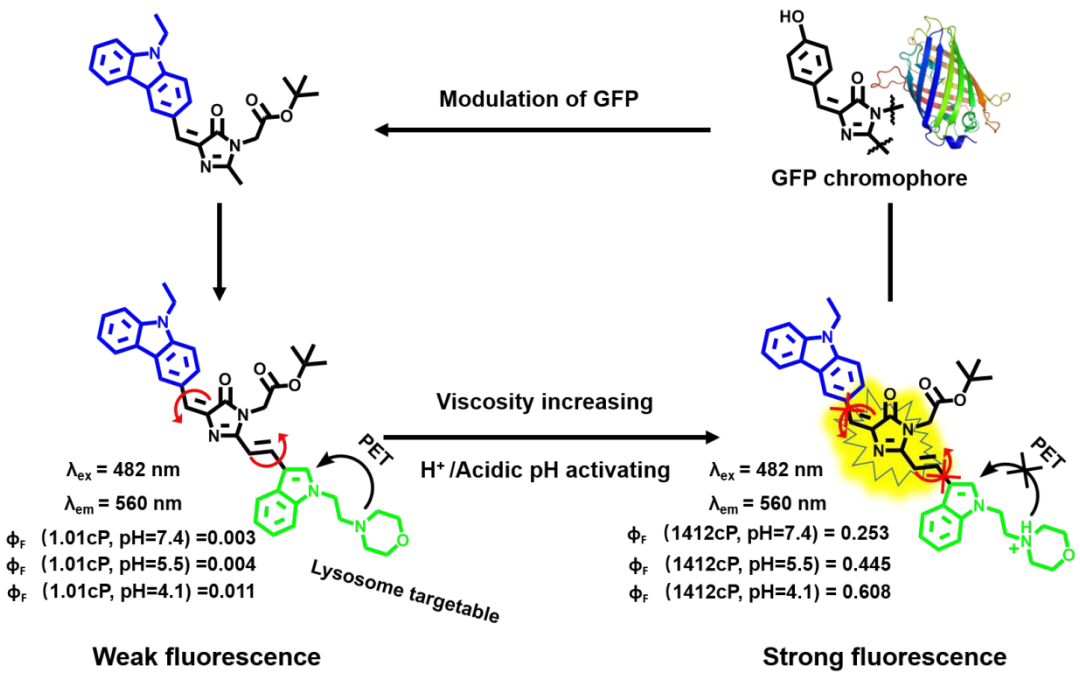
3. Results and discussion

3.1 Design of carbazolyl GFP chromophore analogue



Scheme 2. Design of acidic pH-activatable lysosomal probe Lys-CzFP.

Lys-CzFP, as a fluorescent molecular rotor, the fluorescence of the probe in aqueous solutions was extremely weak that could be attributed to the free rotations of through the single C—C bonds in the π-conjugated bridge.[45] In contrast, these rotations could be inhibited under increasing viscosity solutions, its fluorescence released with the ϕ_F and lifetime increased (Scheme 2), which was very rare compared to most known lysosomal probes (Table. S1). Besides, the low cytotoxicity of the GFP chromophore was a promising performance for applications in living cells. Finally, Pearson's correlation of lysosomal co-labeling was 0.974 that provided specifically lysosomal localization used for tracing viscosity changes in living cells.

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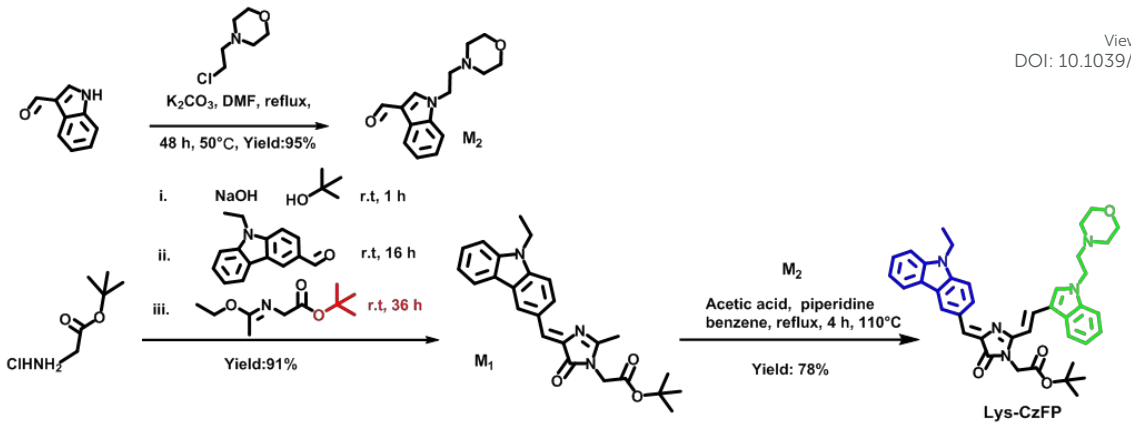

Scheme 3. Synthetic route to afford the Lys-CzFP with high viscosity response.

The existed literature for synthesizing GFP chromophore, and its analogue existed by-products that were difficult in the separation.[46-49] In this work, carbazolyl GFP chromophore analogue was first reported by the following method; the imidate was replaced with tert-butyl (E)-2-((1-ethoxyethylidene)amino)acetate; alcohol was replaced with 2-methylpropan-2-ol, resulting in yield improved remarkably (Scheme 3), which may ascribe the hydrolysis of t-butoxy moiety was inhibited. It will be of great significance for the development of numerous GFP chromophore and its analogue.

3.2 Sensitive responses of Lys-CzFP to viscosity within 5 s

As exhibited in Fig. 1 and Table 1, the maximum absorption wavelength of Lys-CzFP was located at 482 nm, and the corresponding emission peak was located at 560 nm with a large stokes shift of 78 nm (10 μM, pH = 7.4, 25 °C). The molar extinction coefficient of absorption peaks at 482 nm was 2.2×10^4 $cm^{-1}M^{-1}$. Besides, the fluorescence quantum yield of Probe Lys-CzFP was measured. Fluorescence quantum yield of Lys-CzFP was calculated to be 0.253 in glycerol relative to fluorescein in 0.1 M NaOH (ϕ_F = 0.95 in EtOH).

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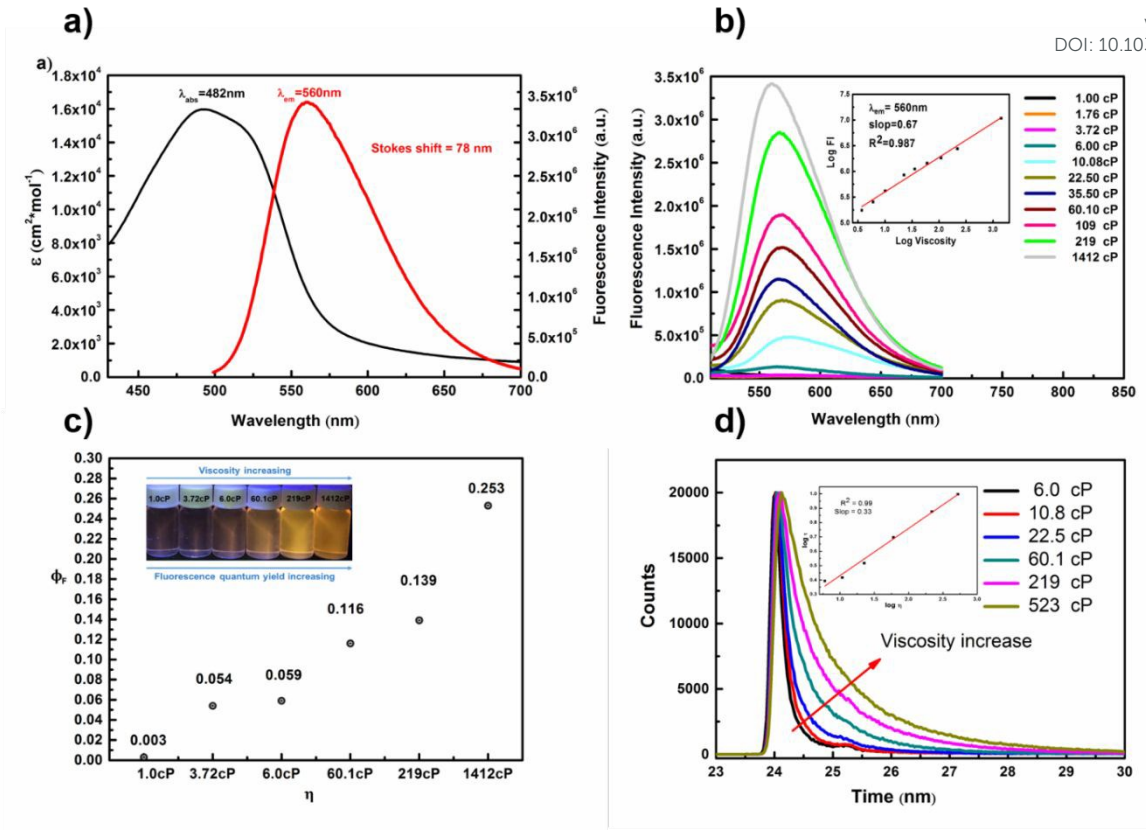

Fig. 1 (a) Molar Extinction Coefficient (ε) and fluorescence spectra of Lys-CzFP (10 μM) in glycerol. (pH 7.4, 20 °C). (b) Fluorescence spectra of Lys-CzFP (10 μM) in glycerol-water mixed solvents (1.00-1412 cP, pH 7.4, 20 °C). Inset line: Linear relationship of fluorescence intensity (560 nm) with viscosity values. (c) Fluorescence quantum yield (ϕ_F) of Lys-CzFP (10 μM) under different viscosity solutions (η). (d) Fluorescence lifetime spectra of Lys-CzFP (10 μM) under different viscosity solutions. Inset line: Linear relationship of fluorescence lifetime with viscosity values.

Fluorescence responses of Lys-CzFP were investigated under different viscosity solutions. As glycerol was gradually added to the solutions mixture, the water and glycerol viscosity gradients increased from 1.00cP to 1412cP; consequently, the fluorescence of Lys-CzFP maximally increased by about 98 - fold at 560 nm (Fig. 1a and Fig. 1b). Moreover, the fluorescence of probe exhibited an excellent linear relationship ($R^2 = 0.987$, slope = 0.67) by fitting the Förrster–Hoffmann equation. This may be ascribed to the fact that the internal rotation of through the single C—C bonds in the π-conjugated bridge was inhibited in the

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viscous environment, resulting in an excited state relaxation of the fluorescent emission. Sequentially, a strong fluorescence emission occurred. The quantum yield of the probe Lys-CzFP was further measured in different solvents. The quantum yield was relatively low (ϕ_F = 0.003) in water. Correspondingly, as the viscosity gradients increasing, the fluorescence quantum yield reached 0.253 maximally (pH 7.4, 20 °C), allowing for observation by the naked eye (Fig. 1c). These results indicated that Lys-CzFP was sensitive to the viscosity changes of the solvents and could be used for tracing viscosity changs.

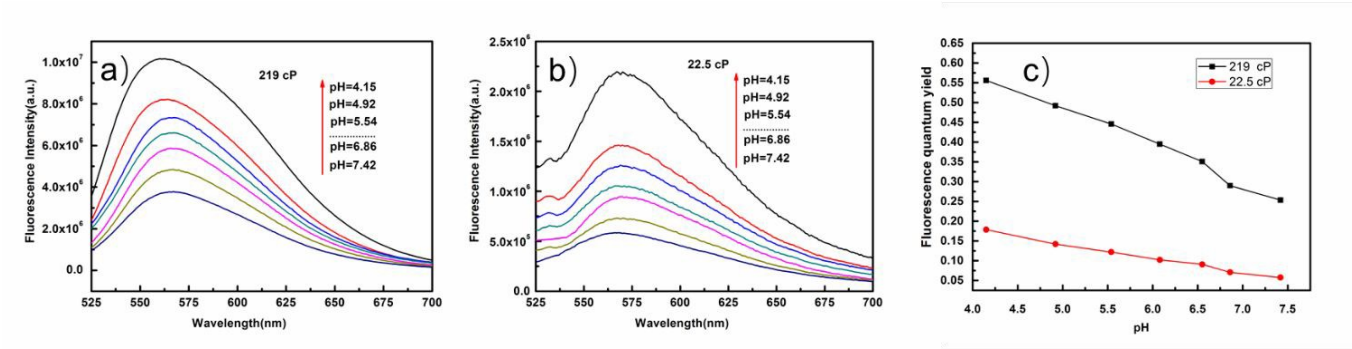

Fig. 2 (a, b) Fluorescence spectra of Lys-CzFP (10 μM) at different pH in ethanol/water/glycerol mixtures at two viscosities: (a) 219 cP and (b) 22.5 cP. (c) Fluorescence quantum yields of Lys-CzFP (10 μM) at different pH.

The fluorescence intensity of Lys-CzFP was very sensitive to both viscosity and pH (Fig 2). For example, a particular viscosity (e.g., 219 cP or 22.5 cP), pH from 7.42 to 4.15; at a viscosity of 22.5 cP, the fluorescence quantum yield (ϕ_F) at pH 4.15 was 0.179, which is almost 18 times that at pH 7.42 (ϕ_F = 0.057). Also, at a fixed pH, the fluorescence intensity increased with increasing viscosity. For instance, at a viscosity of 219 cP , ϕ_F was 0.556, at pH = 4.15, which was 3.1 times than 22.5 cP (ϕ_F = 0.179). Notably, Lys-CzFP's fluorescence was very weak for pH > 6.86, and negligible at lysosomal microenvironment (pH 3.8−6.6, 47−190 cP) (Fig. S10 and S11), which ascribe the effect of PET, the molecule accepted electron from the neutral morpholine, fluorescence was very weak. Whereas in the lysosomal microenvironment (pH 3.8−6.6), it

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released a strong fluorescence. Meanwhile, the background fluorescence was eliminated outside of lysosomes, and that was very conducive to trace the lysosomal locations (pH 3.8−6.6) using confocal microscopy. Besides, the effects of various bio-ions and macromolecules on Lys-CzFP also be studied. As seen in Fig. S9a, in a series of viscosity systems, when 5 eq dexamethasone was added to various viscosities containing 10 μM Lys-CzFP, the fluorescence intensity of the Lys-CzFP was not change significantly, which indicated that the probe was not affected by excessive dexamethasone from outside. According to Fig. S9b, there were no significant effects among water and glycerol (containing 10% water as co-solvent) in bio-ions, responding to viscosity particularly. All of the above characteristics indicated that Lys-CzFP could be used for tracing viscosity changes, and provided acidic pH-activatable property in complex physiological environments.

3.3 Fluorescence spectrums of Lys-CzFP in the different solvents

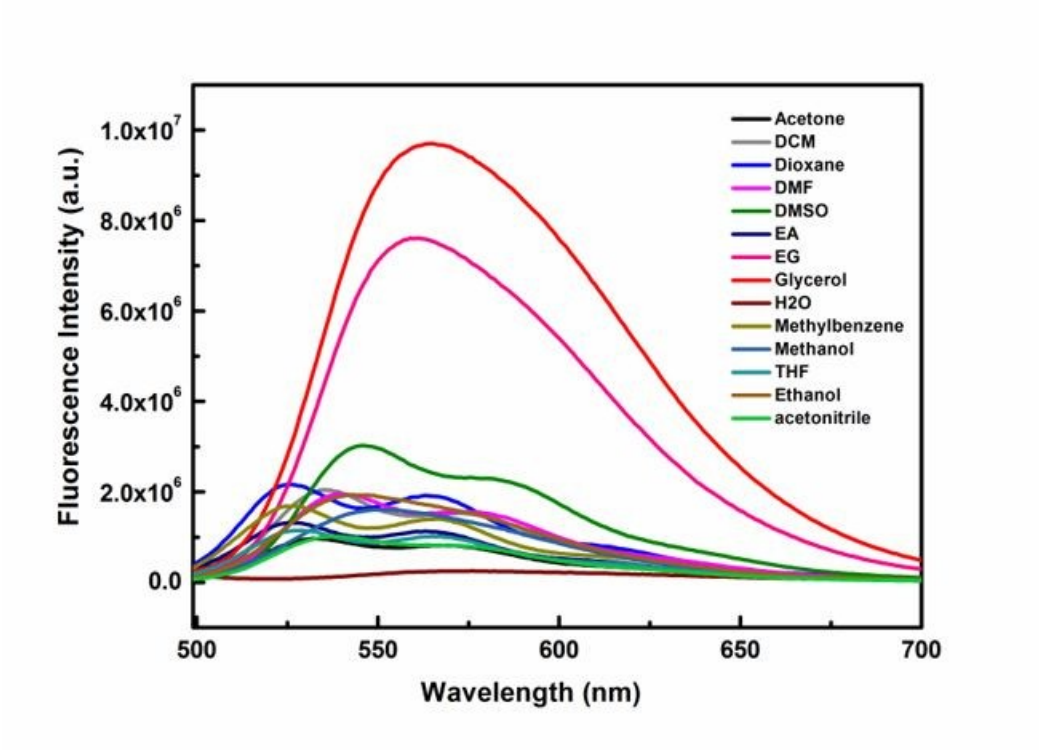

Fig. 3 Fluorescence Intensity in various solvents with different polarities.

Due to the complex cellular environment and numerous influencing factors, solvents polarity could

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be one of the factors affecting probe fluorescence. Therefore, the effect of solvent polarity was also evaluated. According to Fig. 3, the probe Lys-CzFP exhibited strong fluorescence emission at 560 nm in glycerol, which was much higher than those in other solvents. Similarly, in the mixtures of ethanol-glycerol, or ethanediol-glycerol, with different viscosity, Lys-CzFP displayed almost the same fluorescence response in viscous solution. (Figures S6 and S7). In contrast, in other solvents with different polarities, the probe fluorescence was very weak; its fluorescence intensity effects were not obvious in different polar solvents. The quantum yields of the probe Lys-CzFP in various solvents were also calculated. (Table S3 and S4). The quantum yields of the probe Lys-CzFP in other solvents were meager, from 0.003 to 0.026. However, the quantum yield of the probe Lys-CzFP reached to 0.253 in glycerol. Besides, we can see that an obvious upward trend towards the fluorescence intensity of probe occurred as the viscosity of the solvent increases, but the fluorescence had no similar relationship with the increase of polarity. These results indicated that the probe fluorescence intensity response was highly sensitive and selective compared to solvent polarity and that it could be used to trace viscosity changes in complex biological environments.

3.3 Lifetime of Lys-CzFP in various viscosity mixtures

Fluorescence lifetime decays of Lys-CzFP were carried out in a series of viscosity gradient media composed of glycerol and water in a variety of ratios. As shown in Fig. S12a-c, along with solutions viscosity from 6 cP to 523 cP (10 μM, pH 7.4, 20 °C), the lifetime gradually prolonged from 0.25 ns to 1.0 ns. The probe exhibited excellent linear relationship ($R^2 = 0.99$, Slop = 0.33) by fitting the Förrster–Hoffmann equation (Fig. S12d). Hence, these results indicated that the fluorescence lifetime of Lys-CzFP was a sensitive and promising viscosity probe under fluorescence lifetime imaging microscope (FLIM) to trace changes of cells viscosity and focus on alterations in the internal state of the cells in real-time. It is an important significance for future research on in vivo pathology.

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Table 1 The photophysical properties of Lys-CzFP in different solvents

Viscosity value (cP) [a] Solvents	λ_{abs}^{max} [b] (nm)	ε[c] ($M^{-1}cm^{-1}$)	λ_{em}^{max} [d] (nm)	Δλ[e] (nm)	Φ_F[f]	B[g] ($M^{-1}cm^{-1}$)	τ[h] (ns)
1.01 (H_2O)	482	25286	563	83	0.003	76	—
3.72 (H_2O/Glycerol = 3/2)	483	24850	567	84	0.054	1342	0.24
6.00 (H_2O/Glycerol = 1/1)	482	25324	567	85	0.059	1494	0.25
60.10 (H_2O/Glycerol= 1/4)	483	27308	564	81	0.116	3168	0.50
219 (H_2O/Glycerol = 1/9)	482	29080	563	81	0.139	4042	0.75
1412 (Glycerol)	482	35300	560	78	0.253	8931	1.12

[a] Viscosity values with different proportions of water–glycerol systems were obtained from Table S2. [b] Maximum absorbance wavelength. [c] Molar extinction coefficient. [d] Maximum emission wavelength. [e] Stokes shift. [f] Fluorescence quantum yield. Fluorescein in 0.1 M NaOH used as the standard reference with a quantum yield of 0.95 in ethanol. [g] Brightness. Brightness was calculated using $B = \varepsilon * \phi_F$. [h] Fluorescence lifetime was tested by the method of time-correlated single-photon counting.

3.4 Visualize the viscosity changes in Bel-7402 cells

The desirable fluorescence characteristic of Lys-CzFP for detecting viscosity was

conducive to trace viscosity changes intracellularly. Hence, the capability of the probe for cell-viscosity fluorescence imaging was further evaluated. Before the cell experiment, the cytotoxicity of Lys-CzFP was performed by MTT assay in Bel-7402 cells and Fig. S13 revealed that the cell viability was more than 91.5% after 24 h with the negligible cytotoxicity, indicating a favorable property for tracing lysosomal viscosity changes.

The lysosomal localization experiment of Lys-CzFP in Bel-7402 was performed, Bel-7402 cells were co-stained with Lys-CzFP and Lyso-Tracker red, a commercial lysosomal dye, for 30 min at 37 °C. The fluorescence image was obtained under a confocal microscope (Fig. 3a-c). The green and red luminance signals were detected by the green and red channels, respectively, and the yellow signal was the result of the overlap between the Lys-CzFP and Lyso-Tracker red signals. The merged image results showed that two channels tended toward synchronization, and the images overlapped very well (Fig. 4d). In addition, the fluorescence of the cells was extracted, correlation scatters plot and intensity profile showed that the two channels have an excellent relationship (Fig. 4e-f), Pearson's correlation [50] reached to 0.974. All the above results indicated that Lys-CzFP was an excellent lysosomal bio-tracer reagent that could stain lysosomes specifically.

As an essential parameter, the photostability of Lys-CzFP was also investigated under excitation of 482 nm. After 60 min of continuous irradiation, the emission at 560 nm barely changed, which suggested that the Lys-CzFP was very stable. Besides, the photostability of the probe in living cells is a prerequisite for cell viscosity measurement. It can be seen from Fig. S15 that the 10 μM probe was incubated in HeLa cells for 8h, 16h, and 24h. The experimental results showed that there was no apparent change in fluorescence intensity, which indicated that the probe has excellent photostability in cells.

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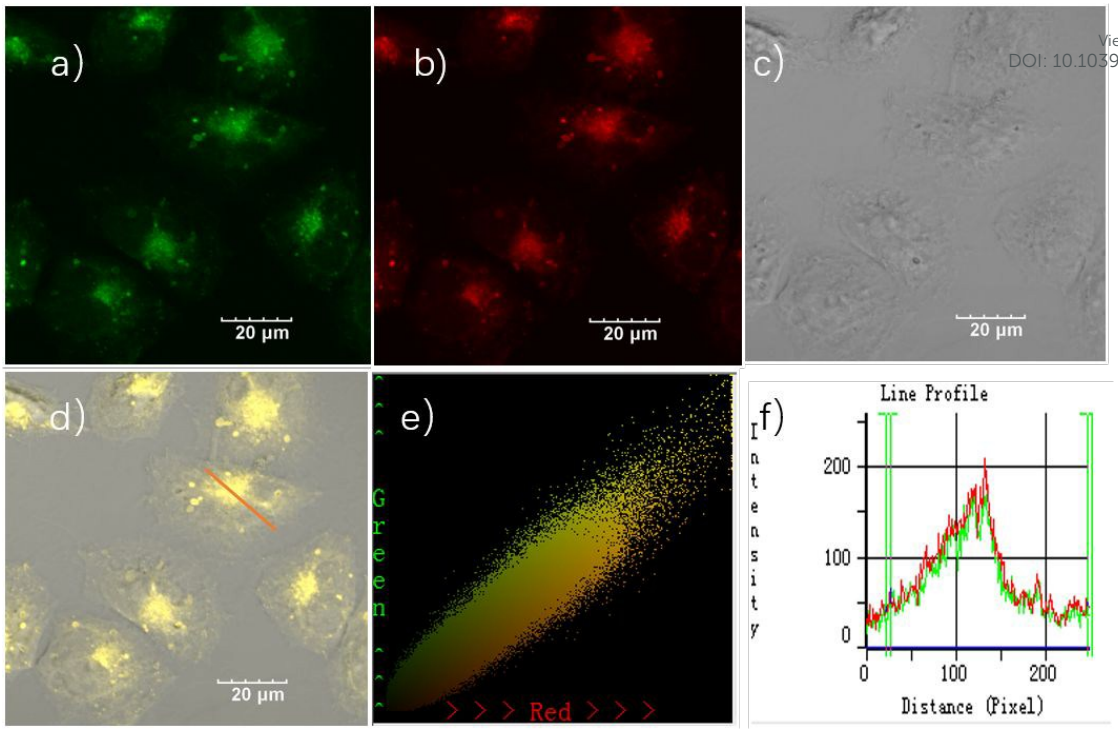

Fig. 4 Colocalization imaging of Bel-7402 cells stained with 10 μM Lys-CzFP and 10 μM Lyso-Tracker Red for 30 min at 37 °C. (a) Confocal image from Lys-CzFP on the green channel; (b) Confocal image from Lyso-Tracker Red on the red channel. (c) Bright-field image. (d) Merged image of (a) and (b). Scale bar is 20 μm. (e) Correlation scatters plot of Lys-CzFP and Lyso-Tracker intensities. (f) Intensity profile obtained from regions of interest.

As shown in Fig. 5, to verify whether lysosomal viscosity changes in living cells could be detected using Lys-CzFP by fluorescence imaging. Dexamethasone, a stabilizer for membranes of lysosomes [51] and an inhibitor of lysosomal enzymatic release, was proved to be effective in stimulating lysosomal viscosity changes in cells.[52] Fig. 5a showed that Bel-7402 cells were treated with 10μM Lys-CzFP for 30 min. The fluorescence was observed in the green channel. Then, dexamethasone (20μM) was added and incubated at 37°C for 60 min. Real-time living cellular images were recorded every 20 min; the fluorescence was significantly enhanced (Fig. 5b-e). To quantify the fluorescence intensity, the relative pixel intensities of each plot were extracted. The results showed that the fluorescence of the cells was significantly increased within 60 min after incubation with dexamethasone. These results

indicated that Lys-CzFP with morpholine moiety could be used to detect lysosomal viscosity changes in living cells.

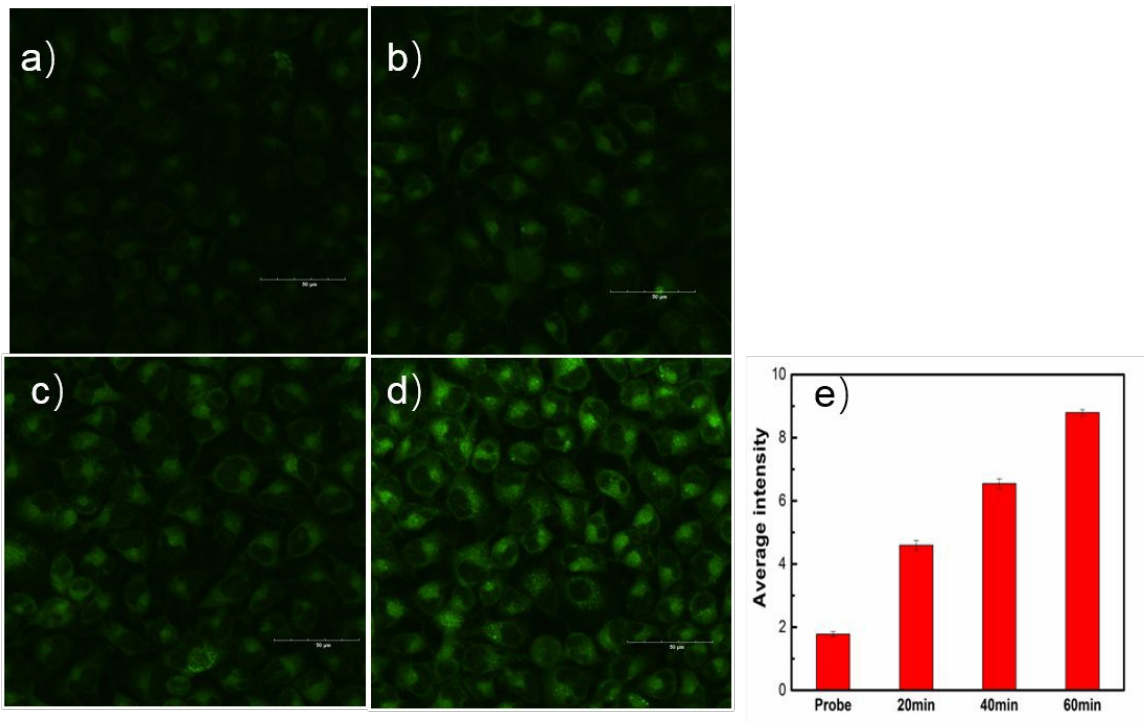

Fig. 5 (a) Real-time living cell images of Bel-7402 stained by Lys-CzFP (10μM) for 30 min. (b - d) Lys-CzFP treated with dexamethasone (20 μM), fluorescence images were taken every 20 min until 60 min later. Scale bar is 50 μm. (e) The average intensity of images a-d.

4. Conclusion

In summary, a novel molecular rotor based on carbazolyl GFP chromophore analogue has been designed for tracing lysosomal viscosity changes. Lys-CzFP showed a remarkable response to viscosity with maximum emission at 560 nm and a large stokes shift of 78 nm. The fluorescence response was maximally increased by about 98 - fold within 5s, with the ϕ_F increasing from 0.003 to 0.253 and the lifetime increasing from 0.25 ns to 1.12 ns, which indicated that using Lys-CzFP via fluorescence lifetime imaging microscope to trace the changes of cells viscosity was allowed. Besides, the electron-rich morpholine moiety weakened fluorescence of the GFP fluorophore via PET, which endued Lys-CzFP with an

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acidic pH-activatable lysosome-targeting fluorescence characteristic that was conducive to eliminate the background fluorescence outside of lysosomes. Lys-CzFP was stable in various bio-ions and small molecules, which showed that the probe has high sensitivity and specificity to viscosity in lysosomes. Finally, the probe Lys-CzFP has been successfully used for tracing lysosomal viscosity changes in living cells. We expect that Lys-CzFP could be exploited as a potential platform to trace viscosity changes in complex biological systems.

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Conflicts of interest

There are no conflicts to declare.

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