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Abstract

Quinoline appended hemicyanine **6MIM** with strong ICT character was successfully synthesized through simple condensation reaction of 6-methoxy-2-chloro-3-formyl quinoline with 2-benzothiazolinium iodide. The photophysical characteristics of synthesized probe revealed that it would selectively detect glutathione (GSH) when it compared with different amino acids including biothiols and the detection limit is found to be 100 nM. The turn off sensor is due to thiol-halogen S_NAr nucleophilic substitution between **6MIM** and thiol group in glutathione. More importantly, the biosensor **6MIM** was effectively applied in the fluorescence bioimaging of GSH in living cells with low cell toxicity. The colorimetric detectable color change of 6MIM-GSH has been effectively integrated with smartphone assisted RGB color value application with lowest detection value of 120 nM.

Keywords

Quinoline derived receptor; hemicyanine; UV-vis and emission spectra; Smartphone integration; fluorescence bioimaging

Introduction

Thiol group containing amino acids are main factors of numerous peptides that work vital tasks in sustaining natural redox homeostasis in genetic methods *via* equilibrium between oxidized disulfide forms and reduced free thiol group.[1] Significantly, many researchers have

established that irregular amounts of these thiol containing amino acids are directly connected with assured infections, including cancer, liver damage, AIDS, Alzheimer's disease, osteoporosis, cardiovascular diseases and inflammatory bowel [2-5]. Among the different biological thiols, glutathione (*L*-γ-glutamyl-*L*-cysteinyl-glycine, GSH) is the largely thiol loaded tri-peptide that serve as eukaryotic and mammalian cellular functions like, xenobiotic metabolism, maintenance of intracellular redox activity, gene regulation and intracellular signal transduction. [5,6] GSH is a crucial endogenous antioxidant that acts an essential function in intercellular defence beside free radicals and toxins.[7] An unusual level of GSH is able to escort aging, heart problems, cancer and other ailments. [6,8] Therefore, it is crucially imperative to develop rapid, simple and selective approaches for detection of GSH and checking the changes of GSH levels under physiological conditions are highly necessary.

Up to now, a lot of tactics have been proposed for the quantification of GSH [9-14]. Even though, most of the analytical methods are highly expensive, labour-intensive, time consuming, need sophisticated instrumental facility and which limits their biological applications. In view of the critical metabolic roles of GSH for intercellular homeostasis, to ascertain the new sensing tactics are still enviable for selective quantification of cellular GSH level [14,15]. Optical visual naked eye sensing approaches are compatible for the quantification of thiol containing biothiols *in vitro* and *in vivo*.[15] Generally, the available sensing tactics are based on the operating by Michael addition,[16] strong nucleophilicity of the thiol group, cleavage of sulfonamide,[17] and disulfide [18] etc. These reaction based sensing approaches were easily identify the biothiols from different amino acids. Though, it is static a difficult to distinguish among the molecules containing thiol group with their analogous reactivities and structures [19,20]. Hence, we have chosen to progress synthesize an organic molecule based fluorescence probe as GSH specific and optically accessible as organic dyes but low cost from a synthetic approach. In addition, we have engaged addicted to account the prospect of utilizing the organic molecules not only for fluorometric but also for colorimetric detection of GSH, raising the selectivity using spectrofluorimetric methods.

On continuing our research interest in the detection of various cations with the application of fluorescence bioimaging in living cells [21, 22], we have decided to synthesize an organic molecule based probe existing of an intramolecular charge transfer compound whose

electronic features like absorption and emission spectra. Hemicyanine dye offers such a features and it is composed of an electron accepter benzothiazolium species and an electron donor that in this case of methoxy group in quinoline core [23, 24]. In this investigation, we focus to develope a simple hemicyanine based fluorescent probe **6MIM** for the detection of GSH under physiological conditions, characteristic merits such as simple preparative method, good photostability, better emission behavior, with super selectivity and sensitivity. It is important to mention that probe **6MIM** shows a high specificity for the quantification of GSH over cysteine and homo-cysteine and that it can be further applied to examine of GSH in cancer cells. In addition, the present colorimetric probe was integrated with smart phone detection of GSH.

2. Experimental section

2.1. Materials

All the chemicals for the probe synthesis of **6MIM** were acquired from commercial suppliers. Solvents were distilled according to previously reported procedures. ^{1}H and ^{13}C NMR spectral studies were recorded in Bruker Avance (400 MHz for ^{1}H and 100 MHz for ^{13}C) in $CDCl_3$ and DMSO-d_6 solvents and chemical shift values were expressed in parts per million using TMS as an internal standard. The reaction path was track by TLC on silica gel coated F254 Merck plates. Elemental analyses were examined by using Perkin Elmer 2400 series II Elemental CHNS analyzer. ESI-MS were recorded on a Thermo Scientific High resolution Magnetic Sector MS DFS by negative ion electrospray ionization (ESI) method.

2.1.1. Synthesis of probe 6MIM

Probe **6MIM** was synthesized through condensation reaction of equimolar solution of 8-methyl-2-chloro-3-formyl quinoline (1 mmol) and 2,3-dimethylbenzothiazolium iodide (1 mmol) in refluxing ethanol. After the completion of reaction monitored by TLC (PE:EA) the obtained precipitate was filtered off and washed with cold ethanol. The purest form of product will be obtained through recrystallization using chloroform: methanol in 1:1 ratio.

2-(2-(2-chloro-6-methoxyquinolin-3-yl)vinyl)-3-methylbenzo[d]thiazol-3-ium **(6MIM)***:* Orange solid; Yield 92 %; mp 234-236 °C; IR(KBr) υ(cm^{-1}): 3407, 2997, 1579, 758; ^{1}H NMR (400 MHz, DMSO-d_6) (ppm) δ: 9.27(s, 1H), 8.49(d, J = 7.6 Hz, 1H), 8.35(d, J = 8.8Hz, 1H), 8.27(s, 1H), 7.96-7.93(m, 2H), 7.89(t, J = 8.0 Hz, 2H), 7.61(dd, J = 2.8 Hz, J = 2.8 Hz, 1H),

7.46(d, *J* = 6.4 Hz, 1H), 4.45(s, 3H), 3.96(s, 3H); ^{13}C NMR(100 MHz, $CDCl_3$) (ppm)δ: 171.4, 148.6, 147.1, 142.6, 141.7, 139.7, 136.3, 133.4, 130.2,129.4, 128.8, 128.7, 127.3, 126.5, 124.9, 118.6, 117.7, 37.3, 36.3; HRMS (ESI-MS) *m/z* 494.1784; Anal.Calcd. for $C_{20}H_{16}ClN_2OS$: C, 65.0; H, 4.38; N, 7.62; S, 8.72; Found: C, 65.1; H, 4.37; N, 7.64; S, 8.73.

2.2. Methods

2.2.1. Absorption and Emission Measurements

UV−vis absorption spectral studies were carried out on a JASCO V-630 UV−visible spectrophotometer with 1.0 cm quartz cuvettes used to monitor the absorption spectral studies. Steady-state fluorescence spectral measurements were performed with a JASCO FP-6600 spectrofluorometer attached with a 1 cm quartz cuvette.

2.2.2. Procedure for GSH sensing

Stock solutions (0.1 M) of GSH were prepared in Tris HCl buffer (pH-7.2). Stock solutions of the probe 6MIM (0.01 M) were prepared in dimethyl formamide (DMF). All UV–vis and fluorescence measurements involved use of 10 μM probe 6MIM in Tris HCl (pH-7.2) and 1 mM GSH. For all fluorescence measurements, the excitation wavelength was 380 nm for the detection of three biothiols and the excitation and emission slit widths were 2.5 and 5 nm, respectively.

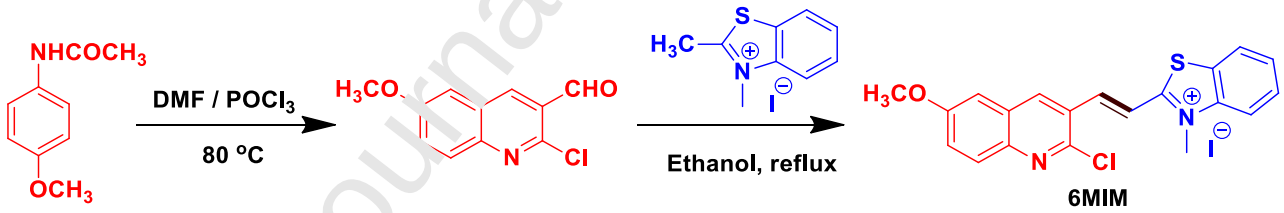
2.2.3. Fluorescence bioimaging of living cells

The skeletal myoblasts C2C12 cells were obtained from national center for cell science (NCCS), Pune, India. These cells were grown in Dulbecco's Eagles medium (DMEM), supplemented with 20% Heat inactivated Fetal Bovine Serum (HI-FBS) and 1% Antibiotics and Antimycoticsat 37 °C in a humidified incubator with 5% CO_2. Once the cells reached 50-60% confluence we have treated these cells with the **probe 6MIM** (100 mM) in presence or absence of GSH (500 mM) for 20 minutes followed by fluorescence imaging with Nuclear counterstain DAPI(4′,6-diamidino-2-phenylindole) in a BX51 Fluorescence Microscopes (Olymbus).The fluorescence signal in the cells were quantified using ImageJ Ver1.52a software (NIH, USA) and represented as the corrected total cell fluorescence (CTCF) using the below mentioned formula.

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

3. Results and Discussion

K. P. Elango *et al*.[25] reported recently the synthesis of quinoline based hemicyanine intramolecular charge transfer (ICT) receptor for the selective detection of cyanide ion in aqueous solution. The synthetic routes for the probe **6MIM** were outlined in **Scheme-1**. To a solution of 2-methylbenzothiazole (1.49 g, 10 mmol) in CH_3CN (20 mL) was added methyl iodide (3.548 g, 25 mmol). The mixture was refluxed under an inert atmosphere for 24 h. After cooled to room temperature, the solid was filtered off and dried under vacuum to afford a white solid with 92% yield, which was then used for next step reaction without further purification. The probe **6MIM** was synthesized through condensation reaction between 6-methoxy-2-chloro-3-formyl quinoline (1 mmol) and 2,3-dimethylbenzothiazolium iodide (1 mmol) in refluxing ethanol. The formation of probe **6MIM** was attested by spectroscopic techniques. In the ^{1}H NMR spectrum of probe **6MIM**, the disappearance of aldehyde and appearance of olefinic protons at δ 8.49 (J = 7.6 Hz) and δ 8.35 (J = 9.8 Hz). The N-methyl (N-CH_3) proton appeared at δ 4.44 confirmed the formation of probe **6MIM**.



Scheme1. Synthetic route of probe 6MIM

3.1. Absorption spectroscopic studies

For the importance of GSH detection, we examine its reactivity **6MIM** towards GSH through UV-visible absorption spectral studies. The UV-vis spectra of free **6MIM** in DMF/Tris HCl buffer (pH-7.2) before and after the addition of GSH is displayed **Fig. 1**. As visualized in **Fig.1**, free **6MIM** compound showed a characteristic peaks at 306 nm and 382 nm due to the π–π* and n–π* transitions, respectively. Upon addition of GSH, the initial absorption peak (382 nm) decreased (**Fig.1**) regularly along with the simultaneous increasing the peak at 306 nm with slight blue shift, while, color also changes from yellow to colorless.

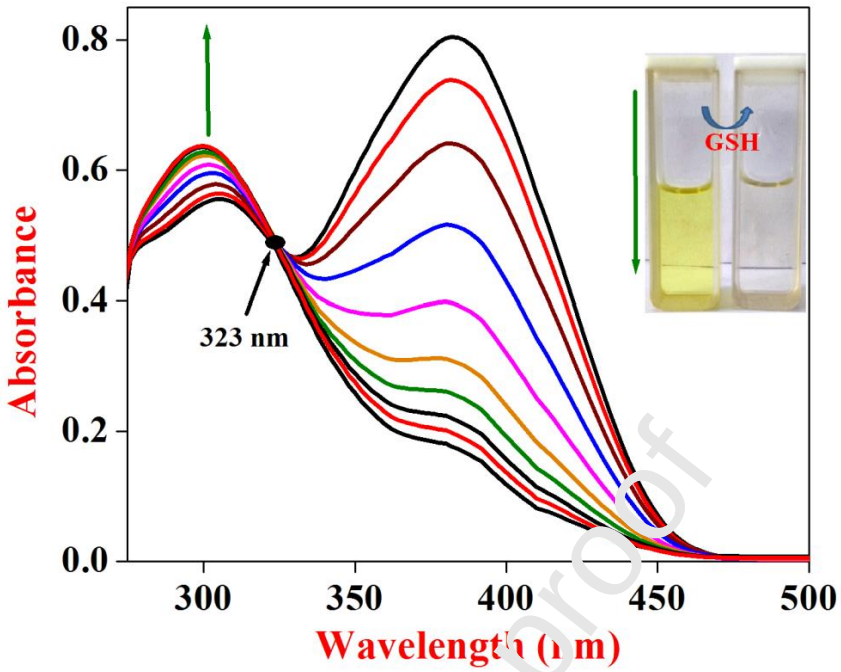


Fig. 1. UV–vis absorption spectral changes of 6MIM in DMF/Tris HCl buffer (pH-7.2) with the increasing concentration of GSH

The decreasing absorption peak at 332 nm and color change is caused by interaction between the **6MIM** and GSH. Furthermore, absorption peak at 306 nm increased with slight blue shift is owing to the chlorine group of **6MIM** is primarily be replaced by the thiol group of GSH and the succeeding intramolecular rearrangement would quickly lead to the increasing absorption peak at 306 nm. Similar kinds of results have been earlier made [26, 27] in coumarin systems. Therefore, in this case we believed that GSH contains the thiol group interacts with chlorine group of the **6MIM**.

3.2. Emission spectral studies

In addition, we examined the emission behavior of **6MIM** in the addition of GSH by using excitation wavelength at 380 nm and corresponding results were displayed in **Fig. 2**. As illustrated in **Fig. 2**, upon the addition of increasing concentrations of GSH to a solution of **6MIM** in buffer media, the emission peak at 522 nm was decreased steadily and at the same time a new band at 428 nm increased with a clear isoemissive point at 452 nm, whereas, color also changes from yellow to colorless. Furthermore, quantum yield (Φ_F) of the **6MIM** before and after the addition of GSH was measured with compared to the quinine sulphate as a standard.

The value of Φ_F also reduced from 15% to 1.3% was noted after the addition of GSH by **6MIM**. This observation further supports to the emission quenching experiments. The obtained isoemissive point solely indicates that reactant and product in equilibrium process. The calibration plot is relative emission intensity ($\Delta F=F_0-F$) as a function of the different amounts of GSH is presented in **Fig.3**. It can be seen from **Fig.3**, the calibration plot demonstrates good linear relationship within the experimental concentration range from 30 μM to 75 μM and yielded detection limit value of 100 nM (S/N=3) [28]. These results are well consistent with the absorption spectral measurements. The observed spectral and possible sensing mechanism is represented in **Scheme 2**.

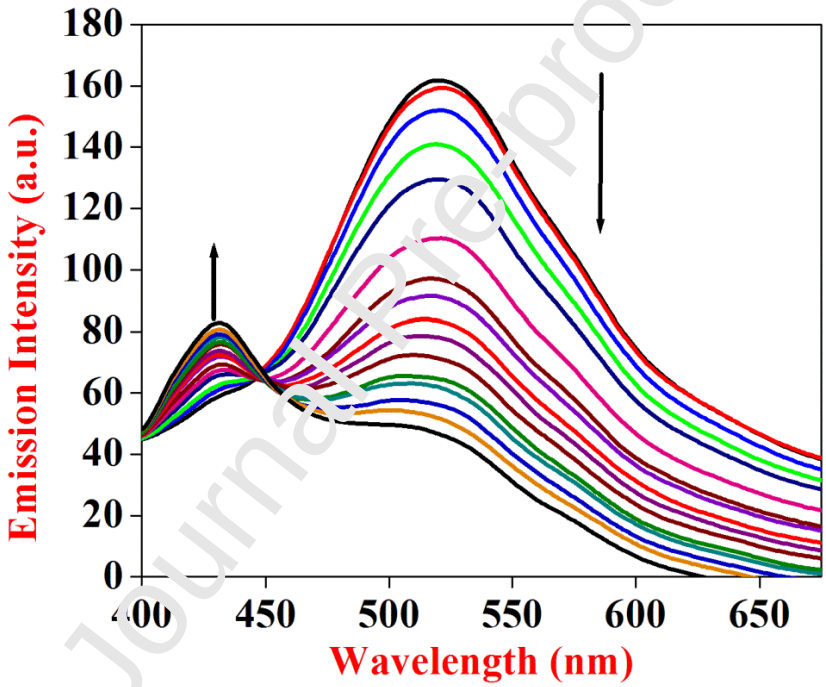


Fig.2. Emission spectral results of 6MIM in DMF/Tris HCl buffer (pH-7.2) with the increasing concentrations of GSH λ_{ex} = 380 nm

The plausible mechanism is depicted in **Scheme-2** and illustrated as follows. Chlorine group in the 2nd position of quinoline would enable the thiol-halogen S_NAr nucleophilic substitution between **6MIM** and thiol (GSH). Thus, it was expected that the chloro group of **6MIM** could initially be replaced by thiol group of GSH to produce thio-quinoline hemicyanine. The $-NH_2$ group in the terminal position of GSH undergoes Michael addition with two pathways to produce **I** or **II** membered ring products thio-quinoline

hemicyanine **I** or **II**. In fact, the hearsay could be supported by a recent article on a large ring product resulted from the reaction of glutathione with acrylate [14]. Overall, if our mechanism suggestion is rational, it would be conclude with discrimination of GSH in terms of the different chemical structures and thus from mass spectrum analysis of **6MIM** receptor with GSH, the molecular ion peak at *m/z* 637.2118 (Figure S5) corresponds to thiol-quinoline hemicyanine **I** or **II**. **(Scheme-3)**

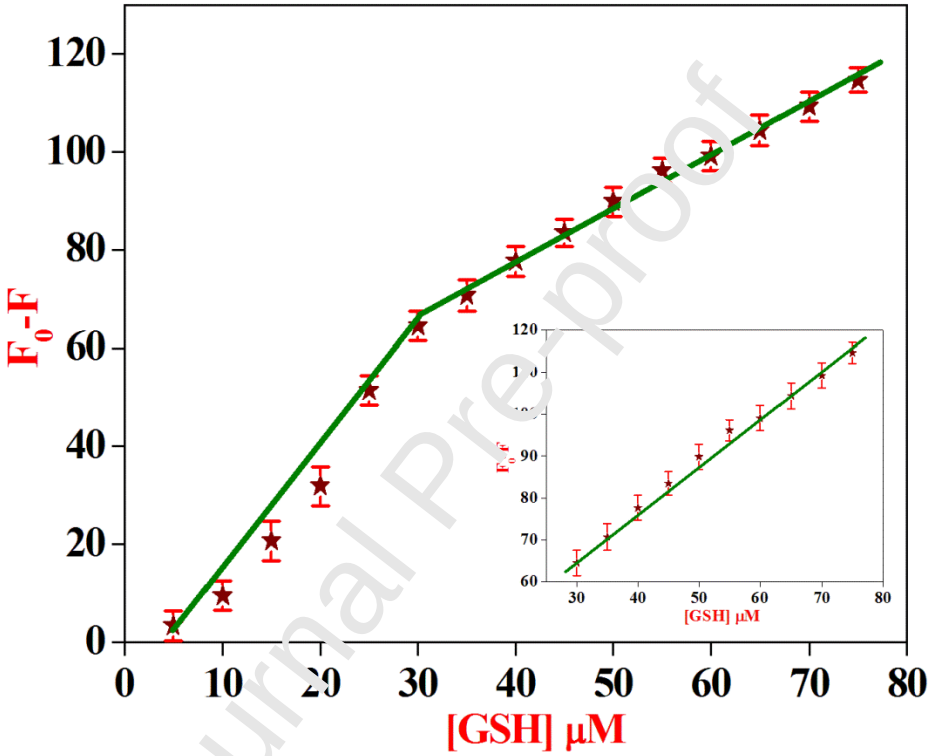
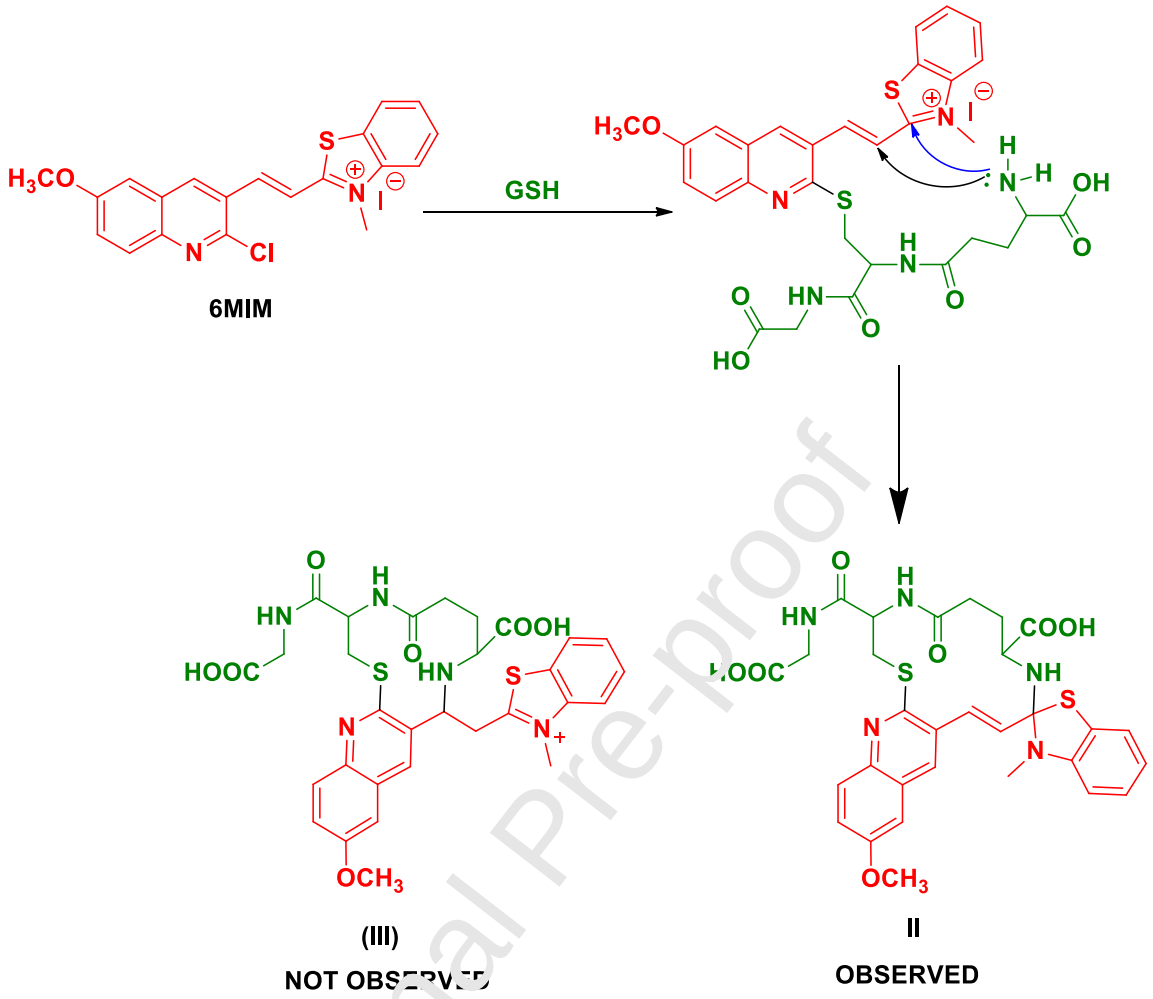
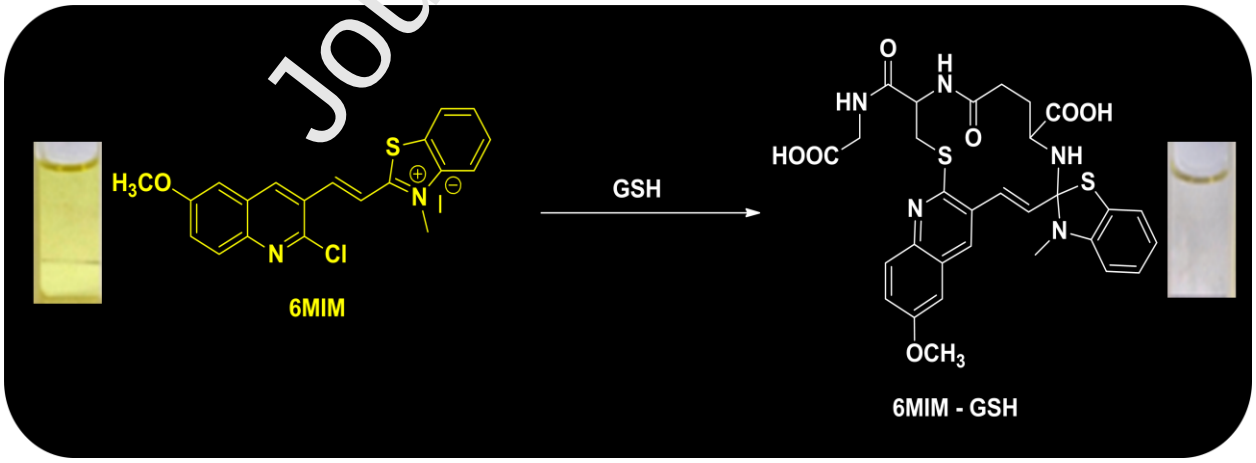


Fig.3. Calibration plot for relative emission intensity of 6MIM versus GSH

To know the stoichiometry of the present method, Job's plot was plotted between the emission spectral change of at 428 nm and mole fraction of GSH concentration and this plot was given in Figure S4. As displayed in Figure S4, the maximum intersection point of the absorption maximum is observed at 0.5, which clearly points out that a 1:1 binding stoichiometry between GSH and probe **6MIM**. This observation was further validated by ESI-MS spectral analysis. ESI-MS spectrum of organic molecule based probe **6MIM** with GSH was displayed in Figure S5. The observed *m/z* value of 637.1052 (**6MIM** with GSH) confirmed the 1:1 binding stoichiometry between GSH and probe **6MIM**.



Scheme-2. Plausible mechanism of hemicyanine 6MIM with GSH selectivity



Scheme-3. Plausible sensing mechanism of 6MIM with GSH

3.3. Quenching mechanism

To find out the emission quenching mechanism, the reduce in fluorescence intensity of 6MIM at 552 nm in the incremental addition of GSH were estimated with the assist of predictable Stern-Volmer equation[29, 30]

$$\frac{F_0}{F} = 1 + k_{SV}[Q] \ldots\ldots\ldots (1)$$

where, F and F_0 are fluorescence intensities of with and without quencher, respectively. [Q] is the concentration of the quencher. k_{SV} is the Stern-Volmer quenching constant which measures the efficiency of quenching. Fluorescence quenching results of **6MIM** in the existence of GSH were plotted as relative emission intensities at 522 nm vs the different amounts of GSH and is visualized in Figure S5. Stern-Volmer plot displays that within the experimental GSH concentrations, it showed an excellent linear association. Generally, a Stern-Volmer linear plot displays that simply single kind of emission quenching process take places, in either energy transfer or ground state complex formation pathway [31]. Furthermore, the value of k_{SV} was calculated from intercept and the slope of the Stern-Volmer plot and it was established as 3.33×10^4 L/ mol (R = 0.9959). The noted k_{SV} value clearly indicates that the strong binding interaction involved between the probe **6MIM** and GSH.

3.4. Determination of binding constant and free energy

The binding constant of **6MIM** in the presence of GSH was calculated by modified double logarithmic equation (Eqn. 2)

$$\log\left[\frac{F_0 - F}{F}\right] = \log K_b + n \log [Q] \ldots\ldots\ldots (2)$$

where, F_0 and F are same as in Eqn. (1), and K_b is the binding constant. The double logarithmic plot of log $[F_0\text{-}F]/F$ *vs* log [GSH] was shown in Fig. S6. As visualized in Fig. S6, the plot exhibits well fitted straight line within the investigated concentrations. The binding constant K_b value was calculated as 1.80×10^5 L/ mol (R = 0.9914). The collected high binding constant value demonstrated that the strong binding force occupied between the probe and analyte. The free energy change (ΔG) of the present case for 6MIM with GSH was computed by the consequent equation ($\Delta G = -2.303$ RT log K_b, R shows the universal gas constant, K_b denotes the binding constant, which can be collected from the double logarithmic plot) [32] and the value is

-29.993 kJ/mol. The observed negative free energy change value revealed that the present method is highly favorable and spontaneous.

3.5. Smartphone integration of 6MIM by the RGB value

On account of innovation in mobile phone aspects in idioms of offering authoritative integrated-sensors provides important benefit over usual methods in terms of fast investigation, easy-operation, low-priced, and compacted tool. Therefore, systematic researchers and medicinal experts have exposed their attention to apply this tool as analytic devices for numerous uses [33, 34]. Mostly, for embryonic kingdoms they require of such a tool is highly necessary because of the high priced instrumentation facilities. To achieve the smart phone detection of GSH, the freely accessible portable RGB Color value APP was downloaded in online the Realme 5 Pro and the alteration in red, blue, green (RGB) values of the glass tubes holding **6MIM** solutions before and after the additions of various amounts of GSH was monitored using back camera of mobile phone. In general, the model RGB range is characterized by whole number value for each of the three colours like, red, green and blue (RGB) from 0 to 255. In this RGB scale, the whole number [255,255,255] matches to accurate white while [0,0,0] to utter black. To

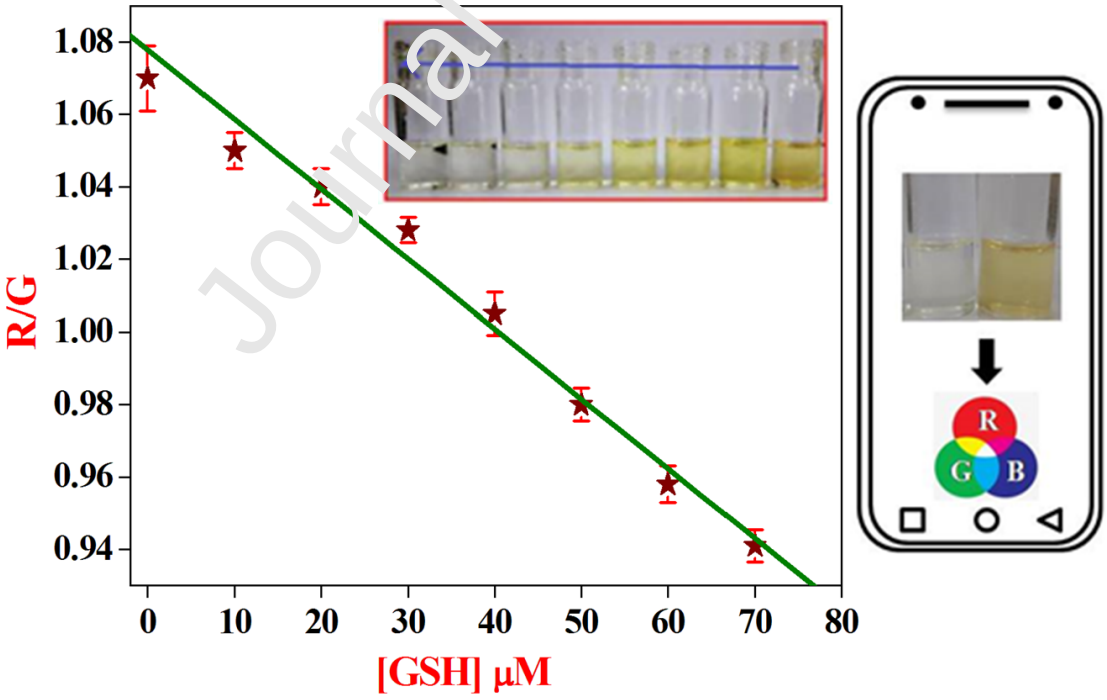


Fig. 4. Smartphone assisted RGB response for the detection of GSH biothiols

appraise the colour change of **6MIM** with the incremental additions of GSH for on-site quantification[35-37], we have incorporated the present organic molecule based probe with a smartphone to observe the RGB value changes when the yellow colour of **6MIM** changes to colorless after the mixing of GSH. Smartphone based sensing of GSH by RGB value, the colour change was easily monitored by tracking the RGB values. The ratio of R/G value was plotted against the concentrations of GSH and represented in Figure 4. As illustrated in Figure 4, a well fitted linear relationship range was noted with the concentration range from 20 μM to 50 μM and the value of LOD is calculated as 120 nM. This assist of the **6MIM** with the smartphone, a low priced visual naked eye approach was demonstrated for the on-site quantification of GSH.

3.6. Selectivity

The fluorescence spectral measurements were also monitored to study the reactivity of probe **6MIM** with other amino acids. The emission spectral data of the **6MIM** in the presence of other amino acids (10-fold higher concentrations) counting GSH, Cys, Hcy, Ala, Asn, Trp, Lys, Leu, Thr, Met, Tyr, Asp, Ser, Gln, Phe, Arg, His, Glu, Pro and Gly were shown in Fig. 5. From Fig.5, the emission intensity at 522 nm was did not affected even in the presence of 10-fold higher concentrations of all GSH, Cys, Hcy, Ala, Asn, Trp, Lys, Leu, Thr, Met, Tyr, Asp, Ser, Gln, Phe, Arg, His, Glu, Pro andGly. Also, the current assay **6MIM** is sensitive sufficient to determine GSH even at very low level (nM range) in same physiological medium.

In addition, GSH can be simply distinguished by the naked eye when introduction of GSH to the solution of 6MIM results in a color change from yellow to colorless. These colorimetric changes can be easily visualized by naked eyes (inset of **Fig. 4)**. Therefore, the colorimetric probe **6MIM** is serving as a micromolar level "naked-eye" indicator for GSH. The observed high selective detection of GSH is due to discrimination of GSH in terms of the different chemical structures and thus the distinct photophysical properties of the corresponding thiol-quinoline hemicyanine formation. In the case of other thiol containing amino acids such as, Cys and Hcy might form stable amino-substituted covalent complexes with **6MIM** through a sequential thiol substitution and rearrangement reaction.

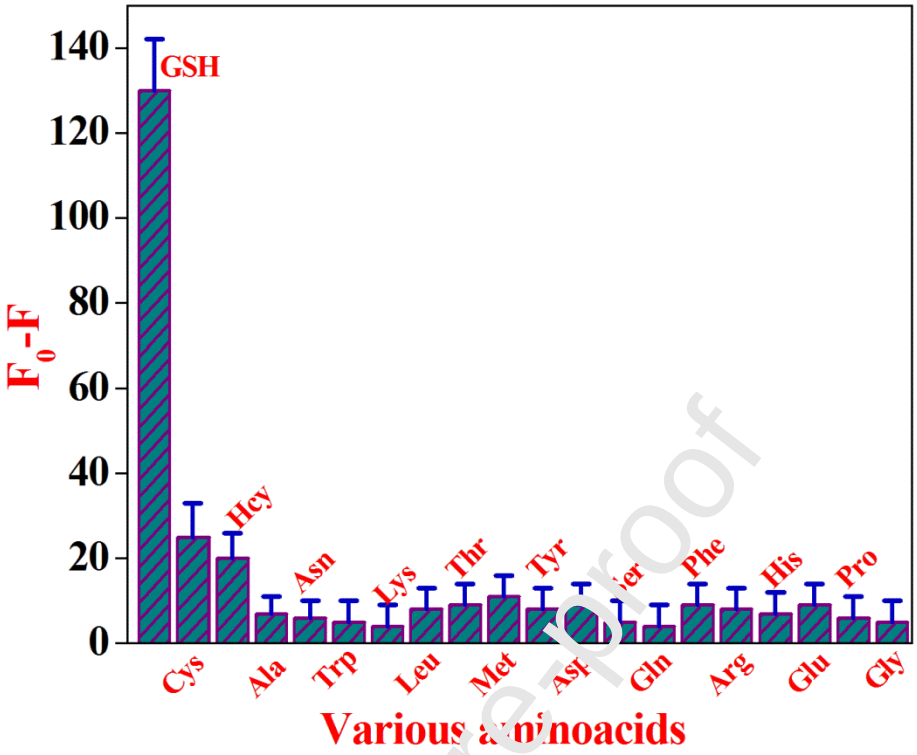


Fig.5. Relative fluorescence intensity changes (λ_{em} = 522 nm) of 6MIM upon addition of 10 folds of various amino acids

3.7. Fluorescence Bioimaging

GSH is synthesized in mammalian cells and plays a crucial role in cellular protection from oxidative damage and toxicity [38]. It also important to maintain redox homeostasis in living cells and any alterations in GSH homeostasis leads to progression of various diseases including cancer [39]. Thus, detecting the level of GSH in mammalian cells in real-time will be useful to understand these normal cellular functions [40], disease progression and management. Thus, we have evaluated the potential of probe **6MIM** to GSH in biological system. We have used this **6MIM** in cell culture studies to determine its biological relevance. First, we treated skeletal muscle cells C2C12 with or without **6MIM** for 20 minutes and subjected to fluorescence imaging. The results showed that, the probe is capable to cross the membrane and enter to the live cells and fluorescence signal present predominantly in cytoplasm not in the nucleus. We have observed quenching of **6MIM** fluorescence signal when we incubate the cells with GSH. We have also noticed the change in cell morphology when we treated with the **6MIM (Fig. 6A)**. Then we have analyzed the potential of 6MIM to detect endogenous GSH. It is known that

oxidative cellular insult leads to increased productions endogenous antioxidant GSH to protect the cells from damage [38]. We have tested the ability of 6MIM to detect oxidative insult induced endogenous GSH. For this we have treated cells with H_2O_2 a strong oxidizer and probed endogenous GSH induction with **6MIM**. Here we have observed **6MIM** fluorescence quenching in H_2O_2 treated cells compared to untreated control **(Fig. 7)**. Hence these results suggest that probe **6MIM** can act as a GSH biothiol sensor in mammalian live cells.

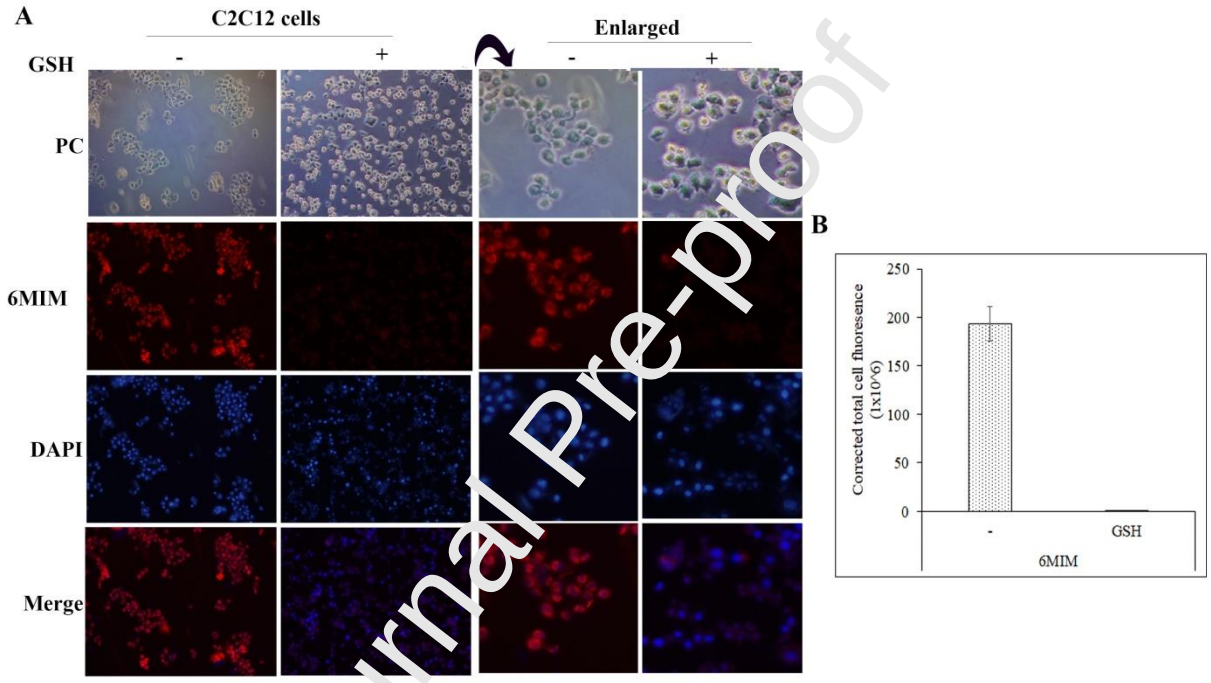


Fig.6. GSH sensing in mammalian cells. *Fluorescence microscope images of C2C12 cells treated with **6MIM** (10 μM) in presence or absence GSH (500 μM) and the cells were also counter stained with nuclear stain DAPI (A). Cell images were obtained for **6MIM** and DAPI using an excitation filter BP545-580 and BP330-385 respectively. Magnification 10X. Quantification of fluorescence signal in these cells was shown in a graph (B). Error bar indicates +/- Standard Error Mean (SEM)*

Conclusion

Quinoline bearing hemicyanine fluorescence probe **6MIM** with strong ICT character was synthesized and investigated. The synthesized probe will selectively detect glutathione over cysteine / homocysteine and the detection limit was found to be nanomolar range. The initial probe **6MIM** having yellow in color in solution, with the addition of GSH led to quenching of yellow color to colorless. Moreover, the cell studies further illustrated that **6MIM** could serve as

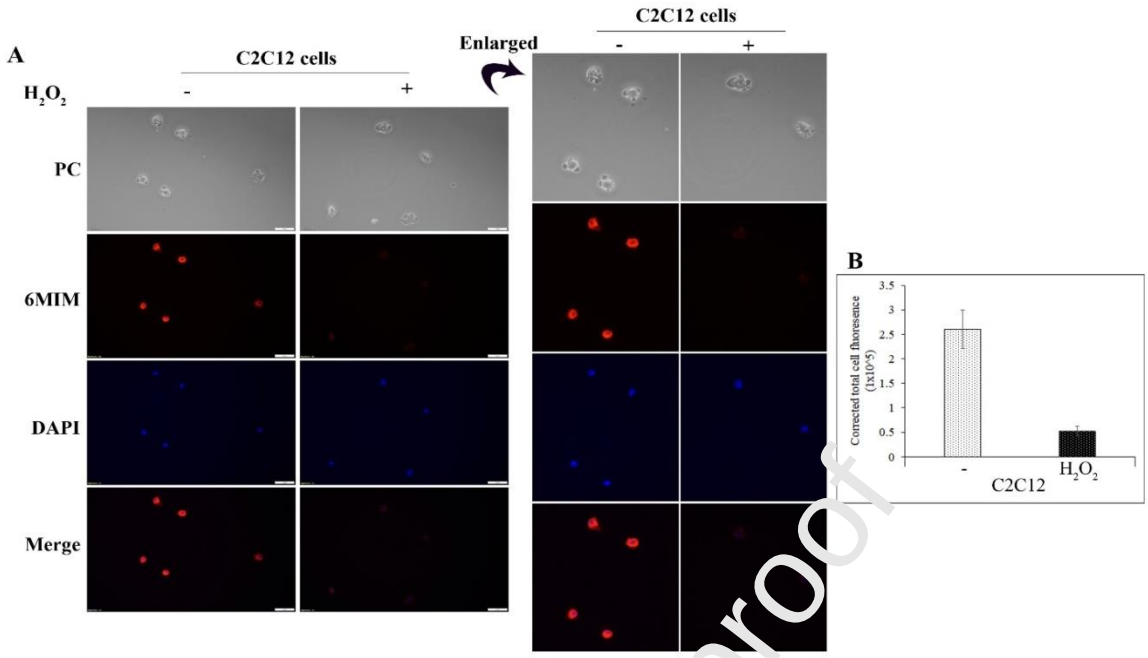


Fig.7. Endogenous GSH sensing in mammalian cells. *Fluorescence microscope images of C2C12 cells treated with* ***6MIM*** *(100 μM) in presence or absence H_2O_2 (150 μM) and the cells were stained with nuclear stain DAPI (A). Cell images were obtained as indicated in Fig 6. Magnification 20X. The quantified fluorescence signal in these cells was shown in B. Error bar indicates +/- SEM.*

a potential fluorescent probe for the detection of GSH in living cells. Finally, probe has been successfully integrated with smartphone RGB color value application for the real-time online analysis of GSH with the detection limit of 120 nM.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

The NMR spectra, mass spectra, UV absorbance and fluorescence spectra and additional data (.doc) file will be available as Supporting Information.

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Smart phone assisted Quinoline-hemicyanine based fluorescent probe for the selective detection of glutathione and the application in living cells

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Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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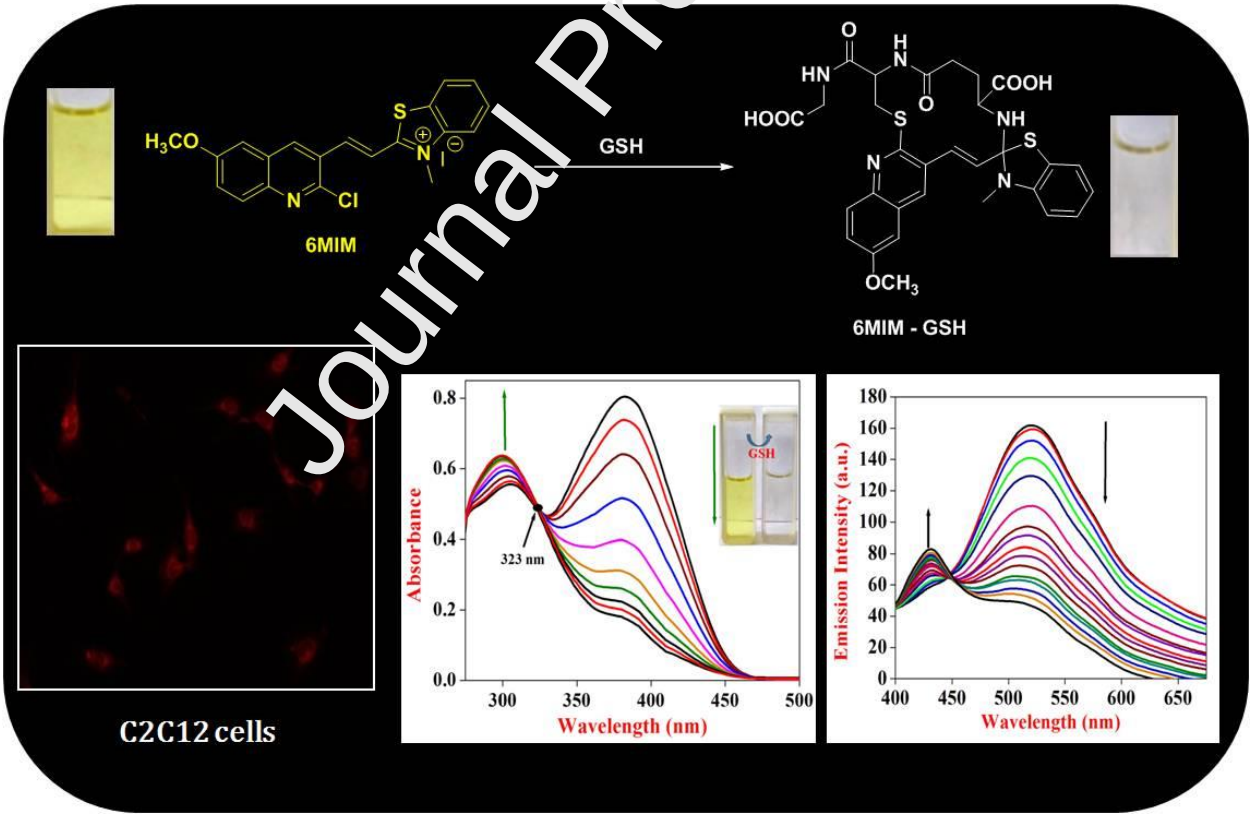
Graphical Abstract



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Research Highlights

1. Quinoline appended hemicyanine fluorescence probe **6MIM** was synthesized and attested through a range of spectroscopic techniques
2. The synthesized probe **6MIM** detects glutathione (GSH) selectively over cysteine and homocysteine through fluorescence quenching.
3. The quenching will be visualized through naked eye from yellow color to colorless and limit of detection will be 120 nM.
4. The colorimetric detectable color change of 6MIM-GSH has been effectively integrated with smartphone assisted RGB color value application with lowest detection value of 120 nM.
5. The biosensor **6MIM** was effectively applied in the fluorescence bioimaging of GSH in living cells with low cell toxicity