Electronic Supplementary Information

Rhodamine derivative as "lock" and SCN^- as "key": Visible light excitable SCN^- sensing in living cell

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Materials and methods. Rhodamine B and thiophen-methaylamine has been purchased from Sigma Aldrich (India). Spectroscopic grade solvents have been used. Either Na^+ or K^+ salts of anions, and NO_3^- or Cl^- salts of cations were used. Other chemicals are of analytical reagent grade and used without further purification. Mili-Q 18.2 MΩ cm^{-1} water has been used throughout all the experiments. A JASCO (model V-570) UV-vis spectrophotometer has been used for measuring the absorption spectra. FTIR spectra are recorded on a JASCO FTIR spectrometer (model: FTIR-H20). Mass spectra are obtained using QTOF Micro YA 263 mass spectrometer in ES positive mode. ^{1}HNMR spectra have been recorded using Bruker Advance 300 (300 MHz) instrument in $CDCl_3$ and ^{13}CNMR spectra have been recorded using Bruker Advance 500 (500 MHz) instrument in $CDCl_3$ or DMSO-d_6. Elemental analysis has been made using a Perkin Elmer CHN analyser with first 2000-analysis kit. Steady-state fluorescence experiments are performed using Hitachi F-4500 spectrofluorimeter. Time-resolved fluorescence life time measurements were performed using a picosecond pulsed diode laser-based time-correlated single photon counting (TCSPC) spectrometer from IBH (UK) at λ_{ex} = 540 nm and MCP-PMT as a detector. The emission from the sample was collected at a right angle to the direction of the excitation beam maintaining magic angle polarization (54.71). The full width at half maximum (FWHM) of the instrument response function was 250 ps and the resolution was 28 ps per channel. The data were fitted to multi exponential functions after deconvolution of the instrument response function by an iterative reconvolution technique using IBH DAS 6.2 data analysis software in which reduced w2 and weighted residuals serve as parameters for goodness of fit. pH measurements have been carried out on a Systronics digital pH meter (model 335, India).

Imaging system. The imaging system is composed of an inverted fluorescence microscope (Leica DM 1000 LED), digital compact camera (Leica DFC 420C), and an image processor (Leica Application Suite v3.3.0). The microscope is equipped with a mercury 50 W lamp.

Preparation of cells. Pollen grains were obtained from freshly collected mature buds of *Tecomastans*, a common ornamental plant having a bright yellow flower, by crashing stamens on a sterile petri plate and suspending them in normal saline. Debris has been removed by filtering through a thin layer of non-absorbent cotton. Suspended pollens are collected by centrifugation at 5000 rpm for 5 min. The pollen pellet is then washed twice in normal saline and incubated in a 15% DMSO buffered solution of the probes (50 μM) for 1 h at room temperature. After incubation they were washed again with normal saline and observed under florescence microscope using green filter.

To detect intracellular SCN^-, *Candida albicans* cells (IMTECH No. 3018) from an exponentially growing culture in a yeast extract glucose broth medium (pH 6.0; incubation temperature 37 °C) were centrifuged at 3000 rpm for 10 min, washed twice in normal saline, and then treated with SCN^- salt at 50 μM for 30 min in normal saline. After incubation, the cells were washed again in normal saline, incubated with **RTA** (1 μM) for 15 min, and observed under a high power fluorescence microscope with a green filter. Cells loaded with **RTA** but not with SCN^- were used as the control.

Squamous epithelial cells were collected from the inner side of lips by scratching with a sterile cover slip. The cells were then washed in normal saline and re-suspended in normal saline.

UV-vis and fluorescence titration. The path length of cells used for absorption and emission studies was 1 cm. For UV-vis and fluorescence titrations, stock solution of **RTA** (10 μM) was prepared in DMSO:H_2O (4:1 v/v, pH 7.4). Working solutions of **RTA** and SCN^- were prepared from their respective stock solutions. Fluorescence measurements were performed using 2.5 nm × 2.5 nm slit width. Except time dependent spectra, all the fluorescence and absorbance spectra were taken after 40min of mixing of SCN^- with **RTA**.

Quantum yield measurements. The fluorescence quantum yields were determined using Rhodamine B as a reference with a known ϕ_{ref} value of 0.65 in basic EtOH.[1] The area of the emission spectrum was integrated using the software available in the instrument and the quantum yield was calculated according to the following equation:[2]

$$\phi_{sample} = \phi_{ref} \times [A_{sample}/A_{ref}] \times [OD_{ref}/OD_{sample}] \times [\eta_{sample}^2/\eta_{ref}^2],$$

where ϕ_{sample} and ϕ_{ref} are the fluorescence quantum yield of the sample and reference, respectively; A_{sample} and A_{ref} are the area under the fluorescence spectra of the sample and the reference, respectively; OD_{sample} and OD_{ref} are the corresponding optical densities of the sample and the reference solution at the wavelength of excitation; η_{sample} and η_{ref} are the refractive index of the sample and reference, respectively.

Calculation of the detection limit. To determine the detection limit, fluorescence titration of **RTA** with SCN^- was carried out by adding aliquots of micro-molar concentration of SCN^-. From the concentration at which there was a sharp change in the fluorescence intensity multiplied with the concentration of **RTA** gave the detection limit.[3] Equations used for the calculation of detection limit (DL):

$DL = C_L \times C_T$,

where C_L is the concentration of **RTA**, C_T is the concentration of SCN^- at which fluorescence enhanced.

Thus:

$DL = 1\ \mu M \times 0.01\ \mu M = 0.01\ \mu M = 10^{-8}\ M$

Cell viability studies. Candida cells were collected from 24 h old broth culture by centrifugation (6000 rpm for 5 min). The collected cells were then washed in normal saline and re-suspended in normal saline and distributed into four tubes as described below:

1) Candida control (candida cells in normal saline);

2) Candida + salt (candida cells were suspended in normal saline, containing 1 mg/mL of the SCN^- salt);

3) Candida + **RTA** (candida cells were suspended in normal saline, containing 1 mg/mL of **RTA**);

4) Candida + salt + **RTA** (candida cells were suspended in normal saline, containing 1 mg/mL of the SCN^- salt and 1 mg/mL of **RTA**).

These tubes were then incubated for 1, 2, 4 and 8 h and after each interval; dilution plating was performed using spread plate technique. After 24 h incubation at 37 °C, the appeared colonies were counted to check the viability of the Candida cells.

MTT cell toxicity assay of RTA.[4–6] For assaying the toxicity of **RTA** *in vitro* cell culture experiment, the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay has been performed. It is commonly known as cell viability and proliferation assay. The assay depends on the reductive cleavage of the yellow tetrazolium salt, MTT, to a water soluble purple color formazan by metabolic active cells which can be measured by spectrophotometry at 570 nm.

Materials: Phosphate buffer saline (PBS); MTT (5 mg/mL in PBS) - filtered and kept in dark; freshly prepared solution of DMSO:*i*PrOH (1:1, v/v); stock solutions of the SCN^- salt and **RTA**, respectively.

Procedure: Exponentially growing broth culture of *Candida albicans* (IMTECH No. 3018) (1.5 mL) grown in the yeast extract glucose broth medium (pH 6.0, incubation temperature 37 °C) was centrifuged at 3000 rpm for 10 min and the pellet was washed twice with normal saline and finally suspended in normal saline at the cell density of ~ 10^8 cell/mL. Five experimental sets (each set composed of three tubes) were prepared as described below:

1) Set I: To each tube *Candida* cell suspension (2 mL) and normal saline (0.2 mL) were placed (this set is considered as a negative control);

2) Set II: To each tube *Candida* cell suspension (2 mL) and tetrazolium salt solution (0.2 mL) were placed (this set is considered as a positive control);

3) Set III: To each tube *Candida* cell suspension (2 mL), tetrazolium salt solution (0.2 mL) and SCN^- salt solution (0.2 mL) from a stock solution of 1mg/mL were placed;

4) Set IV: To each tube *Candida* cell suspension (2 mL), tetrazolium salt solution (0.2 mL) and **RTA** solution (0.2 mL) from a stock solution of 1 mg/mL were placed;

5) Set V: To each tube *Candida* cell suspension (2 mL), tetrazolium salt solution (0.2 mL), SCN^- salt solution (0.2 mL) from a stock solution of 1 mg/mL and **RTA** solution (0.2 mL) from a stock solution of 1 mg/mL were placed. All sets were incubated at 37 °C for 4 h in dark. After incubation a freshly prepared DMSO:*i*PrOH (1:1, v/v) solution (2 mL) was added to each tube of all five experimental sets. Contents were mixed and incubated 37 °C for another 1 h in dark. After incubation, absorbance (OD) of all solutions was measured at 570 nm.

Measurement of the concentration of SCN^- in blood plasma (serum). Fresh sheep blood was collected in a sterile bottle and allowed to clot without disturbance. The straw yellow color serum was carefully aspirated in a separate clean tube. Then it was centrifuged at 3000 rpm for 10 min and collected the liquid portion. A stock solution of serum (10 mL) was prepared by diluting serum (1 mL) with HEPES buffer (0.1 M; DMSO–H_2O, 7:3 v/v; pH 7.4). Varying the amount of serum concentration, three different samples were prepared. The fluorescence intensity of these samples was recorded in the presence of **RTA**. Similarly, three other samples were prepared where SCN^- solution (3 μM) spiked externally. The SCN^- concentration of these samples was calculated using the equation, obtained from the inset plot of Fig. S4 (see below): Intensity = 17.77 + 17.95 × [SCN^-].
Results are listed in Table S2 (see below). The close proximity of the results obtained by standard addition method clearly indicates the non-interference of the complex components present in the blood plasma. The enzymes or proteins, which are present in human body fluid, may have fluorescence due to the presence of amino acids (like tryptophan, histidine, *etc.*). However, these amino acids have an excitation wavelength in the UV region. The excitation wavelength of **RTA**, being 540 nm (visible region), the interference of these enzymes or proteins may be ruled out and, hence, we were able to measure successfully the concentration of SCN^- in the sheep blood serium.

Measurement of the concentration of SCN^- in the living cell.
Fresh scquamus cells have been collected and incubated with four different concentration of SCN^- (10 μM, 25 μM, 50 μM and 75 μM). The fluorescence microscope images of these samples have been recorded, using the same exposure time of 50 ms. The corrected total cell fluorescence (**CTCF**) has been calculated using the following equations:[7]
Whole cell signal = sum of the intensity of the pixels for one cell
Background signal = average signal per pixel for a region selected just beside the cell.
CTCF = whole cell signal (number of pixels for the selected cell = surface selected × background)
All measurements have been performed using the ImageJ software.
The **CTCF** values of the above four samples were then plotted *vs.* their respective SCN^- concentration (Fig. S18). Using this calibration plot, the SCN^- concentration of cells before and after smoking has been found to be ~22 μM and ~60 μM, respectively.

Synthesis of RTA. To a solution of Rhodamine B (1 g, 2 mmol) in EtOH (20 mL), 2-thiophenmethaylamine (0.904 g, 8 mmol) was added. The mixture was refluxed for 48 h. The solvent was evaporated and the crude product was

purified by column chromatography with *n*-hexane:EtOAc (82:18, v/v). X-ray suitable crystals were obtained from MeOH solution. Yield: 0.807 g (75%). ^{1}H NMR, δ: 1.0 (t, $^{3}J_{H,H}$ = 7.2 Hz, 12H), 3.3 (m, 8H), 4.2 (s, 2H), 6.1 (d, $^{3}J_{H,H}$ = 9 Hz, 2H), 6.1 (m, 2H), 6.3 (m, 3H), 6.5 (m, 1H), 7.0 (m, 1H), 7.1 (m, 1H), 7.5 (m, 2H), 7.8 (m, 1H) ppm. ^{13}C NMR, δ: 12.8, 44.2, 97.6, 105.1, 108.6, 122.8, 124.1, 125.6, 126.2, 127.7, 128.8, 128.9, 130.8, 133.3, 140.8, 148.7, 148.9, 153.2, 154.0, 154.1, 167.4 ppm. QTOF-MS ES^{+}, *m/z* (*I*%): 538.21 (100) $[M + H]^{+}$, 560.18 (27) $[M + H]^{+}$. *Anal*. Calc. for $C_{33}H_{35}N_3O_2S$ (537.72): C 73.71, H 6.56, N 7.81. Found: C 73.61, H 6.66, N 7.73%.

X-ray crystallography. The X-ray data of **RTA** were collected at 173(2) K on a STOE IPDS-II diffractometer with graphite-monochromatised Mo-K$_\alpha$ radiation generated by a fine-focus X-ray tube operated at 50 kV and 40 mA. The reflections of the images were indexed, integrated and scaled using the X-Area data reduction package.[8] Data were corrected for absorption using the PLATON program.[9] The structure was solved by a direct method using the SHELXS-97 program[9] and refined first isotropically and then anisotropically using SHELXL-97.[10] Hydrogen atoms were revealed from $\Delta\rho$ maps and those bonded to C were refined using appropriate riding models. The hydrogen atom bonded to N was freely refined. Figures were generated using the Mercury program.[11] $C_{33}H_{35}N_3O_2S$, M_r = 537.70 g mol^{-1}, triclinic, space group *P*–1, *a* = 10.8119(7), *b* = 12.1454(8), *c* = 12.1718(8) Å, α = 104.339(5), β = 110.802(5), γ = 100.215(5)°, *V* = 1384.10(16) Å^3, *Z* = 2, ρ = 1.290 g cm^{-3}, μ(Mo-Kα) = 0.153 mm^{-1}, reflections: 15643 collected, 5157 unique, R_{int} = 0.0804, R_1(all) = 0.0704, wR_2(all) = 0.1532.

CCDC 848215 (**RTA**) contains the supplementary crystallographic data. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk.

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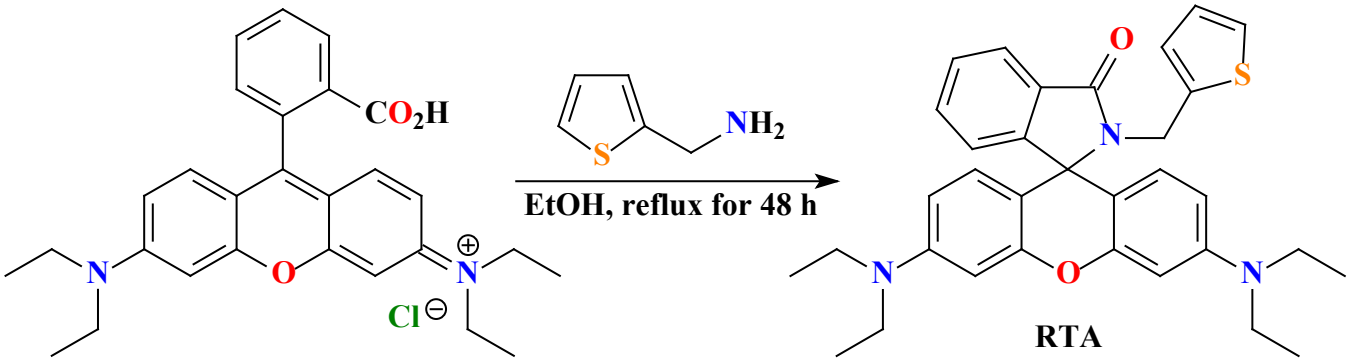

Scheme S1

Table S1 Selected bond lengths (Å) and bond angles (°) for **RTA**

Bond lengths

S(1)–C(6)	1.711(3)	N(3)–C(37)	1.460(4)	C(11)–C(16)	1.385(3)	C(27)–C(28)	1.512(4)
S(1)–C(3)	1.722(2)	N(3)–C(39)	1.509(5)	C(12)–C(13)	1.390(3)	C(29)–C(30)	1.511(3)
O(1)–C(1)	1.224(3)	N(3)–C(39')	1.554(7)	C(13)–C(14)	1.384(3)	C(31)–C(32)	1.389(3)
O(2)–C(32)	1.377(3)	C(1)–C(12)	1.484(3)	C(14)–C(15)	1.396(4)	C(31)–C(36)	1.396(3)
O(2)–C(22)	1.381(2)	C(2)–C(3)	1.500(3)	C(15)–C(16)	1.382(3)	C(32)–C(33)	1.387(3)
N(1)–C(1)	1.360(3)	C(3)–C(4)	1.370(3)	C(21)–C(22)	1.386(3)	C(33)–C(34)	1.397(3)
N(1)–C(2)	1.456(2)	C(4)–C(5)	1.420(4)	C(21)–C(26)	1.403(3)	C(34)–C(35)	1.414(3)
N(1)–C(7)	1.488(2)	C(5)–C(6)	1.339(4)	C(22)–C(23)	1.385(3)	C(35)–C(36)	1.380(3)
N(2)–C(24)	1.380(3)	C(7)–C(31)	1.513(3)	C(23)–C(24)	1.405(3)	C(37)–C(38)	1.501(4)
N(2)–C(27)	1.459(3)	C(7)–C(21)	1.514(3)	C(24)–C(25)	1.410(3)	C(39)–C(40)	1.527(8)
N(2)–C(29)	1.461(3)	C(7)–C(11)	1.527(3)	C(25)–C(26)	1.374(3)	C(39')–C(40')	1.505(9)
N(3)–C(34)	1.385(3)	C(11)–C(12)	1.381(3)				

Bond angles

C(6)–S(1)–C(3)	91.79(13)	C(4)–C(3)–C(2)	126.7(2)	C(13)–C(12)–C(1)	129.37(19)	N(2)–C(27)–C(28)	112.9(2)
C(32)–O(2)–C(22)	118.11(15)	C(4)–C(3)–S(1)	110.74(18)	C(14)–C(13)–C(12)	117.5(2)	N(2)–C(29)–C(30)	112.1(2)
C(1)–N(1)–C(2)	122.27(17)	C(2)–C(3)–S(1)	122.58(16)	C(13)–C(14)–C(15)	120.7(2)	C(32)–C(31)–C(36)	115.84(18)
C(1)–N(1)–C(7)	114.30(15)	C(3)–C(4)–C(5)	112.4(2)	C(16)–C(15)–C(14)	121.2(2)	C(32)–C(31)–C(7)	121.84(18)
C(2)–N(1)–C(7)	122.35(16)	C(6)–C(5)–C(4)	113.0(2)	C(15)–C(16)–C(11)	118.0(2)	C(36)–C(31)–C(7)	122.32(18)
C(24)–N(2)–C(27)	121.30(19)	C(5)–C(6)–S(1)	112.1(2)	C(22)–C(21)–C(26)	116.10(18)	O(2)–C(32)–C(33)	114.30(18)
C(24)–N(2)–C(29)	122.47(18)	N(1)–C(7)–C(31)	111.93(15)	C(22)–C(21)–C(7)	122.68(17)	O(2)–C(32)–C(31)	123.35(17)
C(27)–N(2)–C(29)	115.79(19)	N(1)–C(7)–C(21)	110.50(15)	C(26)–C(21)–C(7)	121.21(17)	C(33)–C(32)–C(31)	122.3(2)
C(34)–N(3)–C(37)	120.8(2)	C(31)–C(7)–C(21)	110.04(16)	O(2)–C(22)–C(23)	114.30(17)	C(32)–C(33)–C(34)	121.4(2)
C(34)–N(3)–C(39)	118.0(2)	N(1)–C(7)–C(11)	99.82(15)	O(2)–C(22)–C(21)	122.47(18)	N(3)–C(34)–C(33)	121.3(2)
C(37)–N(3)–C(39)	121.1(2)	C(31)–C(7)–C(11)	112.99(16)	C(23)–C(22)–C(21)	123.23(18)	N(3)–C(34)–C(35)	121.8(2)
C(34)–N(3)–C(39')	114.6(3)	C(21)–C(7)–C(11)	111.21(15)	C(22)–C(23)–C(24)	120.12(19)	C(33)–C(34)–C(35)	116.92(19)
C(37)–N(3)–C(39')	106.0(3)	C(12)–C(11)–C(16)	120.70(18)	N(2)–C(24)–C(23)	121.47(19)	C(36)–C(35)–C(34)	120.19(19)
C(39)–N(3)–C(39')	45.1(3)	C(12)–C(11)–C(7)	110.39(17)	N(2)–C(24)–C(25)	121.36(18)	C(35)–C(36)–C(31)	123.31(19)
O(1)–C(1)–N(1)	125.62(18)	C(16)–C(11)–C(7)	128.86(18)	C(23)–C(24)–C(25)	117.17(19)	N(3)–C(37)–C(38)	113.4(3)
O(1)–C(1)–C(12)	128.35(18)	C(11)–C(12)–C(13)	121.74(19)	C(26)–C(25)–C(24)	121.19(18)	N(3)–C(39)–C(40)	106.6(4)
N(1)–C(1)–C(12)	106.03(16)	C(11)–C(12)–C(1)	108.87(17)	C(25)–C(26)–C(21)	122.11(19)	C(40')–C(39')–N(3)	104.6(4)
N(1)–C(2)–C(3)	114.05(18)						

Table S2

Amount of serum solution added from the stock [mL]	Fluorescence intensity [a. u.]	SCN^- found in free serum [μM]	Fluorescence intensity [a. u.] of serum after spiking 3 μM SCN^-	SCN^- found in serum [μM] after spiking 3 μM SCN^-
1.0	46.0	1.57	101.0	4.63
1.5	60.0	2.35	115.0	5.41
2.0	74.0	3.13	130.0	6.25

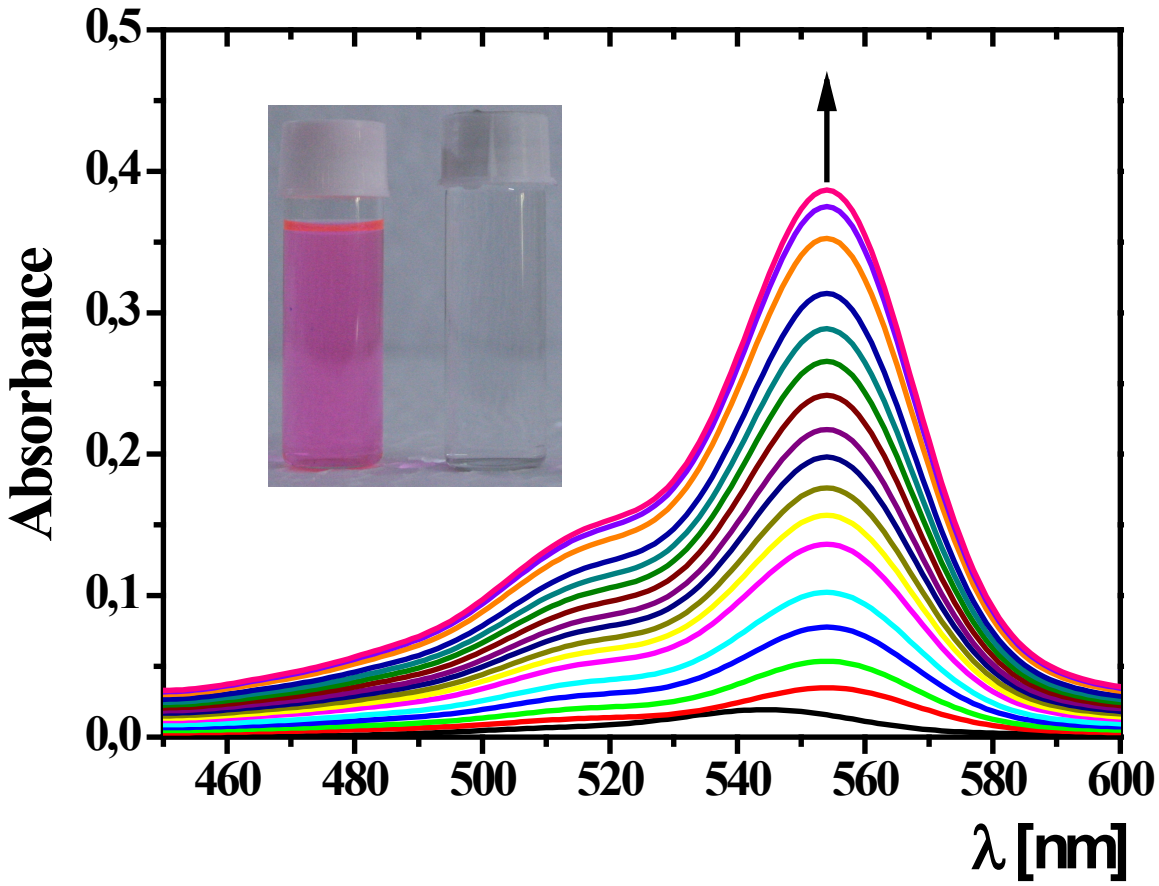

Fig. S1 UV-vis spectra of **RTA** (10μM) in HEPES buffered (0.1 M; DMSO–H_2O, 7:3 v/v; pH 7.4) solution upon gradual addition of SCN^- (1.0, 2.5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 250, 500 μM) at room temperature. Inset shows the naked eye color of free **RTA** and the [**RTA**–SCN^-] system.

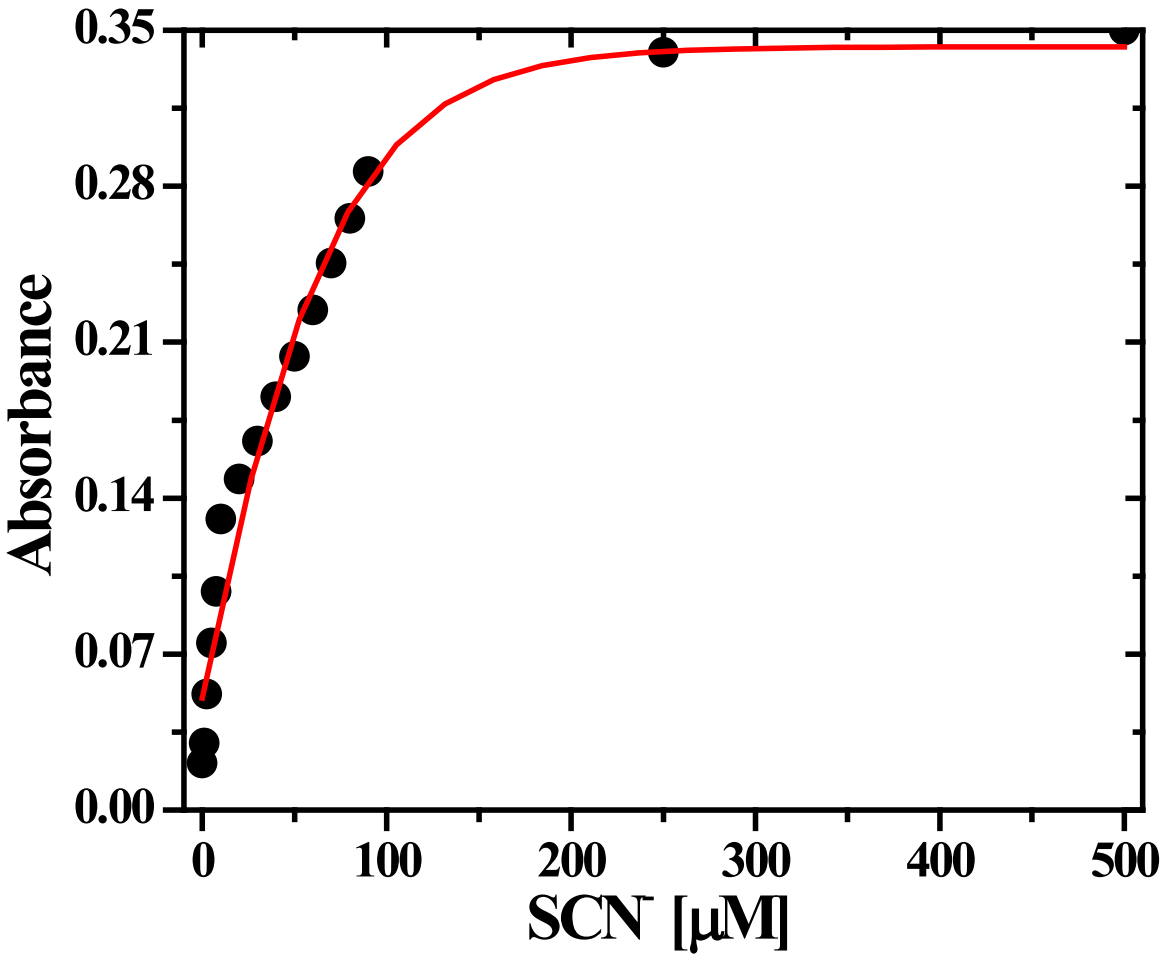

Fig. S2 Variation of absorbance of **RTA** in HEPES buffered (0.1 M; DMSO–H_2O, 7:3 v/v; pH 7.4; λ = 555 nm) solution as a function of externally added SCN^- (1–500 μM).

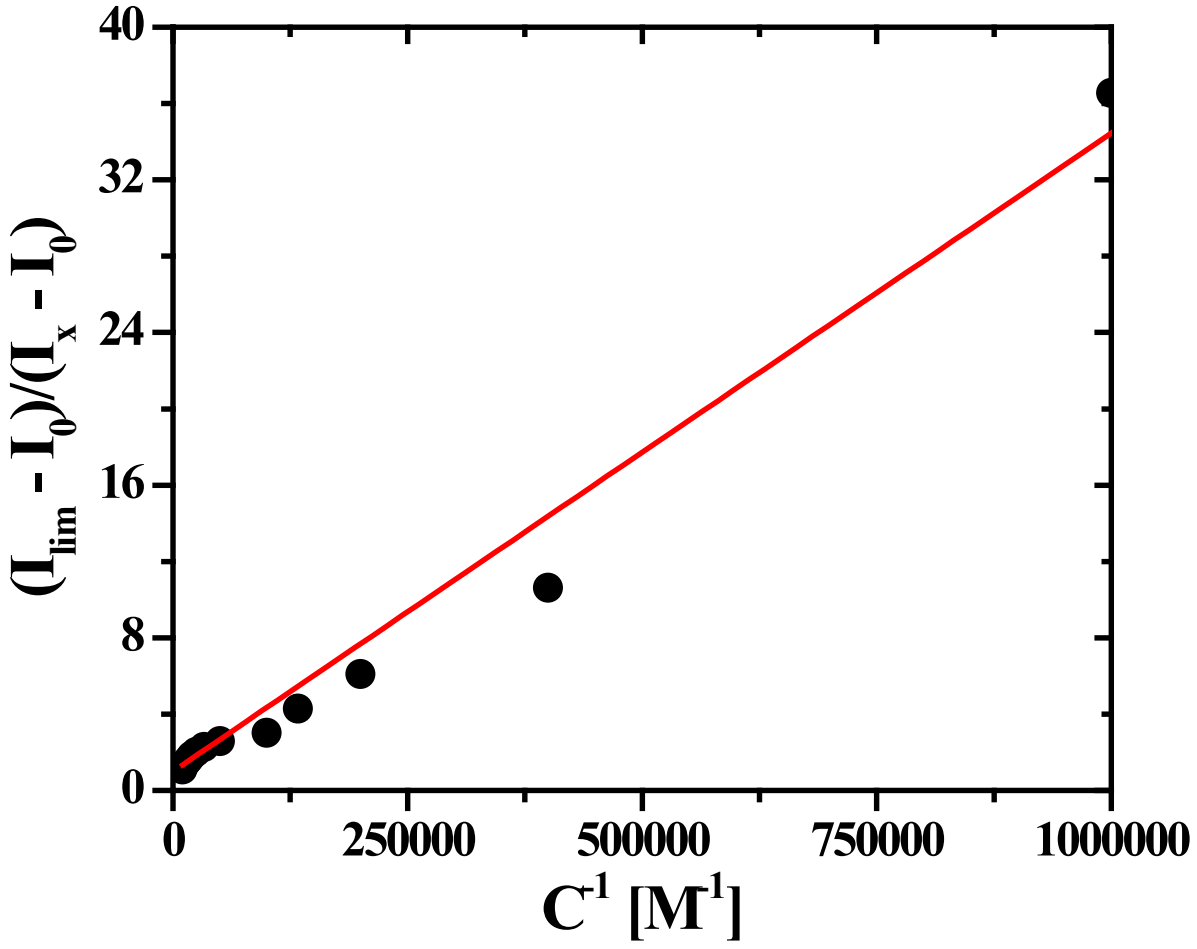

Fig. S3 Determination of the binding constant of **RTA** with SCN^- using the UV-vis technique.

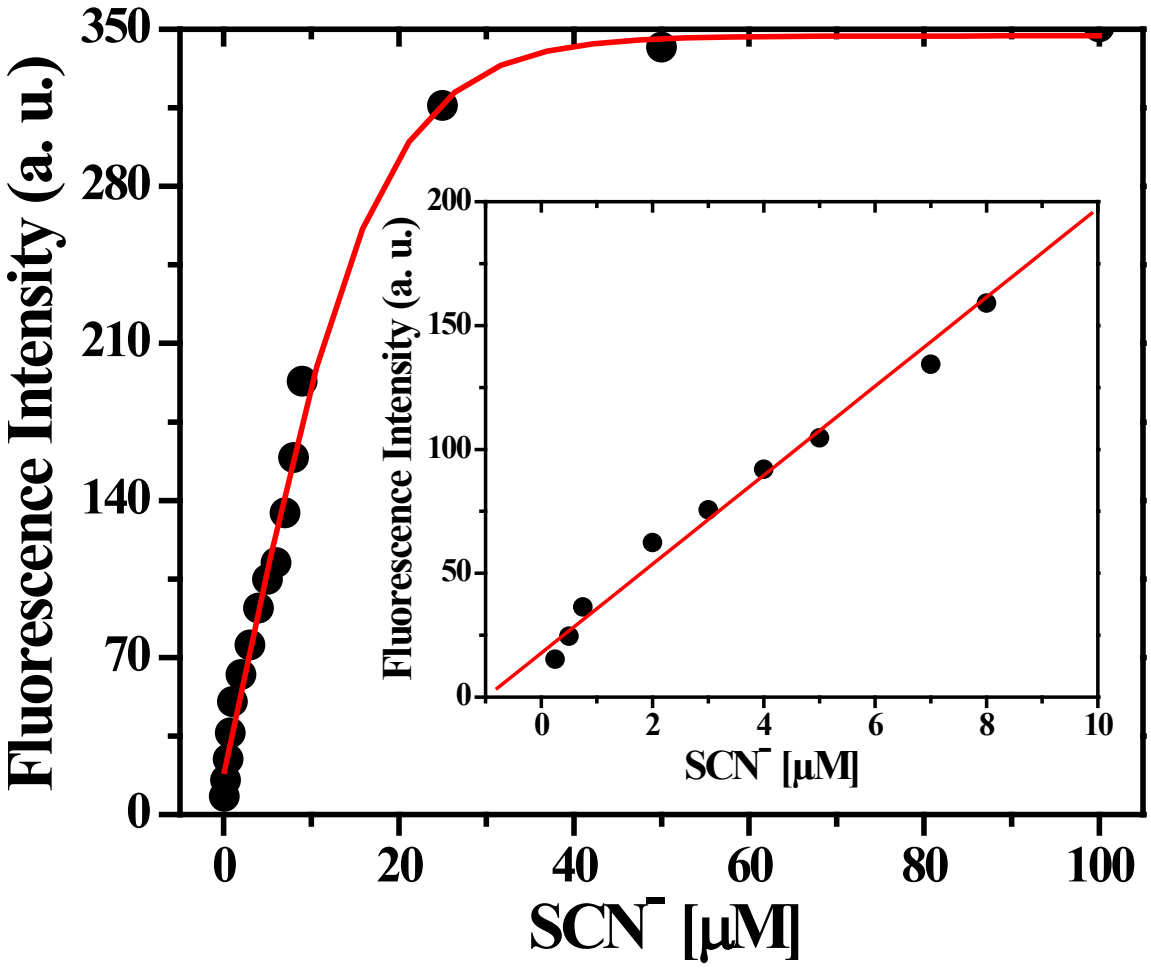

Fig. S4 Fluorescence intensity *vs.* externally added SCN^- (1–100 μM). Inset shows the linearity of the fluorescence intensity, observed up to 10 μM of added SCN^-.

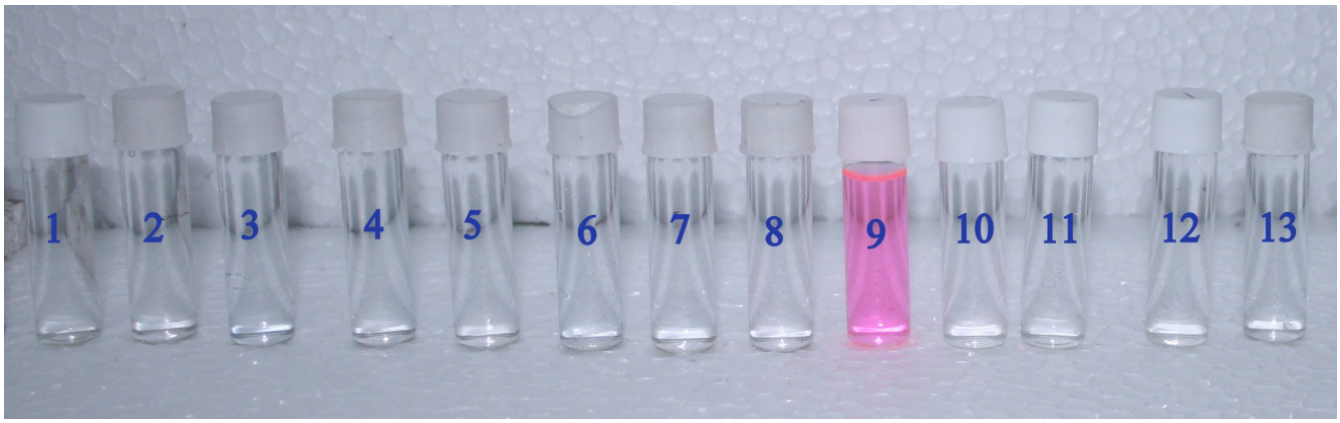

Fig. S5 Naked eye view of **RTA** (1) and in the presence of different anions: Cl^- (2), OCl^- (3), N_3^- (4), NCO^- (5), NO_3^- (6), CH_3COO^- (7), ClO_4^- (8), SCN^- (9), HPO_4^{2-} (10), SO_4^{2-} (11), S^{2-} (12) and $H_2AsO_4^-$ (13).

Fig. S6 UV light exposure colour of **RTA** (1) and in the presence of different anions: Cl^- (2), OCl^- (3), N_3^- (4), NCO^- (5), NO_3^- (6), SCN^- (7), CH_3COO^- (8), ClO_4^- (9), HPO_4^{2-} (10), SO_4^{2-} (11), S^{2-} (12) and $H_2AsO_4^-$ (13).

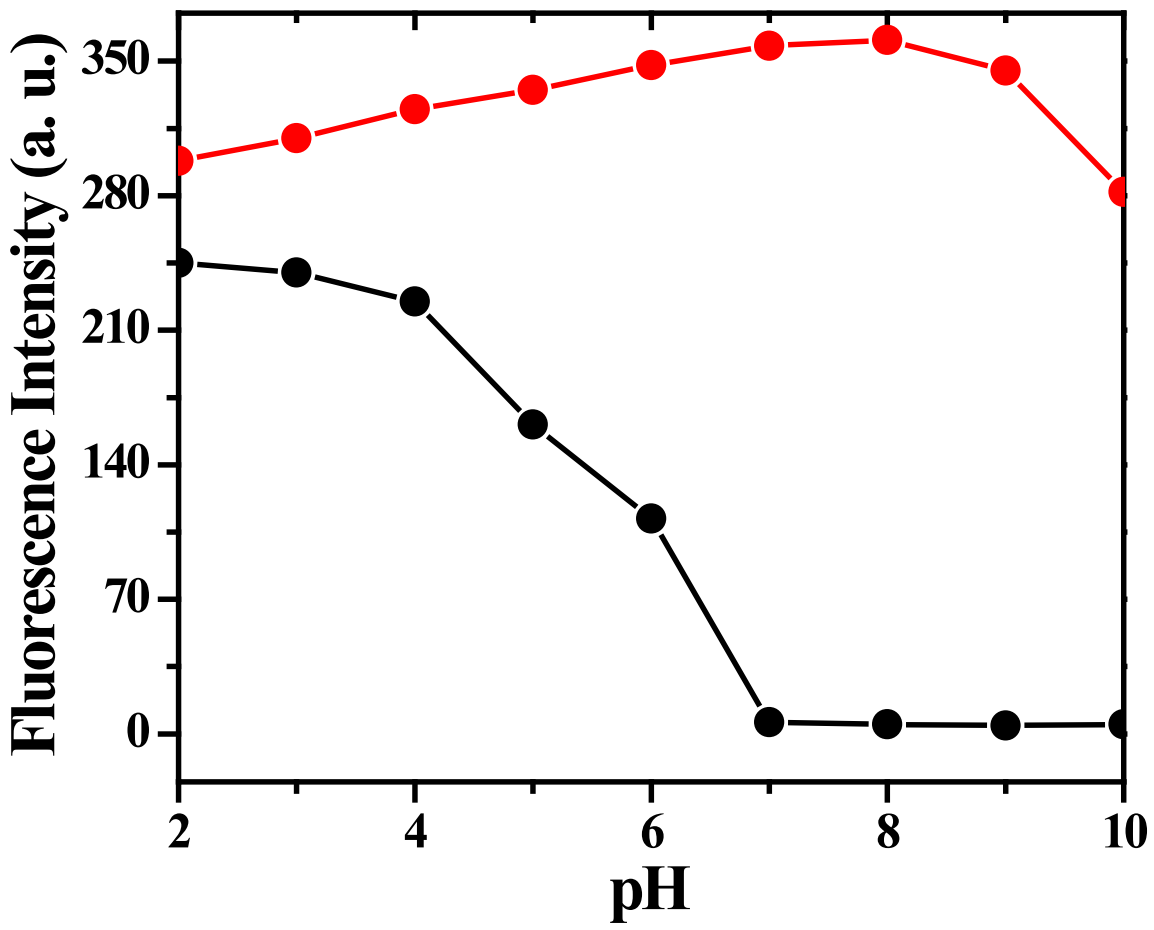

Fig. S7 Effect of pH on the emission intensity of **RTA** (black) and the [**RTA**–SCN^-] system (red).

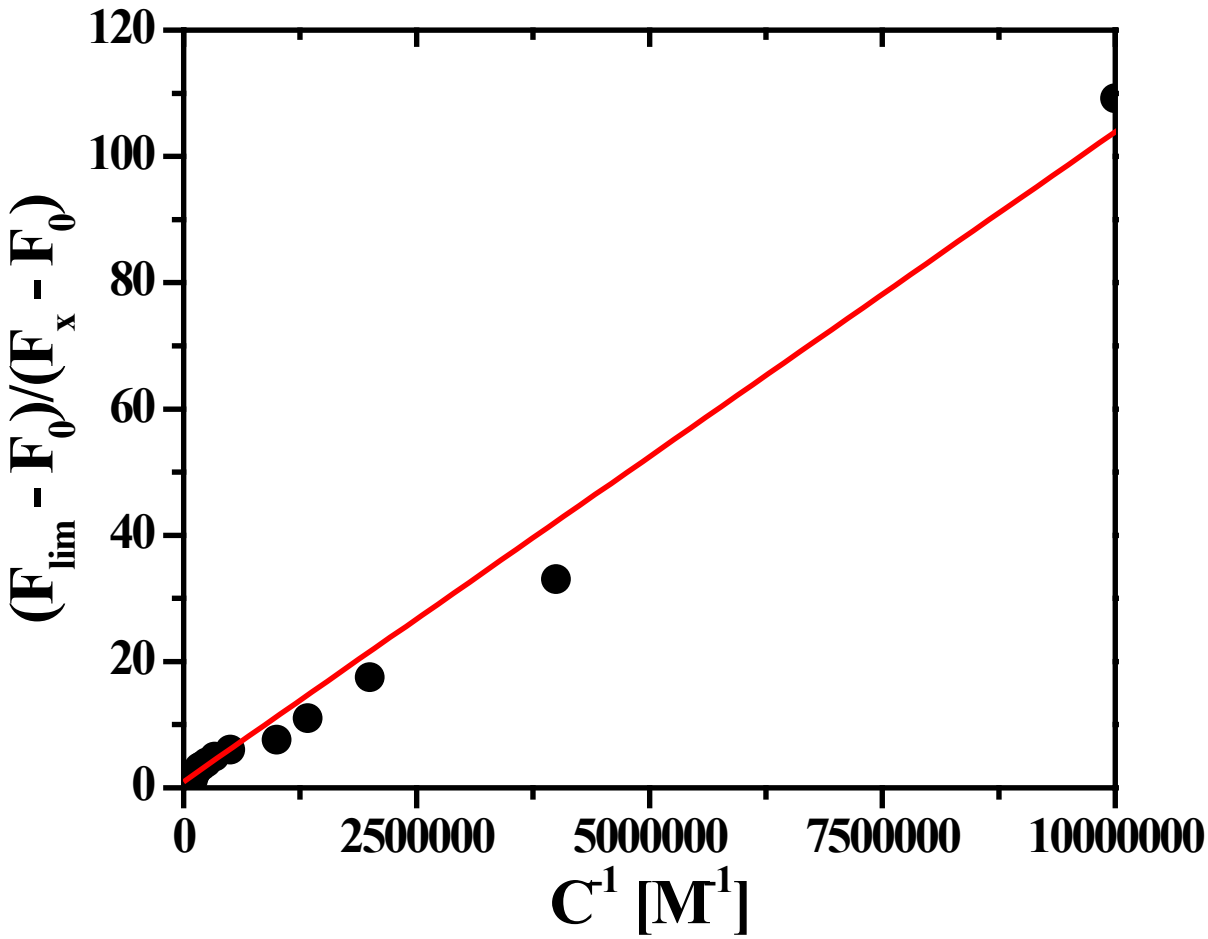

Fig. S9 Determination of the binding constant of **RTA** with SCN^- using $(F_{max} - F_0)/ (F_x - F_0) = 1 + (1/K) \times (1/[M]^n)$, where F_{max}, F_0 and F_x are fluorescence intensities of **RTA** in the presence of SCN^- at saturation, free **RTA** and at any intermediate SCN^- concentration, respectively.

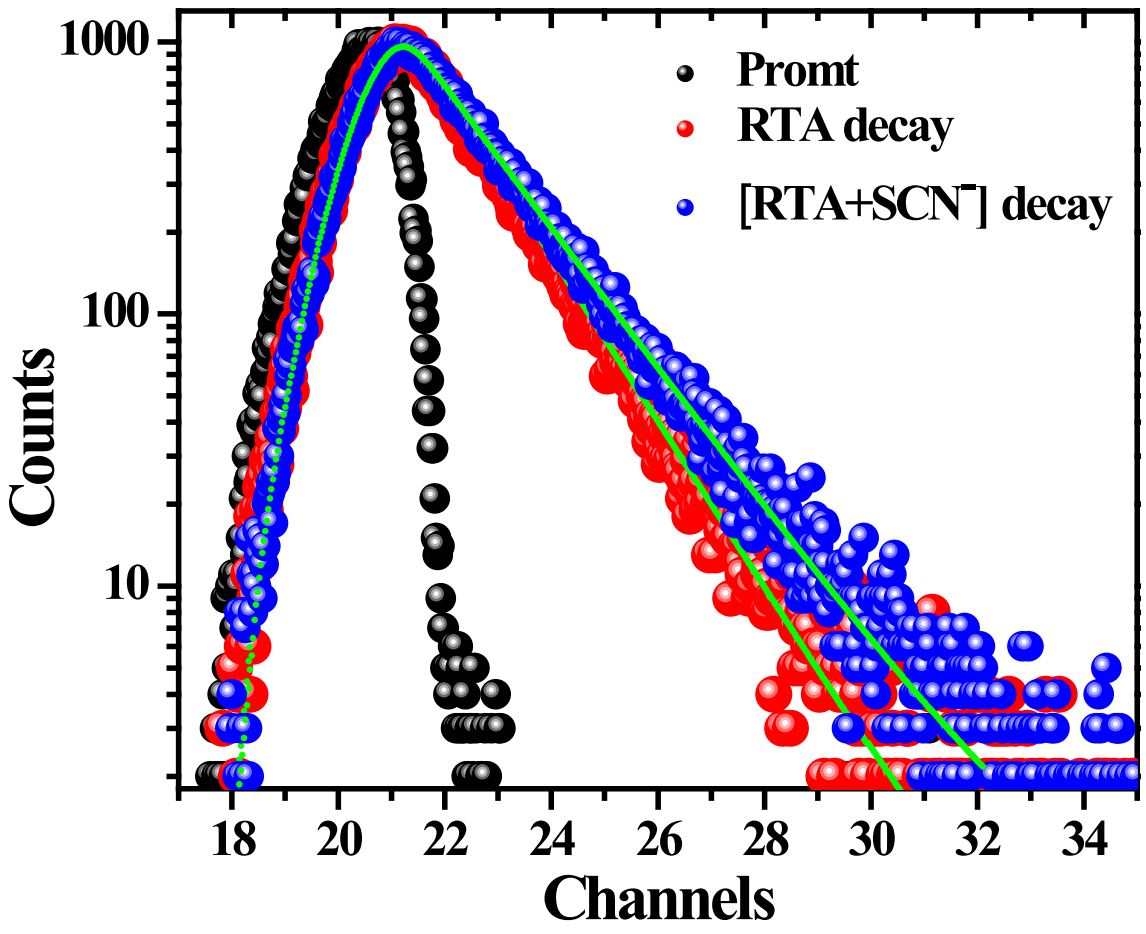

Fig. S9 Fluorescence lifetime decay of **RTA** (1μM) and the [**RTA**+SCN^-] system in HEPES buffered (0.1 M; DMSO–H_2O, 7:3 v/v; pH 7.4) solution (λ_{em} = 577 nm).

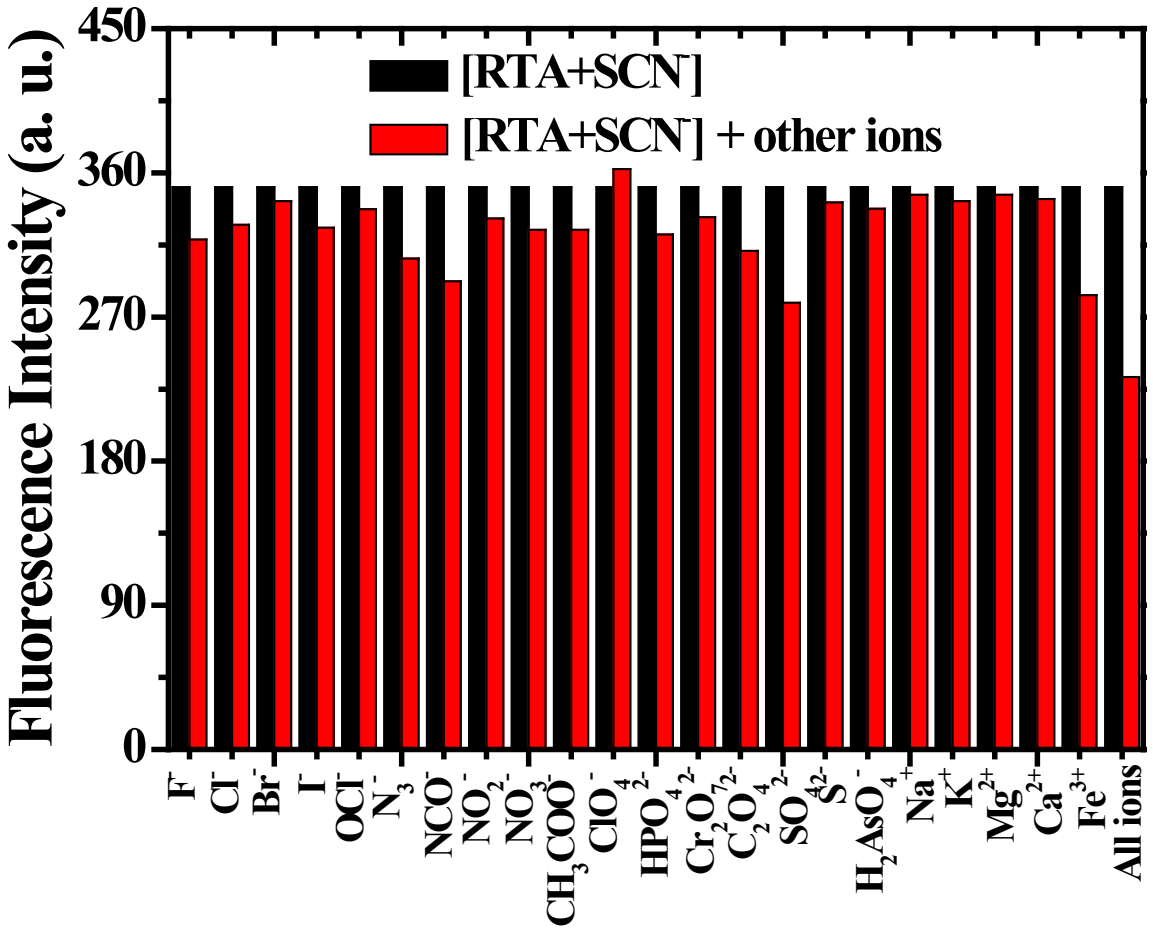

Fig. S10 Ion selectivity of **RTA** (1 μM) in HEPES buffered (0.1 M; DMSO–H_2O, 7:3 v/v; pH 7.4) solution with λ_{ex} = 540 nm. Black bars represent the emission intensity of the [**RTA**+SCN^-] system and red bars show the fluorescence intensity of the [**RTA**+SCN^-] system in the presence of 1 μM of different anions or cations.

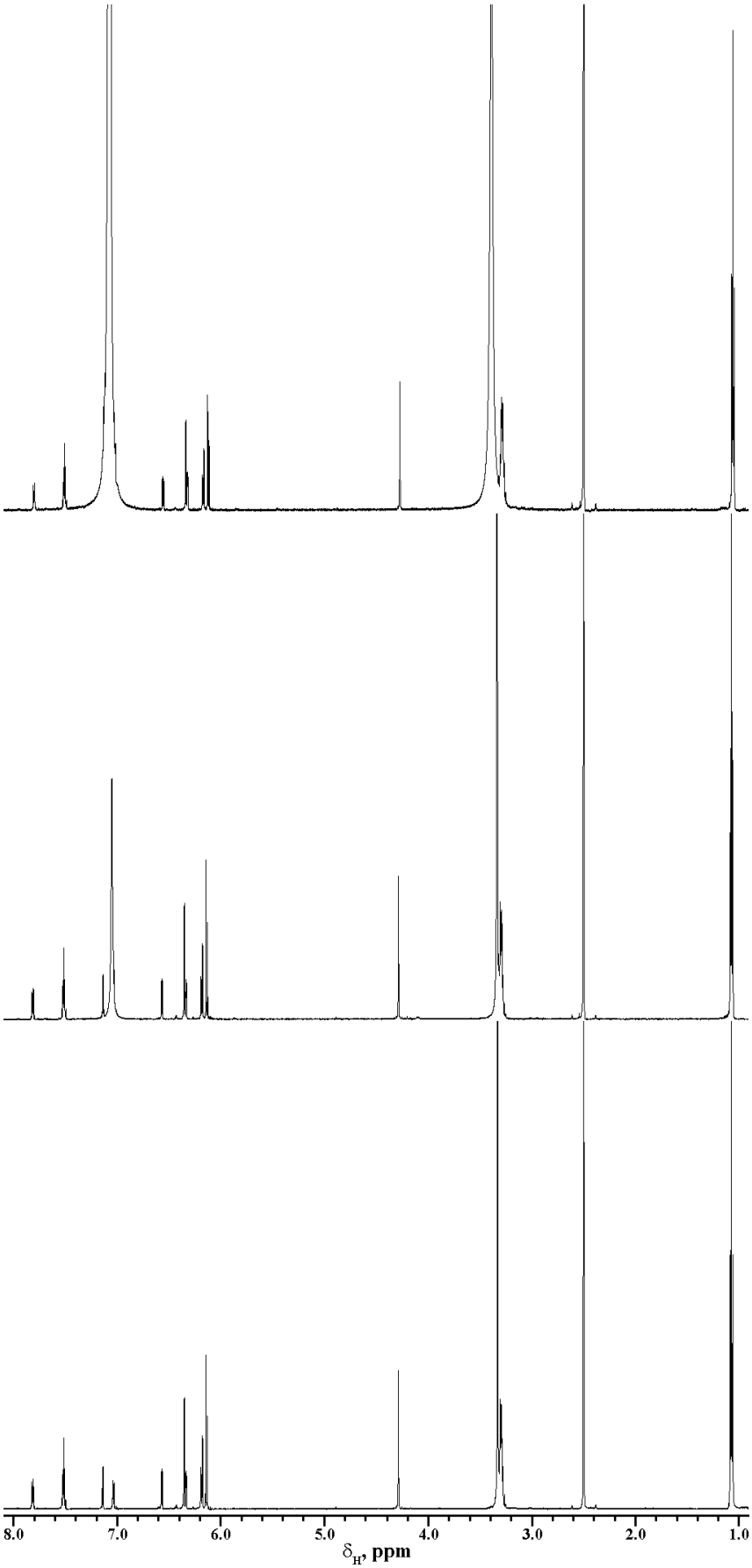

Fig. S11 ^{1}H NMR spectra of **RTA** (bottom), **RTA** + 1 equivalent of SCN^{-} (middle) and **RTA** + 5 equivalents of SCN^{-} (top) in DMSO-d_6.

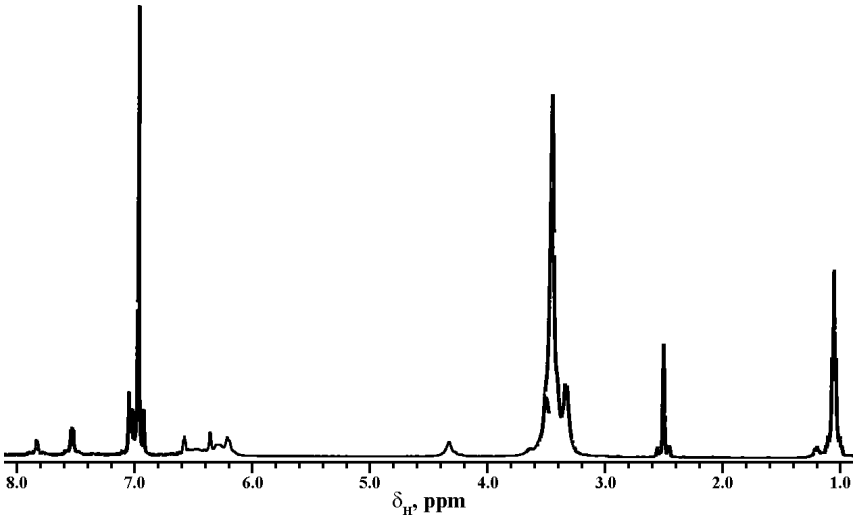

Fig. S12 ^{1}H NMR spectrum of the **[RTA+SCN^{-}]** adduct in DMSO-d_6.

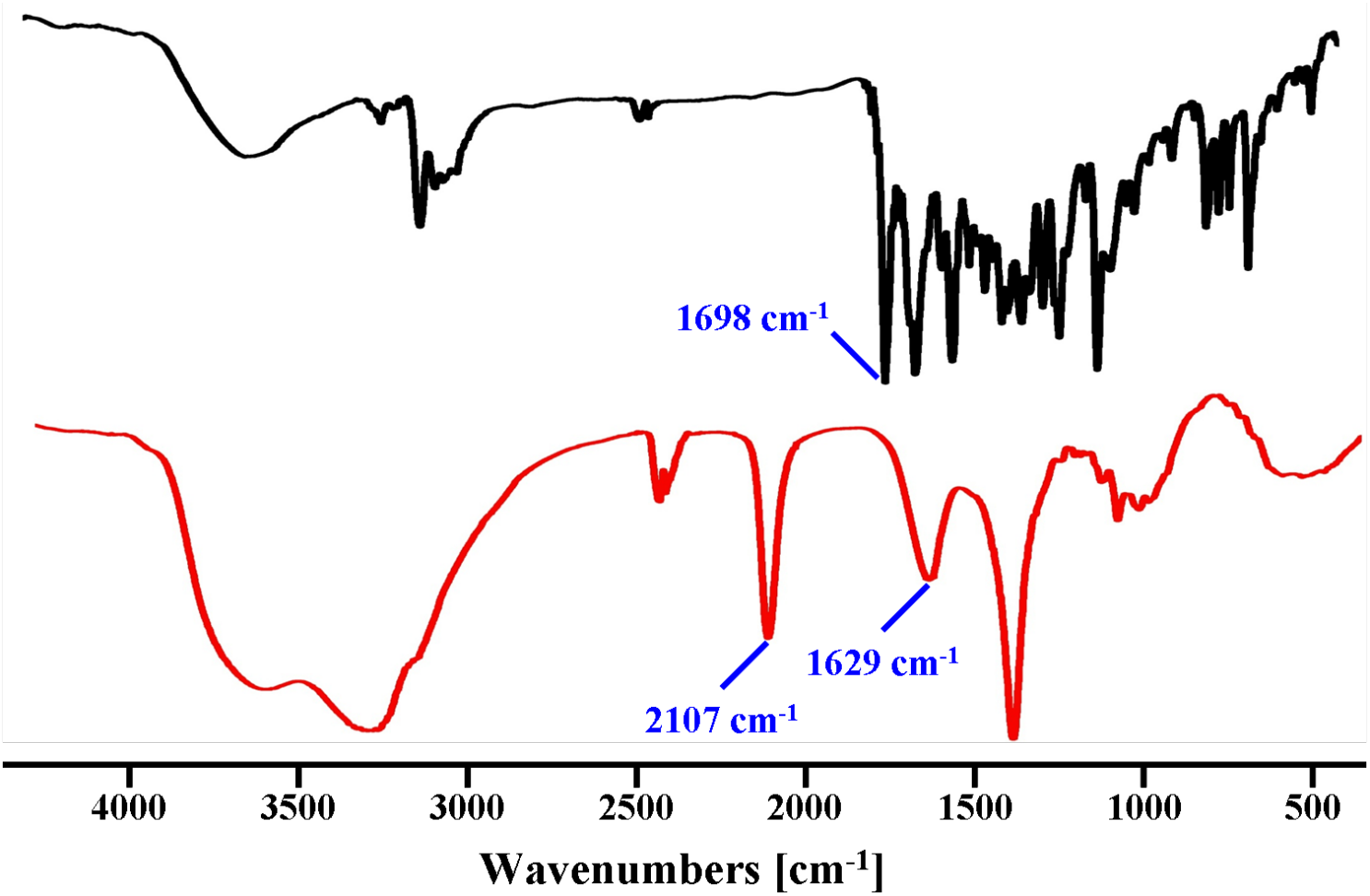

Fig. S13 IR spectra of **RTA** (black) and the **[RTA+SCN$^-$]** adduct (red).

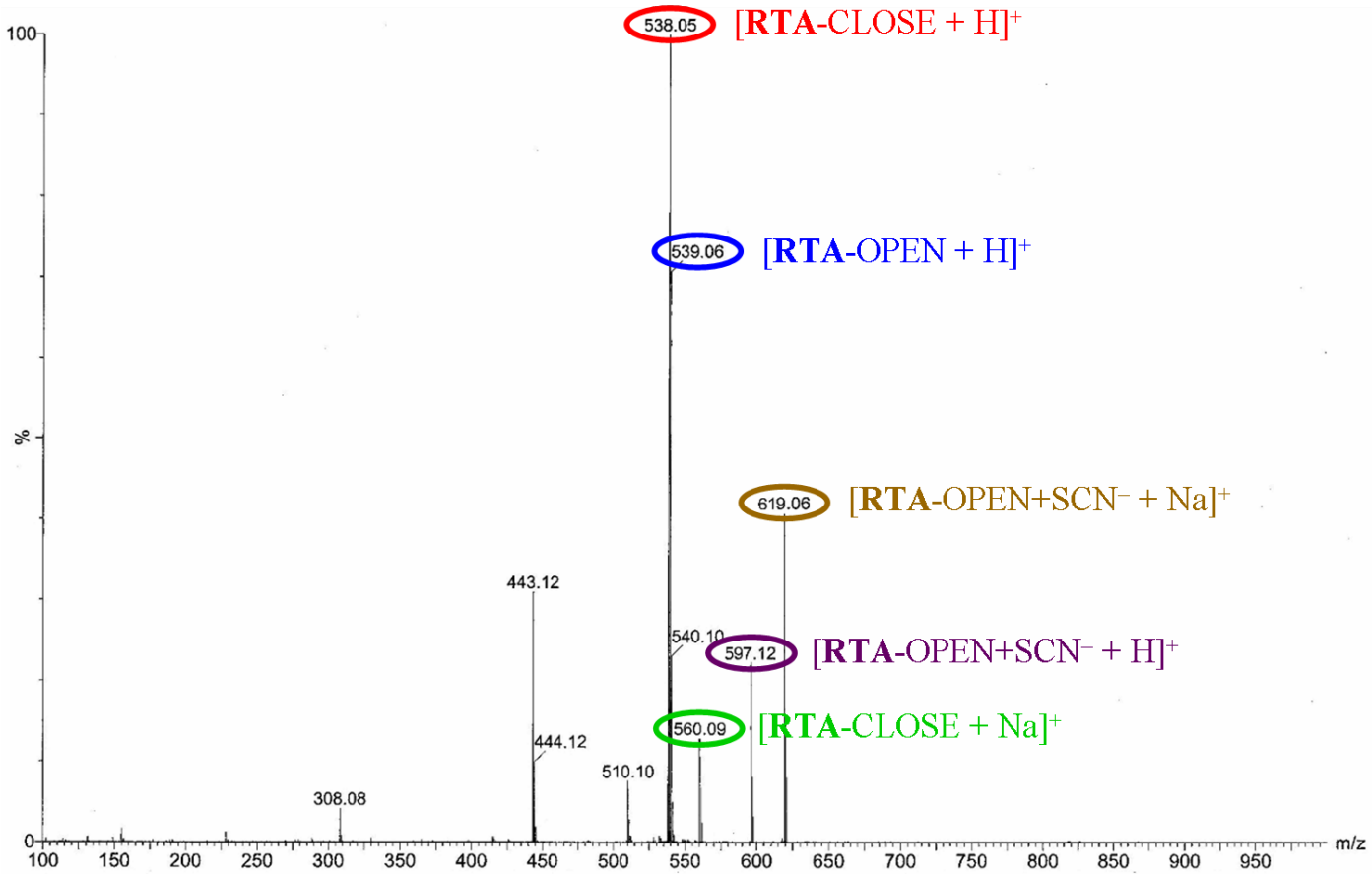

Fig. S14 ESI-TOF MS^+ spectrum of the **[RTA+SCN$^-$]** adduct in CH_3CN–H_2O (7:3 v/v).

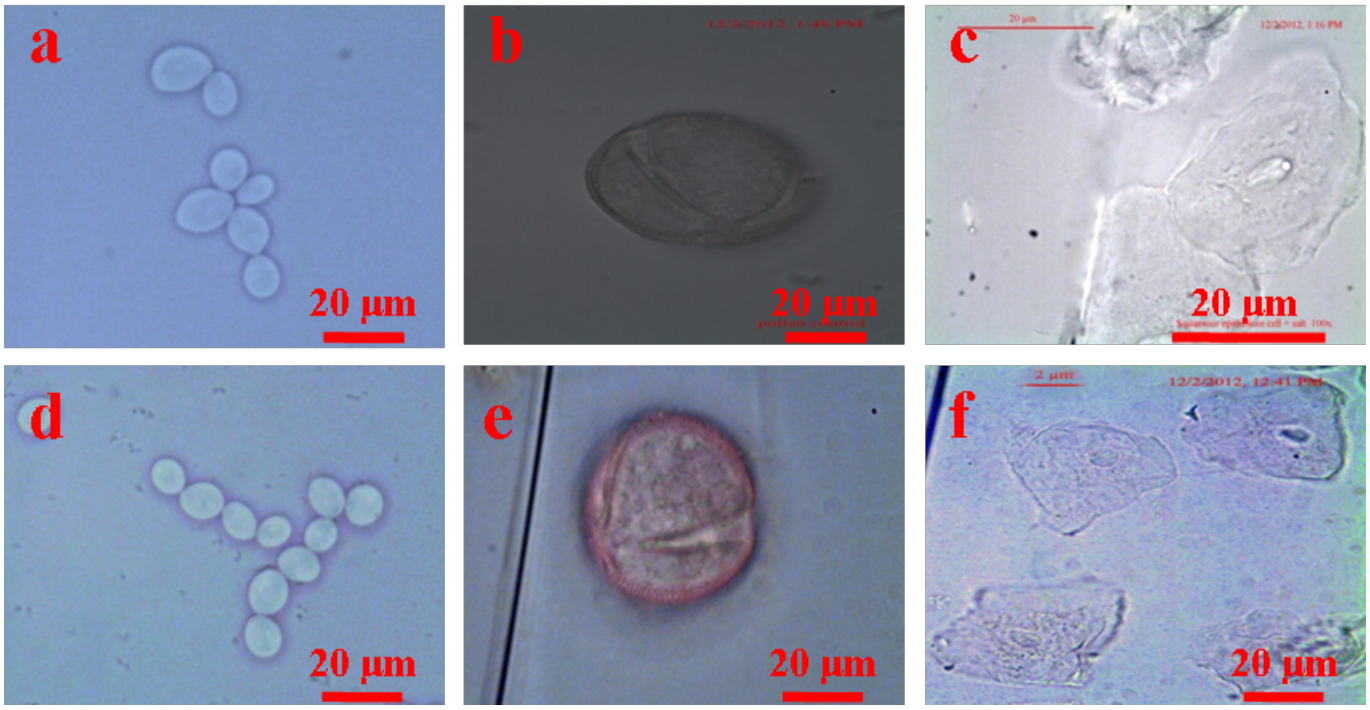

Fig. S15 Bright field images of *Candida albicans* (**a**), pollen grains of *Tecomastans* (**b**) and scquamus epithelium (**c**) cells. Bright field images of *Candida albicans* (d), pollen grains of *Tecomastans* (**e**) and scquamus epthilium (**f**) cells treated with **RTA** (1 µM) and SCN^-.

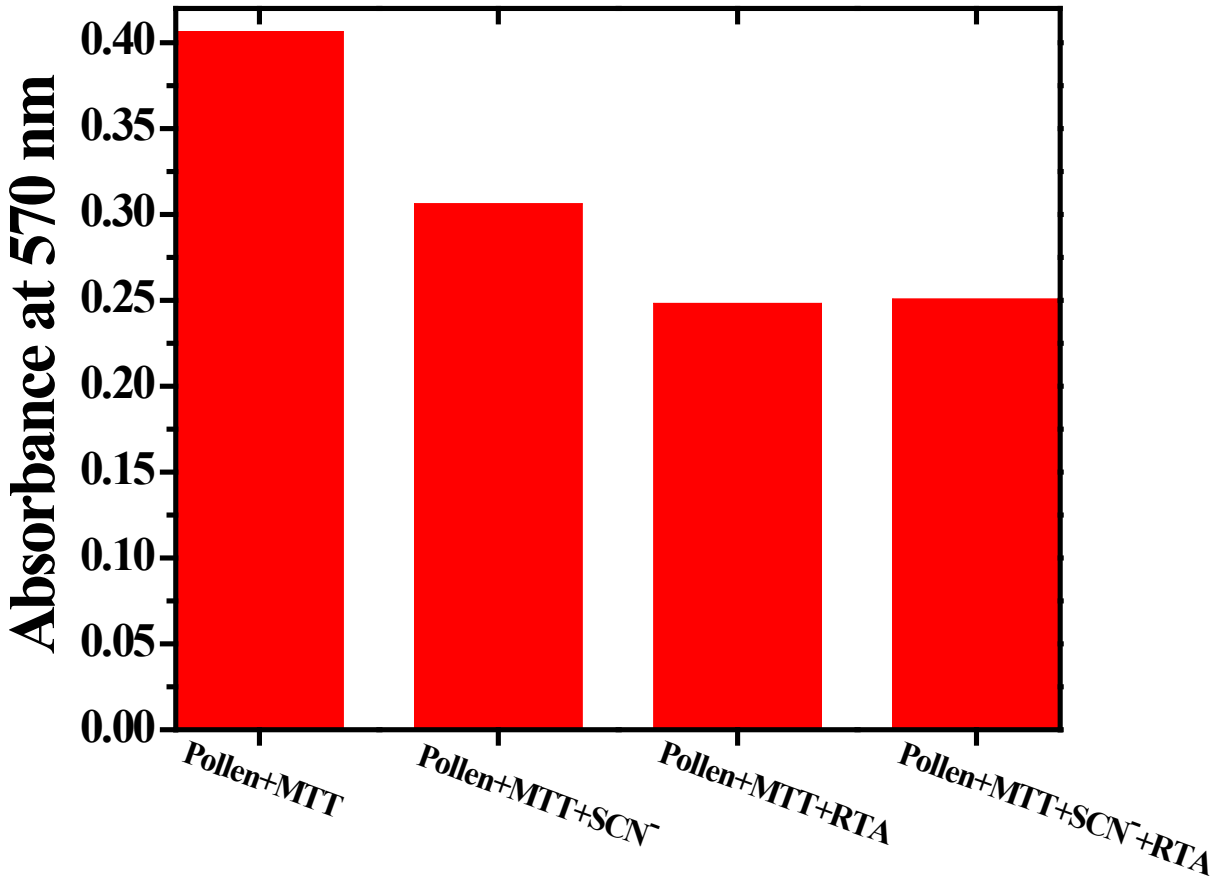

Fig. S16 MTT assay studies of **RTA**.

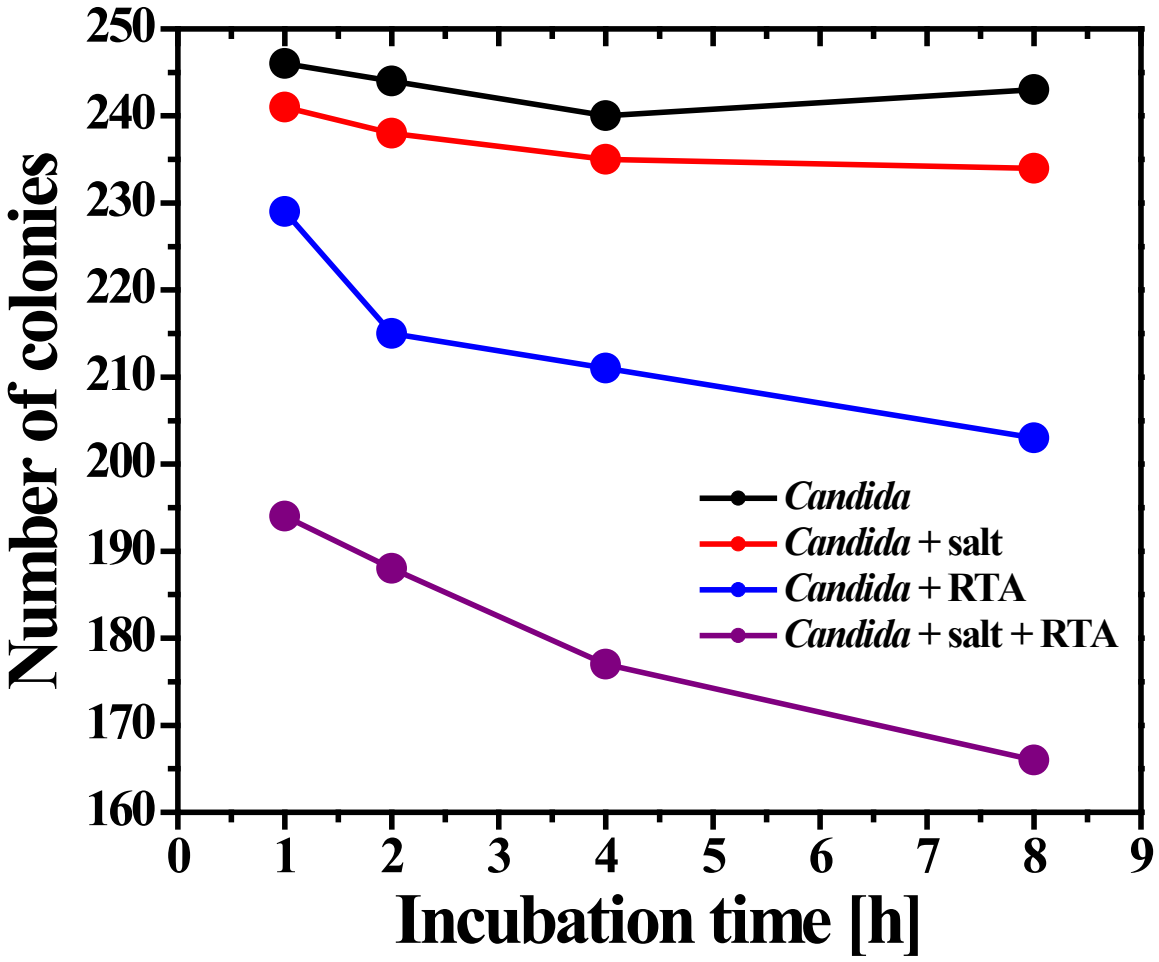

Fig. S17 Cell viability studies of **RTA**.

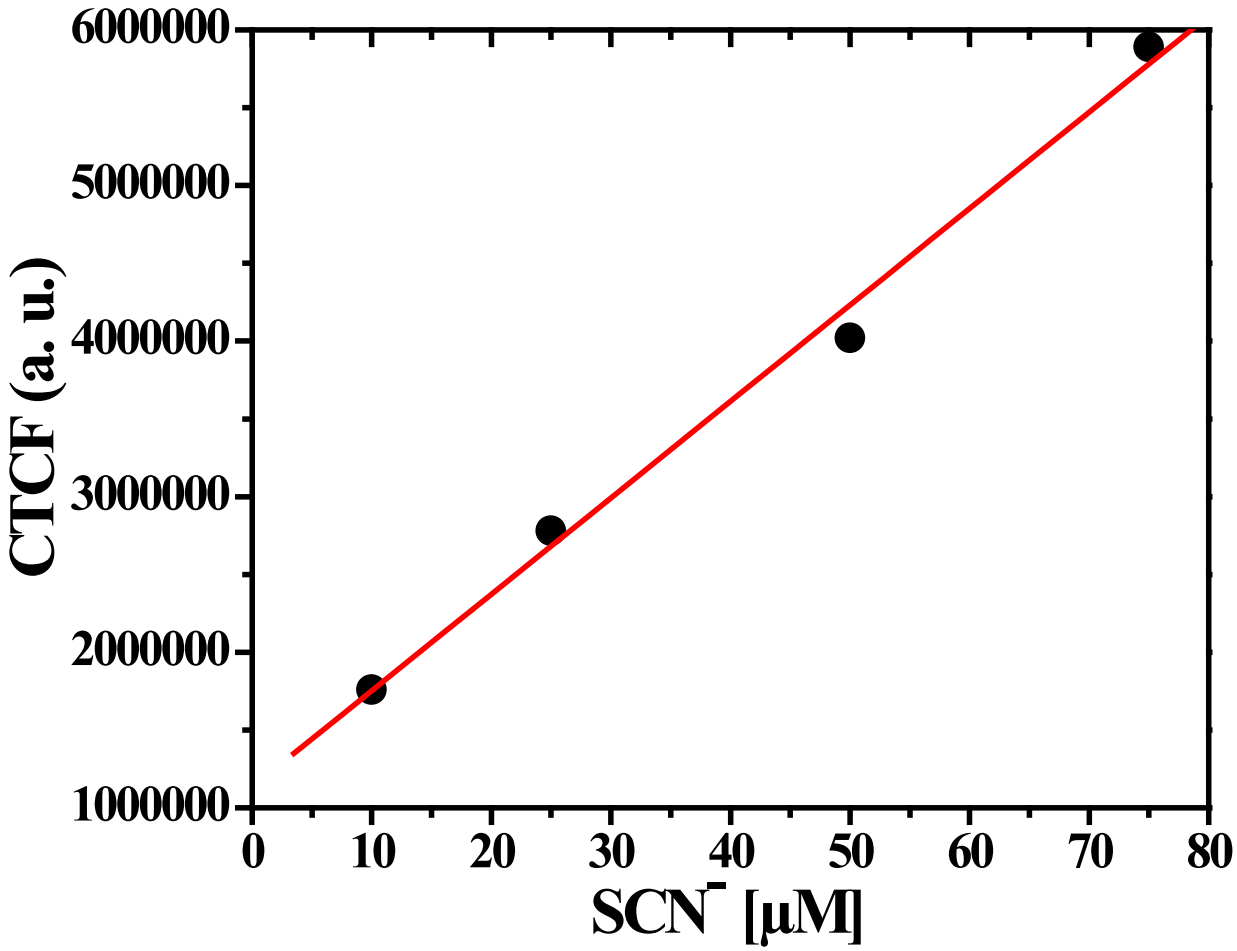

Fig. S18 Corrected Total Cell Fluorescence (**CTCF**) *vs.* concentration of SCN^- (10–75 μM).