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A FRET-Based Ratiometric Chemosensor for *in Vitro* Cellular Fluorescence Analyses of pH

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Abstract

Ratiometric fluorescence sensing is an important technique for precise and quantitative analysis of biological events occurring under complex conditions by simultaneously recording fluorescence intensities at two wavelengths and calculating their ratios. Herein, we design a ratiometric chemosensor for pH that is based on photo-induced electron transfer (PET) and binding-induced modulation of fluorescence resonance energy transfer (FRET) mechanisms. This ratiometric chemosensor was constructed by introduction of a pH-insensitive coumarin fluorophore as a FRET donor into a pH-sensitive amino-naphthalimide derivative as the FRET acceptor. The sensor exhibited clear dual-mission signal changes in blue and green spectral windows upon pH changes. The pH sensor was applied for not only measuring cellular pH, but also for visualizing stimulus-responsive changes of intracellular pH values.

Keywords

pH sensor; Ratiometric sensing; FRET; PET; Biological imaging

1. Introduction

Intracellular pH is an important physiological factor that is tightly regulated by a complex interaction of proton (H^+) transport, H^+ -consuming, H^+ -producing reactions, and H^+ buffering [1]. For instance, intracellular acidification has been reported to be a concomitant of apoptosis in cancer cells [2,3]. Intracellular acidification is also an early feature of apoptosis. Thus, knowledge of changes in pH in complex biological samples is crucial for understanding and quantifying these processes. Optical sensors have been intensively studied as they can be non-invasive, disposable, easily miniaturized (down to sub-micrometer levels), and easy to process for environmental analysis, medical diagnosis, and process control [4,5]. Many optical pH chemosensors have been used for cell and tissue imaging [6,7]. Most pH sensors are based on pH dependent intensity changes in single emission windows. However, fluorescence signals are readily perturbed by environmental factors, such as temperature, solvent polarity, and inhomogeneous cellular distribution and unknown concentrations of analytes. All these factors make it difficult to analyze

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fluorescence signals in quantitative manner [8]. Consequently, single-emission detection is problematic for precise analyses under biological conditions.

To alleviate the above problems, ratiometric measurements were developed through simultaneously recording fluorescence intensities at two wavelengths and calculating their ratios [9]. The ratiometric measurement provides greater precision than that of using a single wavelength. In general, two types of fluorescent sensors are suitable for ratiometric investigation. Type one sensors are based on internal charge transfer mechanism using a single fluorophore. A typical example is seminaftorhodafluor-1 (SNARF-1) [10]. Type two sensors are based on chemical conjugation of two different fluorophores, which can undergo fluorescence resonance energy transfer (FRET) to improve the sensitivity [8,11]. Because of the high flexibility of the design of FRET sensors and wide choices of fluorophores, FRET has been widely utilized for the design of ratiometric biosensors, such as protein-based biosensors [12–14], pH sensors [15], and zinc ion sensors [16].

We recently developed a few amino-naphthalimide derivatives as fluorescent pH sensors [5]. These amino-naphthalimides exhibited large Stokes shifts, less susceptibility to interference by metal ions, and higher photostability than fluorescein derivatives. They displayed turn-on single emission intensity changes by H^+ [5]. Herein, we report the design, synthesis, investigation, and application of a FRET-based ratiometric pH sensor (**SR1**) by integrating an amino-naphthalimide pH probe as a FRET acceptor with a pH-insensitive coumarin fluorophore as a FRET donor. Besides the study of pH dependent dual emissions of **SR1**, we use **SR1** to monitor intracellular pH changes induced by chloroquine and dexamethasone for mouse macrophage J774A.1 and human cervical cancer HeLa cell lines, respectively.

2. Materials and methods

2.1. Materials

All chemicals and solvents were of analytical grade and were used without further purification. Triethylamine, trifluoroacetic acid, tetrahydrofuran (THF), 1,2-ethanediamine (compound **1**), 4-bromo-1,8-naphthalic anhydride (compound **3**), 7-diethylamino coumarin-3-carboxylic acid (compound **6**), di-*tert*-butyl dicarbonate (Boc_2O), *N*-(2-hydroxyethyl)piperazine (compound **8**), *N,N'*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), 1-hydroxybenzotriazole hydrate (HOBt), *N*-(3-dimethyl aminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI), dexamethasone, and chloroquine were commercially available from Aldrich (St. Louis, MO) and were used without further purification. Double distilled water was used for the preparation of the buffer solutions. The pH values were determined with a digital pH meter (Thermo Electron Corporation, Beverly, MA) calibrated at room temperature (23 ± 2 °C) with standard buffers of pH 10.01, 7.00, and 4.01. Britton-Robinson (B-R) buffers composed of acetic acid, boric acid, phosphoric acid, and sodium hydroxide were used for tuning pH values.

Hoechst 33342, LysoTracker Red[®] DND-99, Eagle Minimum Essential Medium (EMEM), and Keratinocyte medium were purchased from Invitrogen (Carlsbad, CA). EMEM was used for HeLa and J774A.1 cells' culture. Keratinocyte medium was used for human esophagus precancer CP-A cells' culture.

2.2. Syntheses

2.2.1 Synthesis of compound 2—To a 250 mL of round-bottom flask was added ethylenediamine (**1**, 6.06 g, 100.8 mmol) in $CHCl_3$ (50 mL). A solution of di-*tert*-butyl dicarbonate (2.2 g, 10.1 mmol) in $CHCl_3$ (30 mL) was added dropwisely over a period of 1

h at 0 °C and then stirred at room temperature for 24 h. After poured into water, the organic layer was separated, washed with brine, dried over anhydrous Na₂SO₄, and concentrated in vacuo to afford compound **2** (1.42 g, 8.86 mmol) as a colorless oil in 88% yield. ¹H NMR (CDCl₃, δ, ppm): 1.27 (s, 9H), 1.83 (s, 2H), 2.72 (t, 2H), 3.10 (q, 2H), 4.86 (br s, 1H).

2.2.2 Synthesis of compound 4—Compound **2** (1.40 g, 8.74 mmol) was dissolved in 10 mL of ethanol in a round bottom flask. After stirring for a few minutes, 4-bromo-1,8-naphthalic anhydride (compound **3**, 2.42 g, 8.74 mmol) was added and the reaction mixture was refluxed for 2 h. The reaction was cooled to room temperature. The precipitated solid was filtered and washed with cold ethanol and dried under a vacuum to give an off-white solid of **4** (3.3 g, 7.87 mmol). Yield: 90%. ¹H NMR (CDCl₃, δ, ppm): 1.30 (s, 9H), 3.49–3.55 (q, 2H), 4.34 (q, 2H), 7.84 (dd, 1H), 8.02–8.04 (d, 1H), 8.40–8.42 (d, 1H), 8.50–8.53 (dd, 1H), 8.64–8.66 (dd, 1H).

2.2.3 Synthesis of compounds 5 and 7—Compound **4** (3.0 g, 7.16 mmol) was placed into a round bottom flask to which dichloromethane (30 mL) and trifluoroacetic acid (5 mL) were added. The mixture was stirred vigorously and the reaction was monitored by TLC (15:1 of dichloromethane/methanol by volume). On completion of reaction, excess trifluoroacetic acid and dichloromethane were removed under reduced pressure to give compound **5** (2.2 g, 6.89 mmol). Yield: 96%. Compound **5** was used for the next step without further purification. To a solution of compound **6** (0.4 g, 1.57 mmol) in DMF (3 mL) was added HOBt (0.2 g, 1.57 mmol) and EDCI (0.3 g, 1.57 mmol). The mixture was stirred for 15 min at room temperature and then compound **5** (0.5 g, 1.57 mmol) was added. The mixture was stirred overnight at 50 °C and then poured into water (10 mL). The precipitated solid was filtered, washed with water, and dried under a vacuum to give a bisque solid, which was further purified by column chromatography (dichloromethane/methanol: 15/1 by volume) to get 0.35 g (0.62 mmol) of compound **7** as a yellow solid. Yield: 40 %. ¹H NMR (DMSO-*d*₆, δ, ppm): 1.05 (t, 6H), 3.40–3.55 (q, 4H), 3.60–3.70 (q, 2H), 4.20–4.30 (q, 2H), 6.55 (s, 1H), 6.75–6.85 (d, 1H), 7.55–7.65 (d, 1H), 7.90–8.00 (t, 1H), 8.20–8.40 (m, 2H), 8.50 (s, 1H), 8.55–8.60 (d, 1H), 8.70–8.80 (dd, 1H).

2.2.4 Synthesis of compound SR1—Compounds **7** (0.1 g, 177.8 μmol) and **8** (0.023 g, 533.4 μmol) were mixed in methoxyethanol (3 mL) and heated under reflux overnight. After the solvent was removed under reduced pressure, the residue was purified by column chromatography using dichloromethane/methanol (20:1 by volume) as the eluent. 0.058 g of yellow product was obtained. Yield: 53%. ¹H NMR (CDCl₃, δ, ppm): 1.19–1.23 (t, 6H), 2.69–2.72 (t, 2H), 2.84 (q, 4H), 3.29 (q, 4H), 3.40–3.43 (q, 4H), 3.68–3.70 (t, 2H), 3.81–3.83 (d, 2H), 4.46–4.48 (d, 2H), 6.44 (d, 1H), 6.61–6.63 (d, 1H), 7.18–7.20 (d, 1H), 7.36–7.39 (d, 1H), 7.65 (t, 1H), 8.37–8.39 (d, 1H), 8.50–8.52 (d, 1H), 8.56–8.59 (d, 1H), 8.65 (s, 1H), 8.89–8.92 (dd, 1H). ¹³C NMR (CDCl₃, δ, ppm): 164.55, 164.05, 163.50, 162.50, 157.57, 155.71, 152.37, 148.03, 132.70, 131.28, 131.05, 130.16, 129.97, 126.15, 125.66, 123.15, 116.78, 114.97, 110.43, 109.76, 108.37, 96.52, 59.32, 57.77, 52.99, 52.95, 45.02, 39.32, 38.40, 12.39. HRMS (APCI): *m/e* calculated for C₃₄H₃₇N₅O₆ (M + H) 611.2744; found. 611.2725.

2.3. Instruments and characterization

A Varian liquid-state NMR operated at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR was used for NMR spectra measurements. High resolution mass spectrometry (HRMS) was performed by the Mass Spectrometry Laboratory at Arizona State University. A Shimadzu UV-3600 UV-Vis-NIR spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) was used for absorbance measurements. A Shimadzu RF-5301

spectrofluorophotometer was used for fluorescence measurements. Nikon Eclipse TE2000E C1Si (Melville, NY) was used for recording fluorescence images.

2.4 Culture of cells for intracellular bioimaging

CP-A cells (kindly provided by Dr. Brian J. Reid at Fred Hutchinson Cancer Research Center, Seattle, WA) were cultured in Keratinocyte-serum free medium (Invitrogen, Carlsbad, CA), supplemented with Bovine Pituitary Extract (BPE) and human recombinant Epidermal Growth Factor (rEGF, Invitrogen) at 37 °C in a 5% CO₂ atmosphere. HeLa and J774A.1 cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured in EMEM supplemented with 10% fetal bovine serum, 100 unit/mL of penicillin, 2 mM L-glutamine (Sigma-Aldrich), and incubated at 37 °C in a 5% CO₂ atmosphere. Cells were seeded onto 96 well plates at 10,000, 15,000, and 30,000 cells per well for HeLa, CP-A, and J774A.1 cell lines, respectively for overnight. **SR1** dissolved in DMSO was added to the medium to make the sensor concentrations in a range of 100 nM – 5 μM. 10 min of internalization was found to be sufficient for achieving satisfying images. To confirm the sensor's subcellular localization, acidic organelle specific LysoTracker Red[®] and nuclei specific Hoechst 33342 were used to co-stain cells with **SR1**. Cells internalized with **SR1** for 10 minutes were washed with fresh medium. LysoTracker Red[®] was then added. Cells were continuously incubated for an additional 30 min for observation of colocalization of **SR1** with LyoTracker Red[®]. Before adding Hoechst 33342, cells were washed with fresh medium, Hoechst 33342 dissolved in fresh medium was then added to stain cell nuclei for another 30 min. Concentrations of LysoTracker Red[®] and Hoechst 33342 were of 100 nM and 10 μM, respectively. Under the confocal fluorescence microscope, Hoechst 33342 was excited at 402 nm and its blue emission was collected using a 450/35 nm filter set; blue channel of **SR1** was excited at 402 nm and **SR1**'s blue emission was collected using a 450/35 nm filter set; green channel of **SR1** was excited at 440 nm and **SR1**'s green emission was collected using a 515/30 nm filter set; LysoTracker Red[®] was excited at 561 nm and its red emission was collected using a 605/75 nm filter set. When **SR1** and Hoechst 33342 were used simultaneously, because both of these probes exhibit blue emissions, only green emission of **SR1** from the green channel to represent **SR1** was recorded.

2.5 Lysosomal pH changes in mouse macrophage J774A.1 induced by chloroquine

Mouse macrophages J774A.1 internalized with 2 μM **SR1** for 20 min at 37 °C were washed three times with a PBS buffer to remove excess sensor and then chloroquine (100 μM) was added to stimulate lysosomal pH change. Cells were observed using a Nikon Eclipse TE2000E confocal fluorescence microscope. Lysosomal pH values were estimated by comparing the blue/green fluorescence intensity ratios to the calibration curve of **SR1**.

2.6 Intracellular pH changes in apoptotic HeLa cells induced by dexamethasone

HeLa cells internalized with **SR1** (2 μM) were washed with fresh medium and then dexamethasone (2.0 μM) was added. Cells were observed using a Nikon Eclipse TE2000E confocal fluorescence microscope. Intracellular pH values were estimated by comparing the blue/green fluorescence intensity ratios to the calibration curve of **SR1**.

3. Results and Discussion

3.1. Molecular design and synthesis

Figure 1 gives chemical structure of the designed ratiometric chemosensor, **SR1** and its sensing mechanism. **SR1** was synthesized via 5 steps of traditional organic synthesis techniques. The compound **SR1** was characterized using ¹H NMR, ¹³C NMR, and high resolution mass spectroscopy (HRMS). **SR1** possesses a coumarin unit (FRET donor) that is

connected to the amino-naphthalimide (FRET acceptor) through a short linker. At basic solution, for example pH of 10, fluorescence of amino-naphthalimide fluorophore was quenched by the electron lone pair in the amino group of piperazine moiety through photo-induced electron transfer (PET). At this condition, the amino-naphthalimide fluorophore was not an efficient acceptor to receive energy from the coumarin donor. Therefore, **SR1** predominantly showed blue emission from the coumarin unit. At acidic condition, for example pH of 3, protonation of the amino group of the piperazine unit restores the emission of the amino-naphthalimide segment, which acts as an efficient FRET acceptor. Therefore, **SR1** predominantly showed green emission from the amino-naphthalimide unit due to FRET effect at acidic condition.

3.2. Fluorescence sensing of pH

SR1 was soluble in aqueous solution when dispersed from its concentrated DMSO solution (200 μ M) into water, buffers, and cell culture media at a final **SR1** concentration of less than 10 μ M. 5 μ M **SR1** in B-R buffer was used for absorption and emission measurements (Figure 2). Solution of **SR1** showed two distinct absorption maxima at 395 nm at basic condition and at 425 nm at acidic condition, corresponding to the absorption maxima of the coumarin and amino-naphthalimide fluorophores, respectively (Figure 2a). Upon titration, the absorption peak at 395 nm decreased significantly from pH 10.0 to pH 7.0 and the peak at 425 nm increased significantly from pH 7.0 to pH 3.0. When excited at 405 nm, distinct emission maxima at 467 nm and 525 nm from the coumarin and amino-naphthalimide units were observed (Figure 2b). The dual-emission spectra changed in a seesaw manner. The large increase in emission at 467 nm and the concomitant decrease in emission at 525 nm along with increase of pH values from 7 to 10 strongly suggested that FRET from the coumarin unit to the amino-naphthalimide moiety be effectively prohibited by PET effect. This observation indicated the sensing mechanism of **SR1** is an integration of PET and FRET mechanisms (Figure 1).

Ratios of the emission intensities at 467 and 525 nm ($R = F_{525}/F_{467}$) decreased from 2.62 to 0.22 from pH 3 to pH 10 ($R_{\text{pH at 3}}/R_{\text{pH at 10}} = 11.9$; Figure 2c). According to these pH dependent ratios, typical application range of **SR1** can be found to be from 4.0 to 8.0. According to the plot of **R** against pH, pK_a was calculated to be 6.01 by the typical sigmoidal fitting using Boltzmann equation:

$$\frac{F}{F_0} = \frac{m1 - m2}{1 + \exp(\frac{pH - pK'_a}{p})} + m2$$

where, F and F_0 are the fluorescence intensities measured at varying pH values and at the highest pH value (pH 10) used during the calibration, respectively. $m1$, $m2$, pK'_a , and p are empirical parameters describing the initial value ($m1$), the final value ($m2$), the point of inflection (pK'_a), and the width (p) of the sigmoidal curve.

Another way for data processing is to use the reverse ratio of **R** (R'). The ratio R' at 467 nm and 525 nm ($R' = 1/R = F_{467}/F_{525}$) increased from 0.38 to 4.35 ($R'_{\text{pH at 10}}/R'_{\text{pH at 3}} = 11.9$; Figure 2d) from pH at 10 to pH at 3. According to the plot of R' against pH, application range of **SR1** can be found to be from pH 6 to pH 10.

Therefore, using the two ratiometric data analysis approaches of **R** and R' , **SR1** is suitable for pH measurement in a broad range from pH 4 to pH 10.

3.3. Ratiometric imaging of pH in living cells

As mentioned above, the advantage of ratiometric imaging can be effectively exploited under complicated biological conditions, such as those inside cells. **SR1** was applied to ratiometrically visualize $[H^+]$ in mammalian living cells. It's well known that pH of cytoplasm is usually from 7.0 to 7.4 and pH of lysosomes is from 4.5 to 5.0, which is inside the sensing range of the chemosensor. We used Barrett's esophagus premalignant CP-A cells, cervical cancer HeLa cells and reticulum sarcoma J774A.1 cells to study the imaging and sensing of the pH chemosensor in cells. Concentration of the probe in the medium was controlled to be in a range of 100 nM – 5 μ M. DMSO in the medium was controlled to be less than 3% by volume. The sensors with concentrations of 100 nM could stain the cells, but the signal was weak. The fluorescence became much stronger with an increase of sensor concentration. To achieve images with satisfactory signal-to-noise ratios, a sensor concentration of 1 μ M was usually used for cell internalization study. As expected, confocal fluorescent microscopic images showed that the green fluorescence from naphthalimide is colocalized completely with the blue fluorescence from coumarin, indicating the ratiometric dual fluorescence sensor was delivered into cells and localized within the cell boundaries (Figure 3). Pixel-by-Pixel ratio images of the cells were obtained from the blue and green channels (Figure 3), which showed that the ratios are not constant throughout the cells, implying that the pH is not uniformly distributed inside the cells. These ratios fluorescence intensities of the green channels to blue channels (F_{525}/F_{467}) can be divided into two groups. The ratios of first group are from 2.25 to 2.10, which correspond to the pH from 4.5 to 4.9. The ratios of the other group are from 0.90 to 0.75, which correspond to the pH from 7.1 to 7.4. Therefore, **SR1** can be used not only for acidic but also for neutral intracellular pH measurements.

To determine the subcellular distribution of the sensors, nuclei specific staining probe Hoechst 33342 was used to co-stain the cells with **SR1**, and their colocalizations were studied (Figure 4). The images showed that **SR1** with bright green emissions distributed mainly in the cytoplasm region (Figure 4b, 4e and 4h), confirmed by only minimal colocalization of the green emissions of **SR1** with blue emissions from the nuclei specific Hoechst 33342 (figure 4a, 4d and 4g).

In order to further confirm whether **SR1** can be used to image intracellular pH values of the cytoplasm region, a commercially available acidic compartment (lysosome) specific staining probe, LysoTracker Red[®], was used to co-stain cells with **SR1** (Figure 5). The yellow fluorescence in Figure 5c, 5f and 5i indicated the same subcellular localization of LysoTracker Red[®] with **SR1**. The green fluorescence in Figure 5c, 5f and 5i showed the different subcellular localization of **SR1** with LysoTracker Red[®]. These observations indicated that **SR1** is suitable for pH imaging at both the acidic and neutral ranges. Colocalization percentage was quantified using Pearson's sample correlation factors (R_f) [17,18]. Cell line dependent colocalization of **SR1** with LysoTracker Red[®] was observed with $83.2 \pm 2.5\%$ for CP-A cells, $84.9 \pm 2.2\%$ for J774A.1 cells, and $61.7 \pm 1.7\%$ for HeLa cells. Herein, high R_f indicated a high subcellular distribution in the acidic organelles; low R_f suggested a low subcellular distribution in the acidic organelles, while a high distribution in the neutral organelles. Based on the above understanding, J774A.1 cells were used for investigation of environmentally stimulated acidic lysosomal pH changes, while HeLa cells were used for study on environmentally stimulated intracellular pH changes around the neutral range.

3.4. Monitoring stimuli-responsive changes of lysosomal pH

Lysosome is the first defense line of cells, capable of breaking down biological polymers - proteins, nucleic acids, carbohydrates, and lipid. Furthermore, the lysosomal membrane had

been considered to be the barrier of drug/DNA delivery. To monitor the real-time changes of lysosomal pH values, mouse macrophages (J774A.1) were exposed to an antimalarial drug, chloroquine [19]. Chloroquine could induce a lysosomal pH increase [20] in macrophages and to enhance the drug/DNA delivery efficiency [21].

As discussed above (section 3.3), **SR1** mainly localized in lysosome of J774A.1 cells, therefore, it could be used to study the lysosome pH changes. According to the ratiometric intensity ratios of **SR1**'s blue channel to green channel under confocal fluorescence microscope (Figure 6a), lysosomal pH values were calculated to be in a range of 4.5 to 5.0 before the treatment using chloroquine. Addition of 100 μ M chloroquine caused time dependent decreases of fluorescence intensities at both of blue and green channels (Figure 6). Merging fluorescence in blue and green channels gradually changed from mainly green to blue along with the time increase (Figure 6-a₄, b₄, c₄, d₄, e₄, and f₄). Referring to the titration curve shown in Figure 2b, these changes indicated time-dependent increases of lysosomal pH values stimulated by chloroquine. According to the intensity ratios of F₅₂₅/F₄₆₇ from the green and blue channels, time dependent lysosomal pH changes were plotted in Figure 6g. It should be noted here, there was no influence of chloroquine on **SR1**'s responses to pH in the B-R buffers. Therefore, the intracellular fluorescence changes of **SR1** are due to the increases of lysosomal pH value induced by chloroquine. This study suggested that **SR1** be a ratiometric pH sensor for measuring lysosomal pH changes.

3.5. Monitoring pH changes in apoptotic cancer cells

Intracellular acidification has been proven to be closely related to cell apoptosis [22]. Understanding the role of intracellular acidification in cell apoptosis is of paramount importance when attempting to induce cell acidification and cell apoptosis with drugs. In this study, dexamethasone [23] was used to induce apoptosis in HeLa cells. After treatment with 2 μ M dexamethasone for 9 minutes, the intracellular pH (especially adjacent to the nuclei) became more and more acidic, which resulted in that the blue and green emissions became brighter and brighter (Figure 7). This change was also clearly observed in the merging images of the blue and green channels. Bright loops (indicated by arrows) were observed in the merged fluorescence images. We calculated pH values around the loop area. pH value of these areas before the treatment was around 7.2 to 7.4. After 30 minutes stimulation, pH value of the loop areas was calculated to be 6.75 ± 0.15 . These results showed intracellular acidifications, which may be possibly due to that the release of lysosomal proton in the apoptosis process contributes to the acidification of cytosol [24]. Bright field images showed cell shrinkage with a preservation of an intact plasma membrane around 30 minutes stimulation, suggesting that the cell actually undergo apoptotic changes (Figure 7) [25]. Comparing the changes of fluorescence images and bright field images, it was found cells stimulated by dexamethasone became acidic much earlier than the apoptosis related morphological changes. Lower intracellular pH can activate acidic endonuclease activity, which induces cleavage of genomic DNA into small fragments [3,26]. This process ultimately pushes the cells into an irreversible apoptosis process. These results are consistent with other research work using dexamethasone to induce apoptosis of HeLa cells and produce intracellular acidification [25]. Therefore, **SR1** was demonstrated to be a ratiometric pH sensor that can be used for intracellular pH sensing and imaging.

3.6. Cytotoxicity of SR1

Cytotoxicity of **SR1** to the above cell lines was studied using Trypan blue staining. After 2 hours of cellular internalization of the sensor at a concentration of 5 μ M, more than 97% cells were viable, showing the non-cytotoxicity of **SR1** to cells at our experimental conditions.

4. Conclusion

We have designed a ratiometric dual-emission pH sensor (**SR1**) through a coupling of a pH responsive amino-naphthalimide unit and a pH insensitive coumarin segment. This sensor could be used for measurement of pH values in a broad range from 4.0 to 10.0. **SR1** was applied to visualize intracellular pH values of three cell lines, CP-A, J774A.1 and HeLa. Chloroquine was used to induce pH value increase in lysosome for J774A.1 cells. Dexamethasone was used to induce intracellular acidification of HeLa cells. These environmental stimulations were observed using **SR1**. It should be pointed out that **SR1** has a significant spectral overlap between the coumarin and amino-naphthalimide units and its spectral change is not significant in the pH range of 3 to 7. These facts will affect a wide application of this material. Further investigation to improve the sensors' performance is in progress.

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Figure 1. Synthesis of **SR1** and a schematic illustration of the dual-emission sensing of pH with the chemosensor through FRET and PET turn-on fluorescence sensing mechanisms.

Figure 2.

(a) UV-Vis absorption spectral changes of **SR1** in aqueous solution at different pH values; (b) Fluorescence spectral changes of **SR1** in aqueous solution at different pH values; (c) Plot of the emission ratios of R at F_{525}/F_{467} ; (d) Plot of R' at F_{467}/F_{525} . Measurement conditions: 5 μ M **SR1** in B-R buffers at different pH values, 25 $^{\circ}$ C, λ_{ex} = 405 nm.

Figure 3.

Confocal fluorescence images of **SR1** in CPA (a–c), HeLa (d–f) and J774A.1 (g–i) cells. a, d and g: blue fluorescence from coumarin unit of **SR1**. b, e and h: green fluorescence from amino-naphthalimide segment of **SR1**. c, f and i: overlay of blue and green channels. Scale bars represent 20 μm .

Figure 4. Confocal fluorescence images of **SR1** in CPA (a–c), HeLa (d–f) and J774A.1 (g–i) cells co-stained with nuclei staining Hoechst 33342. a, d and g: blue emission from Hoechst 33342. b, e and h: green emission from amino-naphthalimide segment of **SR1**. c, f and i: overlay of the blue and green channels. Scale bars represent 20 μm .

Figure 5. Confocal fluorescence images of **SR1** in CPA (a–c), HeLa (d–f) and J774A.1 (g–i) cells co-stained with LysoTracker Red[®]. a, d and g: red emission from LysoTracker Red[®]. b, e and h: green emission of **SR1**. c, f and i: overlay of the green and red channels. Scale bars represent 20 μ m.

Figure 6.

Time dependent lysosomal pH changes monitored by **SR1** in mouse macrophages stimulated with chloroquine under confocal fluorescence microscope. a, b, c, d, e, and f indicated times of 0, 10, 15, 20, 25 and 30 minutes, respectively. g showed the time dependent pH values. 1, 2, 3 and 4 represent green channel fluorescence image, blue channel fluorescence image, bright field image, and the overlay of blue and green channels, respectively. Concentration of chloroquine is 100 μ M.

Figure 7.

Time dependent dexamethasone stimulated intracellular pH changes of HeLa cells. a, b, c, d, e, and f indicated times of 0, 3, 9, 15, 21 and 30 minutes, respectively. 1, 2, 3 and 4 represent blue channel fluorescence image, green channel fluorescence image, bright field image, and the overlay of images of blue channel, green channel and bright field, respectively. Concentration of dexamethasone is 2 μ M.