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A radical approach for fluorescent turn 'on' detection, differentiation and bioimaging of methanol

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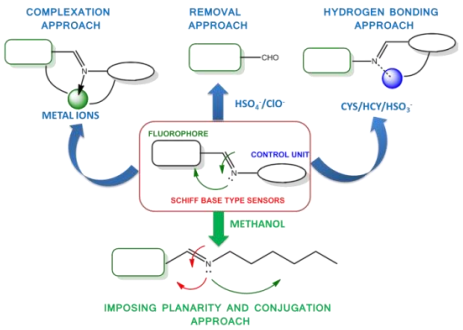
A simple Schiff base (RC) has been explored as a smart example of fluorescent material for selective detection, differentiation and bioimaging of methanol. The nucleophilic attack of methanol over the cyclic control unit of RC leading to its opening and formation of a highly fluorescent moiety RO. The RC displays a good sensitivity for MeOH with a detection limit of 0.042 w% in water.

An age-old challenge for a variety of alcohol sensor is its selectivity towards methanol as the same is colorless, volatile inflammable liquid, simplest of all alcohols[1] with a widespread applications including as a common laboratory solvent[2]/constituent of a number of consumer products.[3] Recent researches also revealed it as a future energy carrier and synthetic feedstock in the name of "methanol economy".[4] However, unlike ethanol, methanol is highly toxic and unfit for consumption.[5] The ingested methanol is metabolized to formic acid or formate salts,[6] which is poisonous to the central nervous system, and may cause blindness, coma and even death also.[7] Thus, for consumer protection, rapid and precise methods to determine the content of methanol in a variety of samples have ever been required. Nevertheless, the co-existence and similar properties of simple aliphatic alcohols (C1-C4)[8] make the detection of individual alcohol particularly of methanol as challenging one. On contrary to the traditional instrumentation methods for alcohol determination,[9] fluorescent materials possess innate advantages because of their high sensitivity, specificity, simplicity, fast response times and offering applications for in vitro/vivo imaging studies.[10]

The literature survey reveals that most of the colorimetric methods reported so far involve indirect measurement of methanol where the same being oxidized and the formaldehyde so formed being determined.[11] Interestingly, even the official methods for methanol determination (by AOAC and ISO) are indirect ones and time consuming also (response time is of more than 4h).[12] In recent past few workers utilized the covalent bond formation strategy for direct detection of alcohols i.e. addition of alcohols to a particular dye *via* reversible chemical reactions.[13] However these methods suffer with the problem of selectivity as well as require high pH/catalyst for better response time. A few cavitand based supramolecular sensors for alcohol have also been reported recently.[14] Their tedious synthetic procedures as well as non-selectivity towards methanol make them poor choice of users.

Thus, the toxicity of methanol and scarcity of effective, simple and selective optical sensors with viability for imaging purposes prompted us for this piece of work. We are reporting hereby a simple Schiff base (*3-(((7-(diethylamino)-2-oxo-2H-chromen-3-yl)methylene)amino)-2-thioxothiazolidin-4-one*; **RC**) derived from the condensation of 7-diethylaminocoumarin-3-carbaldehyde with 3-aminorhodanine. **RC** involves coumarin as a fluorophore while rhodanine serves the role of control unit **(Figure.1)**. The presence of several heteroatoms (N, O, S) on rhodanine as well as its coupling with aldimne moiety introduces the non- planarity, strain as well as the hyperactivity of the carbonyl towards nucleophilc attack of methanol. The same served as the key step towards generation of **RO** (*Methyl 2-((2-((7-(diethylamino)-2-oxo-2H-chromen-3-yl)methylene)hydrazinecarbonothioyl)thio)acetate*) from **RC** leading to selective identification of methanol by **RC** through fluorescence turn **'on'** responses.



Scheme 1: Previous and present approaches utilized for fluorescent detection of analytes through Schiff base type sensors

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Electronic Supplementary Information (ESI) available: Experimental details, ^{1}H NMR, ^{13}C NMR, IR, mass spectrum of RC and RO have been given. Theoretical and crystal refinement data of RO were also supplemented. See DOI: 10.1039/x0xx00000x

Among the several fluorescent sensing mechanisms the C=N isomerization has been actively developed by Wang et al and utilized by other workers as a prime reason for non-emissive nature of Schiff base type receptors.[15,17] Besides C=N isomerization, photoinduced electron transfer (PET) from the lone pair of aldimine N-atom to the fluorophore also contributes towards same.[16] There are three approaches in literature hitherto to suppress above non-radiative processes *viz.,* (a) complexation approach[17] (b) removal approach[18] and (c) hydrogen bonding approach[19] (Scheme 1). To the best of our knowledge, the methanol has never been selectively identified using Schiff base incorporating any mechanistic approach mentioned above.

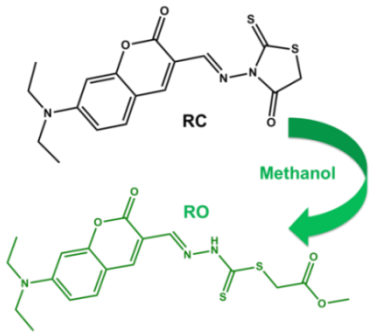

Figure 1: Methanol mediated chemical transformation of **RC** to **RO**

The present strategy involves a simple and a radical approach for the suppression of PET/C=N isomerization in Schiff base type sensor like **RC** through the nucleophilic attack of methanol resulting into planarity and extended conjugation in **RO (Scheme 1; Figure. 1)**. Thus, we utilized the poor sustainability of **RC** towards nuclecophilic attack of methanol as a design principle for constructing a highly smart fluorescent material **RC** for the selective turn **'on'** sensing of a very notorious analyte like methanol from water and from the mixture of other commonly encountered aliphatic alcohols (C1-C4). The recently reported fluorescent methods for methanol detection by Wang et.al and Zhang et. are cumbersome with respect to their applications for the purpose of cell imaging.[20-21] The colorimetric methods developed by Li et al and Ishihara et al. for discrimination of methanol from the mixture of C1-C4 alcohols suffered with the selectivity issue.[22]

Thus, the Schiff base **RC** being reported through this communication is a worthy optical sensor having several edges over all the previously reported ones for the discrimination of methanol from water/mixture of alcohols (C1-C4) in real samples. The cell imaging results further suggest that **RC** is permeable to the cell membrane and can detect intracellular methanol within living cells quite efficiently. To the best of our knowledge this is the very first report of the bioimaging of methanol in living cell and that too using a very simple fluorescent sensor like **RC**.

For studying the photo physical studies of **RC** we primarily tried to optimize the proper solvent system by measuring the absorption spectra of **RC** (1.0×10^{-5} M) in a number of solvents having different polarities *viz.* methanol (MeOH), ethanol (EtOH), propanol, butanol, acetone, ethyl acetate, toluene, dichloromethane (DCM), chloroform ($CHCl_3$), tetrahydrofuron (THF) acetonitrile (ACN), N,N-dimethylformaide (DMF), dimethylsulfoxaide (DMSO) **(ESI; Figure. S5)**. The absorbance spectra portrayed almost same λ_{max} for all solvents, except in case of methanol. **RC** displayed a strong absorption band in the visible region at 445 nm in all the chosen solvents with a meager 3-4 nm variation **(ESI; Figure. S5)**. However in methanol the λ_{max} of **RC** (445 nm) experienced red shifting of ~17 nm and appeared at 462 nm. The same was reflected in terms of naked eye color change as bright yellow in methanol while light yellow in remaining solvents **(ESI; Figure. S6a)**. This unique spectral shift and color response of **RC** selectively in methanol prompted us to check further its emission characteristics in all the above solvents. Surprisingly the methanol solution of **RC** was highly emissive at 520 nm (λ_{ex}=450 nm) while other solutions of the same were either non/very feebly emissive **(Figure. 2; ESI; Figure. S6b)**. The observed emissive change in the characteristics of **RC** in methanol indicated their possible chemical interaction leading to some chemical transformation of **RC**.

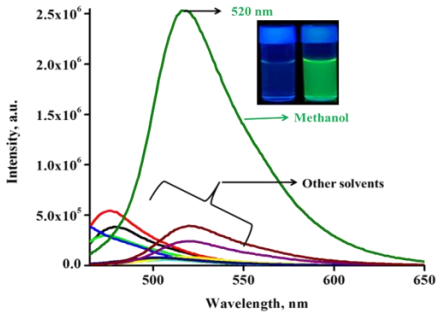

Figure 2: Characteristic effect of methanol solvent over the emission spectra (λ_{ex}=450 nm) of **RC**; Inset: Corresponding fluorescence color changes (Under UV light)

In order to further explore the chemical interaction between **RC** and methanol we tried to isolate the reaction product of the same by dissolving the appropriate amount of **RC** in methanol followed by evaporation of solvent. We carried out the ^{1}H NMR of the same in $CDCl_3$ **(ESI; Figure. S7)**. A comparison of ^{1}H NMR spectrum of **RC** before and after addition of methanol **(Figure. 3)** strengthened our above speculation of its chemical transformation to **RO** as two new peaks at 10.157 δ ppm (1H) and 3.859 δ ppm (3H) were observed along with other peaks of **RC** with slight downfield/upfield shifts. These two new resonances were assigned to –NH and $-OCH_3$ respectively in the open form of **RC** as indicated in figure. 1. This ring opening of **RC** into **RO** may be understood in terms of nucleophilic attack of the methanol at the carbonyl group of strained rhodanine which is supposed to be activated due to vicinal presence of heteroatoms (N and S). The same has been described through **ESI; Figure. S8**.

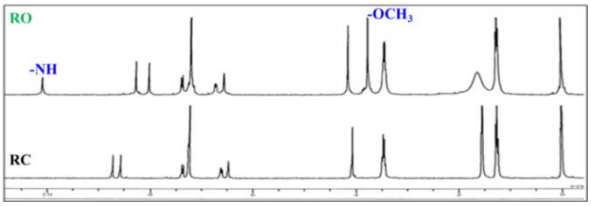

Figure 3: Partial ^{1}H NMR spectra of **RC** and **RO**, showing corresponding changes in their peak positions

The changes in IR spectral pattern of **RC** and **RO** were also compared and confirmed our above views **(ESI; Figure. S9 & S10)**. The mass spectral study of **RO** was also carried out and it showed a molecular ion peak at 430.0888 $(M+Na)^+$ which matches well with that of the calculated one **(ESI; Figure. S11)**. In order to further confirm the above chemical transformation we carried out synthesis of **RC** in methanol instead of ACN following the same synthetic procedure as described above. We successfully isolated the single crystals **RO** from mother liquor and were studied through XRD data (**ESI; Table S1 & S2**). The resulting ORTEP plot **(Figure. 4)** confirmed our above speculation that the **RC** undergoes ring opening at rhodanine leading to the formation of **RO** which was highly fluorescent.

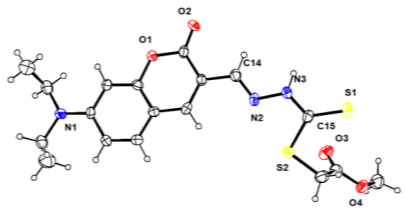

Figure 4: ORTEP view of the **RO** with the partial atom numbering

To explore the miraculous fluorescence behavior of **RO** we took advantage of the density functional theory (DFT) studies. The optimized geometry of **RC** and **RO** (**Figure. 5**) indicated that **RO** possesses less energy (**ΔE= -**1960.11593394 a. u.) in comparison to **RC (ΔE= -**1844.36440774 a. u.) hence the same is over stabilized. The optimised structure showed that **RC** was non-planar as dihedral angles DA3 (C3-N1-N2-C4) and DA4 (N1-N2-C4-S1) equals to 139° and 11° respectively. The non-planarity in the structure of **RC** makes the non-bonding electron on the aldimine nitrogen **(>C=N-)** available for the photo induced electron transfer (**PET**) to the HOMO of the fluorophore resulting into fluorescent quenching. However, the same dihedral angle was found to be 179.64° and 179.73° respectively in **RO** from DFT studies**,** indicating that the whole molecule in **RO** except the diethylamino and ester group has an excellent planarity **(ESI; Table S3)**. The dihedral angles of **RO** found through XRD studies finally supports the DFT results and confirms the planarity in **RO**. This planarity in the system enhances the conjugation and thus the lone pair of the aldimine nitrogen **(>C=N-)** became unavailable for the PET process, consequently **RO** become highly fluorescent. The fluorescent behaviour of **RO** in present study could be corroborated with previously reported thiosemicarbazide based Schiff bases.[23] The very same Schiff bases were reported to detect a variety of analytes through quenching of their fluorescent behaviour. In our case, the ring opening of **RC**, resulted into **RO** which is analogous to Schiff bases incorporating thiosemicarbazide moiety as reported previously by various workers.[23]

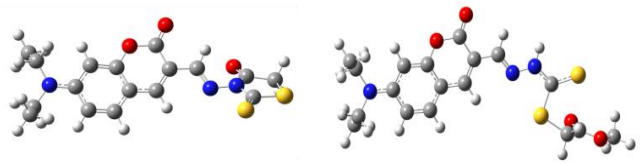

Figure 5: Energy minimized structures of **RC** and **RO**

The photophysical properties of **RC** and **RO** were also been studied through time dependent DFT (TD-DFT) calculations **(ESI; Table S4)** which provided further insight for their UV-visible absorptive characteristics and oscillator strengths. Experimentally **RC** and **RO** absorbs at 445 and 462 nm respectively while the theoretical calculation showed absorption maxima at λ_{max} 428 nm (f= 0.9064) and λ_{max} 449 nm (f= 1.3047), respectively. Thus the theoretical calculation excellently support the absorption characteristics of **RC** and **RO** observed from experimental one **(ESI; Table S4)**. The excited states are described by a combination of transition from occupied to non-occupied molecular orbitals, generally involving mainly the highest occupied orbital (HOMO) and the lowest occupied orbital (LUMO). The energy gap between HOMO-LUMO decreases in **RO** with retention of LUMO as its original level and upgradation of HOMO **(ESI; Figure. S12)** Thus, the ring opening of rhodanine destabilized the HOMO significantly; decreased the band gap by increasing the conjugation length employing **RO** is better for sensor applications.

Furthermore, we thought it worthwhile to check various analytical aspects of **RC** towards selective sensing of methanol. Primarily we investigated the reaction time profile for nucleophilic attack of methanol over **RC** through measurement of emission intensity at several time intervals **(ESI; Figure.S13)**. As it is evident from the bar graph given in **figure. 6a** that although the ring opening followed by methanol addition over **RC** takes ~15 min to complete but the appreciable fluorescent changes could be easily detected within less than 5 min. These results demonstrate that although **RC** is a reaction based probe even than it is rapid enough to have speedy on spot measurement of methanol.

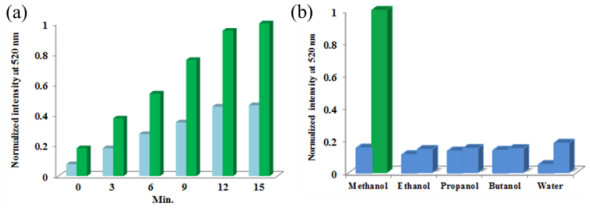

Figure 6: (a) Reaction time profile of transformation **RC** to **RO** in methanol; Blue bars = 5% MeOH in water and Green bars = 100% MeOH; (b) Effect of most interfering solvents on the emission intensity of **RC** (at 0 min. and after 15 min.)

Further we checked the selectivity of **RC** towards the methanol as the efficient and accurate optical sensors for the same are scarce in the literature. We measured the emission intensity of **RC** (at 520 nm) at time interval of 0 and 15 min in water as well as most interfering basic alcohols (C1-C4). We plotted the bar graph from these data **(Figure. 6b)**, which clearly indicated that the transformation of **RC** to **RO** could happen in methanol solution only while in all other solvents it remained non-emissive.

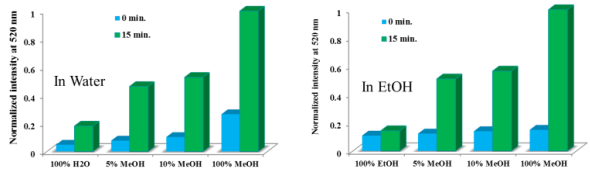

Figure 7: Respective effect of water and ethanol concentrations in Methanol. Normalized intensity (at 0 and 15 min) were shown by varying the mixture content.

We also checked the ability of **RC** towards the minimum possible concentration that it could detect in other miscible solvents *viz.*, water and ethanol. For this we prepared the solution of **RC** by varying the methanol concentration (v/v) by 5%, 10% and 100% in water and ethanol separately). The corresponding bar graph has been presented in **Figure. 7.** These results showed that even the 5% or 10% of methanol content in water or ethanol could be detectable through the appreciable emission enhancement of **RC**. For quantitative purposes the sensitivity of **RC** for methanol was also demonstrated via the determination of lowest detection limit **(ESI; Figure. S14)**. The **RC** displays a good sensitivity for MeOH with a detection limit of 0.042 w% in water.

Encouraged by the fascinating response of **RC** towards methanol, we studied the practical applicability of the same for living cell imaging assay to investigate whether the probe can sense methanol sensitively in biological systems. The E. coli cells were incubated with **RC** (10 μM) for 30 min at 37^0 C showed nearly no intracellular fluorescence. Interestingly, after cells were separately treated with increased concentrations of methanol (5%, 10% and 15 %) with 15 min of incubation, a strong fluorescence signal was observed and collected via fluorescence microscopy in the green channel **(Figure. 8)**. Moreover, the fluorescence intensity of **RC** in E. coli cells depends on the concentration of methanol in the cellular medium. The remarkable green fluorescence upon addition of varying the methanol content was due to the conversion of **RC** into **RO**. Thus, these characteristic features of **RC** were found to be a noble contestant for the sensing of methanol and provide a sensitive and fast response to methanol.

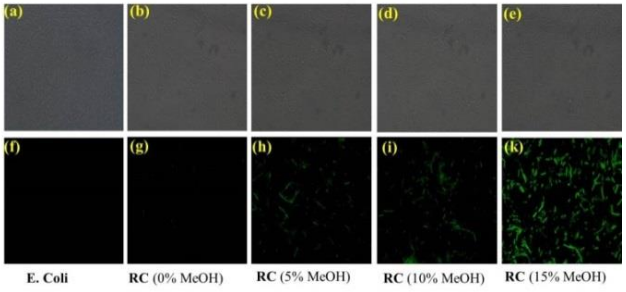

Figure 8: Representative fluorescent images of E. Coli cells incubated with RC and treated with different concentrations of MeOH, in bright (a-e) and dark fields (f-k).

Present work provides a viable solution to the general problem of discriminating methanol from water and other basic alcohols. The fluorescent material **RC** presented through this communication could detect unambiguously the presence of methanol either in ethanol or in water (up to 5%) with a detection limit of 0.042 w% in water. The cell imaging results further suggest that **RC/RO** is permeable to the cell membrane and can detect intracellular methanol within living cells quite efficiently. Hence, the results indicated that **RC** could be a promising sensor for recognizing methanol with excellent selectivity and sensitivity, which makes it a potential candidate for monitoring methanol in biological and environmental areas.

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