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A rhodamine-quinoline based chemodosimeter capable of recognising endogenous OCl^- in human blood cell

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A rhodamine-quinoline based chemodosimeter (RHQ) has been designed, synthesized and characterized in this paper. The structure of the sensor is confirmed through single crystal X-ray study. It detects hypochlorite (OCl^-) selectively among other analytes studied. It showed colorimetric and orange-red fluorescence "turn-on" upon addition of OCl^-. The OCl^- -promoted ring opening of the rhodamine spirolactam ring in RHQ evokes a large absorbance as well as fluorescence enhancement in water/acetonitrile (1/1, v/v) medium with no significant response to other competitive analytes. Furthermore, we demonstrate here RHQ can endogenously detect OCl^- in human blood cell (Peripheral blood mononuclear cell). It also exhibits excellent performance in "dip stick" method. The optimized structure of the probe is calculated by density functional theory calculations. Moreover, the limit of detection of the probe is in 10^{-8} M range.

Introduction

Hypochlorous acid (HOCl) is known to be one of the biologically important ROS (reactive oxygen species), [1] which is weakly acidic. It partially dissociates into the hypochlorite ion (OCl^-) in physiological pH solutions.[2] In living organisms, hypochlorous acid is generated by the reaction of hydrogen peroxide with chloride ions under the catalysis of the heme enzyme myeloperoxidase (MPO), which is synthesized and secreted by activated phagocytes.[3-5]

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$$

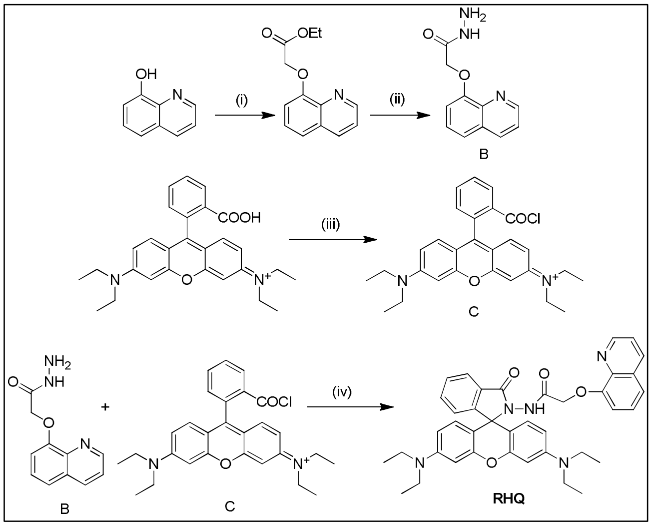
On the other hand, the concentrated hypochlorite solution is a potential hazard to humans and animals. It involves oxidation of important biomolecules such as plasma membrane ATPasaes, collagen, ascorbate, proteins including α_1-antiproteinase, nucleotides, sulfhydryls, thioethers, DNA and DNA-repair enzymes, depletes intracellular ATP and reduced glutathione (GSH), ultimately enhancing cell death.[6] Abnormal levels of hypochlorite lead to a series of complications including cardiovascular diseases, neuron degeneration, diabetes, arthritis, cancer and ageing.[7]

In this paper we have established that RHQ fluorescence probe has successful potential of sensing endogenous HOCl for imaging of living human peripheral blood mononuclear cells (PBMCs).

Colorimetric, luminescent, electrochemical and chromatographic methods have been reported to monitor hypochlorite.[8] In the past few years, fluorescence sensors are widely regarded as one of the most effective way for sensor design due to the high sensitivity, specificity, simplicity of implementation and ability for real-time monitoring. Even though there are a number of HOCl sensors available, real biological applications of probes which enable us to monitor microbe-induced HOCl production are rare. Therefore, it is a challenging task to develop imaging probe for HOCl with a high selectivity and sensitivity, which can be applicable to various biological systems.

In recent years, a large number of sensors based on rhodamine platform have been reported due to their excellent photophysical properties, high quantum yields, good water solubility and high photostability.[9] The sensing mechanism of the rhodamine based dyes involves the opening of the spirolactam ring to give a pink color along with a fluorescence 'turn-on' response. In continuation of our work,[10] herein we present, such a HOCl induced 'turn-on' fluorescent probe (RHQ) using rhodamine-quinoline moiety in aqueous acetonitrile (1/1, v/v, 25^0C) media.

The synthetic scheme of the receptor is shown below (Scheme 1). Intermediate compounds B[11] and C[12] are prepared according to the literature procedures. Treatment of compound B with compound C affords the receptor. The detailed experimental procedure and characterization data are explained in the following.



Scheme 1: Synthetic scheme of the probe RHQ

Reagents and conditions: (i) Ethyl chloroacetate, K_2CO_3, TBAB, Acetone, reflux, 4 h. (ii) Hydrazine hydrate, EtOH, reflux, 2 h. (iii) $POCl_3$, 1,2 dichloro ethane, reflux, 4h. (iv) Acetonitrile, reflux, 12 h.

Experimental

General

Unless otherwise mentioned, materials were obtained from commercial suppliers and were used without further purification. Thin layer chromatography (TLC) was carried out using Merck 60 F254 plates with a thickness of 0.25 mm. Melting points were determined on a hot-plate melting point apparatus in an open mouth capillary and are uncorrected. ^1H and ^{13}C NMR spectra of RHQ were recorded on JEOL 400 MHz and 100 MHz instruments respectively. For NMR spectra, $CDCl_3$ was used as solvent using TMS as an internal standard. Chemical shifts are expressed in δ units and ^1H–^1H and ^1H–C coupling constants in Hz. Fluorescence spectra were recorded on a PerkinElmer LS55 spectrophotometer and UV-vis titration experiments were performed on a JASCO UV-V530 spectrophotometer.

UV-vis method

For UV-vis titration, we used the solution of the host in the order of 10μM. The solution was prepared in CH_3CN: H_2O (1:1, v/v, 25^{0}C). The solutions of the guest analytes using their sodium salts in the order of 2 × 10^{-4} M, were prepared in de-ionized water. Now, different concentrations of host and increasing concentration of analytes were prepared separately and the spectra of these solutions were recorded by means of UV-vis method.

Fluorescence method

Now, for the fluorescence titration the solution of the receptor was prepared (10 μM) in CH_3CN:H_2O (1:1, v/v, 25^{0}C) medium. The solutions of the guest analytes using their sodium salts in the order of 2 × 10^{-4} M, were prepared in deionised water. Here also various concentrations of guest and increasing concentration of analytes were prepared and the fluorescence spectra were recorded.

Synthesis of the receptor (RHQ):

To a stirred solution of rhodamine B (500 mg, 1.04 mmol) in 1,2-dichloroethane (20 ml), $POCl_3$ (0.5 ml, 5.22 mmol) was added in a drop-wise manner over 5 minutes at 0^{0}C. After complete addition whole reaction mixture was refluxed for 4 hours. The reaction mixture was cooled to room temperature and the solvent was evaporated to get the crude acid chloride of rhodamine B (C). The compound was used directly in the next step. Acetonitrile was added to dissolve the acid chloride (C) and a mixture of compound B (250 mg, 1.15 mmol) and triethyl amine (2 ml) in acetonitrile was added drop-wise to it. The reaction mixture was refluxed under N_2-atm for 12 hours. Solvent was evaporated and water was added to it when a pink colored solid was precipitated. The crude solid was purified through column chromatography using 2% CH_3OH in $CHCl_3$ as eluent to get a light pink colored solid (Yield = 400 mg, 60%). **Mp** = 138-140^{0}C.

Solubility: Soluble in $CHCl_3$, CH_2Cl_2, CH_3CN, DMSO, MeOH (Partly), EtOH (Partly), THF.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.20 (t, *J*=5.6 Hz, 12 H), 3.45 (q, *J*=5.6 Hz, 8 H), 4.72 (s, 2H), 10.25 (s, 1H), 6.44 (m, 6 H), 7.11 (m, 4 H), 7.48 (m, 4H), 8.20 (m, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ15.3, 44.5, 66.0, 77.4, 97.6, 104.57, 107.74, 108.42, 109.05, 121.58, 123.38, 123.84, 124.18, 124.87, 126.94, 128.20, 129.09, 129.56, 132.97, 135.85, 148.72, 153.76, 154.03, 154.34, 164.58, 167.18.

HRMS (ESI positive): Calcd for $C_{39}H_{39}N_5NaO_4$ $[M+Na]^+$ (m/z): 664.2900; found: 664.2900

Fluorescence imaging of living human PBMCs:

3ml of venous blood was obtained from volunteer donors (age >50 years) with their informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation by histopaque-1077 obtained by SIGMA. PBMCs were washed and suspended in PBS. RHQ samples were prepared in 50% DMSO and 50% PBS. PBMCs were then incubated with 50μmol/l RHQ sample for 15 minutes at 37^{o}C. Cells were observed under fluorescence microscope (Carl Zeiss HBO 100) with fluorescence emission at 580nm.

Fluorescence life time method:

Fluorescence lifetimes were measured using a time-resolved spectrofluorometer from HORIBA Scientific. The instrument uses a picoseconds diode laser (NanoLed-07, 284 nm) as the excitation source and works on the principle of time-correlated single photon counting. The goodness of fit was evaluated by χ^2 criterion and visual inspection of the residuals of the fitted function to the data.

Method of Crystallization:

An amount of 5 mg of RHQ was dissolved in a vial in 10µl $CHCl_3$ then about 1 ml of CH_3CN was added to it. Keep the vial gently in a cool place without any perturbation. After 2 days fine colorless crystals were obtained.

Results and Discussions

UV-vis study

To examine the selectivity of the sensor (RHQ, 10 µM, water acetonitrile 1/1, v/v, 25^0C), UV-vis and fluorescence titration experiments were performed using common interfering analyts (S^{2-}, N_3^-, NO_2^-, NO_3^-, H_2O_2, $O_2^{\cdot-}$, SO_3^{2-}, Cl^-, I^-, F^- and SO_4^{2-}) in water.

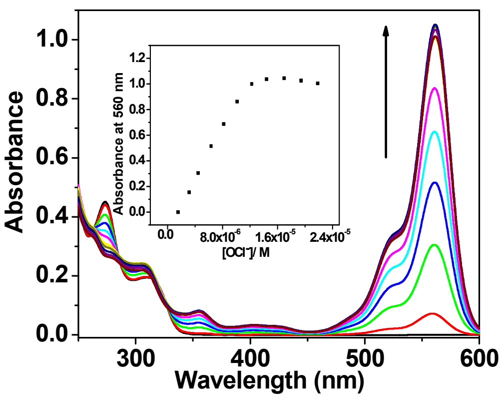
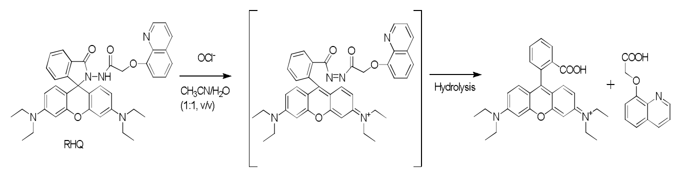


Figure 1: Absorption spectra of RHQ (10 µM) upon titration with OCl^- (0 to 2.5equivalents) in CH_3CN-H_2O (1/1, v/v, 25^0C) solution. Inset: Plot of absorbance of RHQ at 560 nm depending on the OCl^- concentration.

The analyte binding properties of the chemodosimeter are studied by employing the sodium salts of the anions. The solution of the receptor in this mixed aqueous media showed two absorption spectral bands at 270 and 308 nm. There was no detectable band at 500-600 nm to ensure the presence of spirolactam ring of the receptor under the experimental condition (Figure 1). Upon addition of OCl^- to a colourless solution of RHQ (10 µM), a low-energy strong absorption band centred at 560 nm is observed.

The intensity of the band increases regularly as the amount of OCl^- is added progressively (up to 2.5 equiv.). The mechanism behind this kind of sensing phenomenon is the OCl^- promoted oxidation, which actually leaves the rhodamine-B in its open ring platform (Scheme 2). This exhibits a colour change from colourless to pink-red. As shown in figure 2, other competing analytes showed insignificant effect on the absorption spectra of the receptor. A large enhancement of absorbance at 560 nm was observed upon addition of 1.2 equivalents of OCl^-.

This phenomenon indicates that the sensor can be employed conveniently for OCl^- detection by simple visual inspection. From the UV-vis titration experiments, it was illustrated that upon addition of OCl^- up to 1.2 equivalents, the absorbance at 560 nm increases linearly and it reached maxima. Further addition of OCl^- (up to 2.5 equiv.) produces insignificant changes in absorption spectra.



Scheme 2: The chemodosimetric approach of OCl^- after addition in RHQ

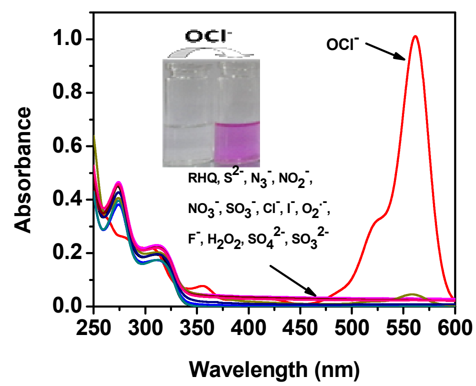


Figure 2: Changes of absorption spectra of RHQ (10 µM) upon addition of different anions (5 equivalents) in (CH_3CN-H_2O, 1/1, v/v, 25^0C) solution. Inset: visible colour change of RHQ upon addition of 2 equivalents of OCl^- in ambient light.

Emission study

The fluorogenic response of RHQ was examined by monitoring the fluorescence behavior upon addition of several analytes (S^{2-}, N_3^-, NO_2^-, NO_3^-, H_2O_2, $O_2^{\cdot-}$, SO_3^{2-}, Cl^-, I^-, F^- and SO_4^{2-} in H_2O, 2×10^{-4} M) in water-acetonitrile (1/1, v/v, 25^0C , pH = 7.2). The free receptor (10 µM) exhibits a very weak emission (Φ = 0.005) band at 580 nm upon excitation at 530 nm. With the addition of OCl^-, there arises a remarkable enhancement of fluorescence with an emission band at 580 nm, which is accompanied with the opening of the spirolactam ring of the receptor to form a intermediate product upon reaction with OCl^- (Scheme 2). In aqueous acetonitrile solution the intermediate product was hydrolyzed and finally gives the rhodamine-B itself which is proved by the mass spectral help (Figure S8, ESI). The fluorescence quantum yield calculated in this stage is 0.64, using rhodamine-B as reference (Φ = 0.68 in ethanol). Notably, addition of other co-existing analytes, even in excess amount, caused insignificant change in the emission intensity of the receptor. Addition of other examined analytes even in excess amount leads no significant change in the emission spectrum of the receptor.

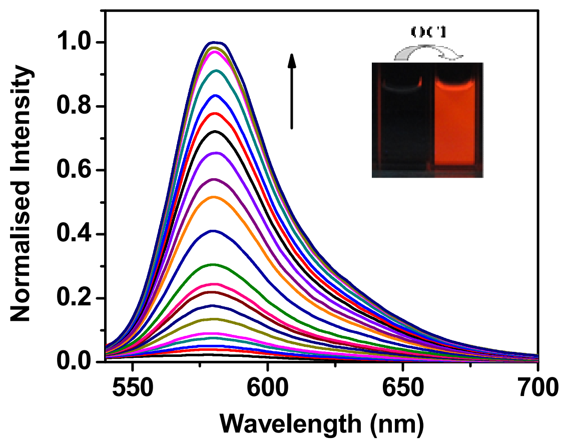


Figure 3: Fluorescence spectra of RHQ (10 μM) upon titration with OCl^- (0 to 3 equivalents) in CH_3CN-H_2O (1/1, v/v, 25^{0}C) solution. λ_{ex} = 530 nm. Inset: Emission colour change of RHQ upon addition of 2 equivalents of OCl- after illumination under UV light.

These do not affect the OCl^- detection of the probe by means of fluorescence spectroscopy. From fluorescence titration experiment (Figure 3) it revealed that a linear enhancement with increasing [OCl^-] up to 16 μM was observed. Higher OCl^- concentrations only caused insignificant emission enhancement for RHQ at 580 nm.

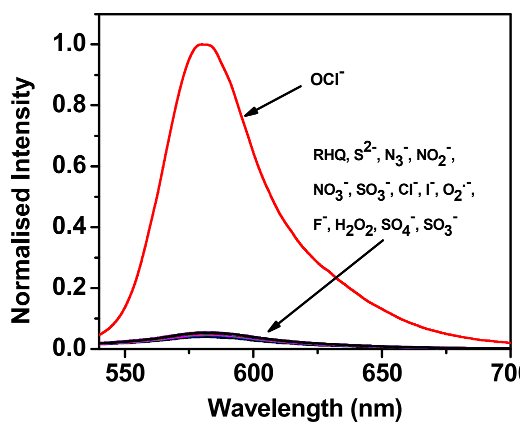


Figure 4. Changes of emission spectra of RHQ (10 μM) upon addition of different metal ions (5 equivalents) in (CH_3CN-H_2O, 1/1, v/v) solution.

The detection limit of the probe for OCl^- was evaluated from the fluorescence titration and determined to be 5.5 X 10^{-8} M, using the equation DL = K × Sb_1/S, where K = 3, Sb_1 is the standard deviation of the blank solution and S is the slope of the calibration curve[13] (see ESI). Figure 4 shows a comparative view of emission intensity of the probe after adding 5.0 equiv. each of the guest analytes.

A nano second time-resolved fluorescence technique has been adapted in order to examine the excited state behavior of our probe RHQ and its reaction based product with hypochlorite in CH_3CN-H_2O (1/1, v/v) solvent (Fig. 6). According to the equations $\tau^{-1} = k_r + k_{nr}$, where $k_r = \Phi/\tau$, the radiative rate constant k_r and the total non-radiative rate constant k_{nr} of RHQ and reaction based species were calculated. For RHQ, τ = 0.573 ns (χ^2 = 1.073) and RHQ + OCl^-, τ = 2.317 ns (χ^2 = 2.084) (Figure S9-S10, ESI, Table S1).

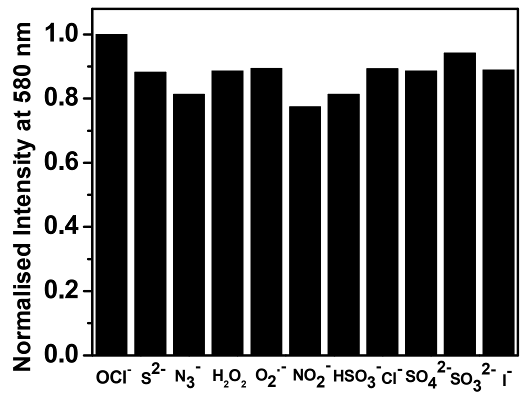


Figure 5. A comparative study of emission intensity after addition of different analytes (5 equivalents) in the solution of RHQ in presence of OCl^- (2 equivalents)

To utilize the receptor as a selective sensor for OCl^-, a competing experiment was also performed by adding OCl^- (2.0 equiv.) in presence of 5.0 equivalents of different analytes in RHQ solution. As shown in figure 5, the studies revealed that OCl^- can be detected by the sensor in presence of almost all the analytes studied. In this way it was concluded that RHQ can be used potentially for the quantitative detection of OCl^- with high selectivity.

Dip-stick method:

There is a number of sensors which can detect OCl^- only in the solution phase, which would restrict their sensitivity. So in order to investigate a practical application of this sensor, we perform an experiment called "dip-stick" method. It is a very simple but very important experiment because it gives instant qualitative information without resorting to the instrumental analysis. In order to perform this experiment, we prepared TLC plates which were immersed into the solution of RHQ (2X10^{-4} M) in acetonitrile, and then evaporating the solvent to dryness.

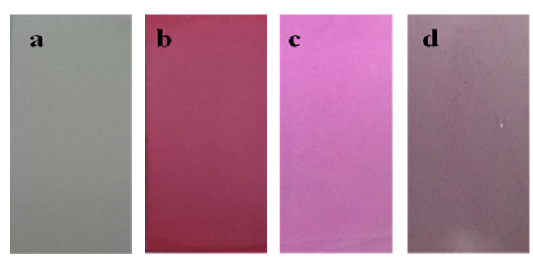


Figure 6. Color changes of RHQ on test paper in the absence (a) and presence of OCl^- (b) 2×10^{-3} M (c) 1×10^{-4} M and (d) 1×10^{-6} M under ambient light.

Now to investigate OCl^-, we immersed the TLC plate to different concentrations of OCl^- (2×10^{-3}, 10^{-4} M, 10^{-6} M) solution and then exposing it in air to evaporate the solvent. The colour of the TLC plates change from colourless to pink and also the fluorescence change from colourless to red. Now this experiment evokes a real time monitoring and devoid of using any instrumental analysis, just *via* naked-eye detection.

Crystal structure study

The molecular structure of RHQ showing 30% probability displacement ellipsoids and atom labelling scheme is depicted in Fig 7. The asymmetric unit of the RHQ contains two crystallographically independent molecules with similar geometries. The xanthenes ring system of molecule A forms dihedral angles of 29.27 and 87.68º with the quinoline and isoindoline ring systems, respectively. The corresponding dihedral angles for molecule B are 35.74 and 88.12º. The dihedral angle between the quinoline and isodoline ring systems for molecules A and B is 58.97 and 60.55º, respectively. The molecule B is stabilized by intramolecular C-H...O hydrogen bond, forming S(6) ring motifs. The crystal packing is consolidated by pairs of intermolecular C-H...O hydrogen bonds, which link the molecules A into centrosymmetric dimmers with $R^2_2(24)$ ring motifs and stacked along the c axis. Intermolecular C-H...O, N-H...O and N--H...N hydrogen bonds further link these dimers with the molecules B, generating $R^2_1(5)$ and $R^2_2(8)$ ring motifs.

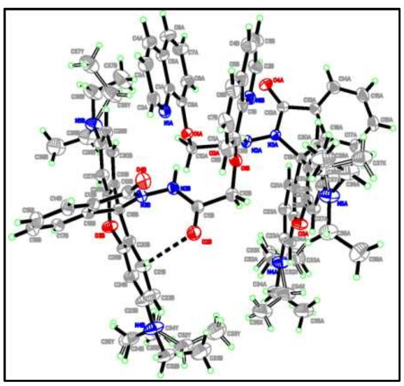


Figure 7. The molecular structure of RHQ. The minor disorder components are indicated with open bonds. Intramolecular hydrogen bonds are drawn as dashed lines

Computational study

The calculated spectroscopic quantities obtained for RHQ is reported in ESI Table S2. As can be seen, the absorption peak appears in the near ultraviolet region with moderate intensity of transition. The molecular orbitals involved in the electronic transition are displayed in ESI Figures S11 & S12. The lobes of HOMO are mostly concentrated over the two benzene rings and on the two adjacent nitrogen atoms around the central xanthene ring. The LUMO extends on the xanthene ring and the carbon atoms of two adjacent benzene rings. Thus the lowest energy transition involves nitrogen lone pair → π^* charge transfer within a short range. The uppermost moiety does not have any effect on this electronic transition.

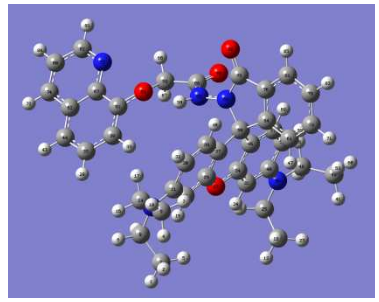


Figure 8. B3LYP/6-31G** optimized electronic structure of RHQ obtained in acetonitrile solution.

Bio imaging:

Use of RHQ probe for imaging of biological samples is a significant addition to the technical knowhow for certain reasons: First of all, it is a non-invasive technique, thus small volume of blood sample or other tissue sample is well enough to estimate the generation of HOCl within the biological system, and subjects do not necessarily require consuming the product, which is a safe, non- toxic technology. Secondly, a very small amount of sample is required (as minimum as 3ml of blood sample).

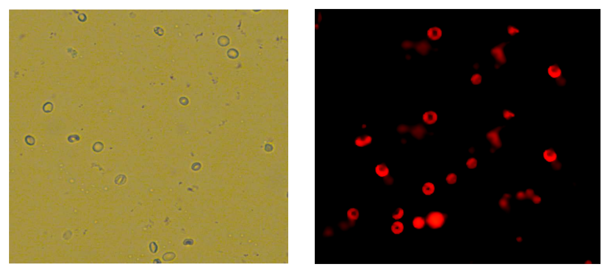


Figure 9. (Left) Bright field image of PBMCs (40X), (Right) Fluorescence image of PBMCs (40X) treated with 50 μM RHQ (fluorescence emission 580nm)

Thirdly, the dye is easily taken up by the cells without causing lysis or morphological changes. Finally, and most importantly, HOCl, an important oxidative stress product can be detected using this technique separately from the other reactive oxygen species. Till now, there are few suitable techniques to detect total ROS of cells, collectively like detection of cellular ROS by DCFDA dye [14] and lucigenin [15] but there is less evidence to detect the cellular HOCl directly. Thus, we report that RHQ fluorescence probe is highly useful for the imaging of biological sample as well as an effective technique to detect cellular HOCl, a toxic ROS product.

Conclusions

In summary, we report here the design, synthesis and sensing property of a rhodamine-quinoline based probe. It showed highly selective and sensitive response towards OCl^- over other competing analyts in acetonitrile/ water (1:1, v/v, 25^{0}C) media. A pink coloration and large enhancement of emission intensity was observed after the chemodosimetric approach of OCl^-. The detection limit was found to be 10^{-8} M level, which indicates our probe RHQ is a highly efficient sensor of OCl^- in mixed aqueous media. Moreover, RHQ can detect OCl^- endogenously in human blood sample.

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† Electronic Supplementary Information (ESI) available: [Detection limit determination, ^{1}H NMR, ^{13}C NMR, HRMS spectroscopy, X-ray data, Computational data. CCDC reference no. 982181]. See DOI: 10.1039/b000000x/

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