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Di-oxime based selective fluorescent probe for arsenate and arsenite ions in purely aqueous medium with living cell imaging application and H-bonding induced microstructure formation

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A 2-hydroxy-5-methyl-benzene-1,3-dicarboxaldehyde dioxime based turn-on blue emission fluorescent probe was found to recognize both AsO_2^- and $H_2AsO_4^-$ in purely aqueous medium in intra and extra-cellular conditions. Self-organization of ligand in absence and presence of AsO_2^- and $H_2AsO_4^-$ were investigated by DLS, optical microscope, optical Fluorescence microscope and FE-SEM methods.

The abundance of arsenic (As) compounds in the environment poses a huge global public health concern. Arsenic is a widely distributed element, exists in both organic and inorganic forms in minerals and soils that can easily percolates into water where it exists exclusively as arsenite (As^{III}) and arsenate (As^{V})[1], along with lower amounts of monomethylarsonic acid (MMA) and dimethylarsonic acid (DMA).[2] In general, organoarsenic compounds are less toxic than corresponding oxyacids.[3] These compounds are also introduced in water body through the mining of sulfide ores,[4] industrial operations,[5] and agricultural activities.[6-8] Arsenite (AsO_3^{3-} or AsO_2^-) and arsine (AsH_3) dominate in reducing atmosphere while arsenate (AsO_4^{3-}) is more stable in oxygenized environments.[9] However, inorganic arsenic species (As^{III} and As^{V}) are present in a greater extent in natural water and the erosion of arsenic containing rocks is probably responsible for its occurrence in water supplies.

Arsenic oxidation states range from −3 to +5, with the trivalent As^{III} state being the most toxic of the environmentally accessible compounds.[10] In mammals, As^{III} compounds like arsenite = $As(OH)_3$ show a strong affinity for thiol biomolecules like cysteine and glutathione [$K_f = 32.0$ for $As(SG)_3$],[11] which lead to the disruption of key enzymes such as pyruvate dehydrogenase etc,[12,13] whereas, the As^{V} compound like $HAsO_4^{2-}$ interrupts the Kreb's cycle by acting as a phosphate mimic.[14] Moreover, arsenite and arsenate have resemblance with phosphite and phosphate ions and probably is the cause of their toxicity.[15] Arsenite and arsenate hinder the conversion of ATP to ADP by permanently replacing the phosphate groups.[16]

Human exposure to arsenic is primarily through drinking water and contaminated food[17] and leads to an increased risk of liver, bladder, and lung cancer.[18] Additionally, chronic arsenic exposure causes a skin condition known as arsenicosis.[17,18] Concerns over As exposure caused the U.S. EPA and the WHO to lower the maximum contaminant level (MCL) for As in drinking water from 50 to 10 ppb in 2001.[19] This lower MCL also stimulated researcher to develop new methods for monitoring As.

Current methodologies for As detection either generate or use toxic chemicals or require sophisticated equipment like hydride generative inductively coupled plasma atomic emission spectrometry (HG-ICP-AES),[20] capillary electrophoresis, inductively coupled plasma mass spectrometry (CE-ICP-MS),[21] high performance liquid chromatography-inductively coupled plasma mass spectroscopy (HPLC-ICP-MS)[22] etc and a long analysis time is required for all these techniques.[23] On the other hand, colorimetric Gutzeit method utilizes a strong reducing agent to reduce As compounds to AsH_3 (arsine gas) coupled with its subsequent reaction with mercuric bromide to afford a coloured salt. Though this method is inexpensive and can be performed with ease, it produces highly toxic gas, AsH_3. However, fluorescence methods are less expensive, non-destructive, and easy to operate exhibiting low detection limit and become very successful as an alternative in environmental chemistry, medicine and biology for fast and simple tracking of As-species. However, $H_2AsO_4^-$ or AsO_2^-

selective fluorescent sensors have scarcely been reported, either as $H_2AsO_4^-$ or AsO_2^- sensor[24-27] but not for both the toxic ions together.

Again, in recent years there grows a considerable research interest on the nano/microstructures derived from small organic molecules due to their potential applications in numerous fields like colour-tunable display, field-effect transistors, chemical sensors and optical wave guides.[28] The properties of such organic nano/microstructures are found to be intimately related to their morphologies. As a result the fabrication of such materials is gaining intense research interests of chemists, biologists and physicists. Much effort has been devoted to synthesize organic nano/micro particles using synthetic strategies like *reprecipitation*[29], *solvent evaporation*[30], *physical vapor deposition* (PVD)[31] and *template-directedmethods*.[32] However, self organization of organic molecules through non-covalent interactions or in presence of other molecules or ions (organic/inorganic) that reinforce this organization towards the fabrication of nano/microstructures are relatively rare.

Here we are going to report, for the first time, an oxime based selective fluorescent probe for arsenate and arsenite in purely aqueous medium with living cell imaging applications along with the nano/microstructures formed by H-bonding interactions in absence and also in presence of these ions in aqueous phase.

The ligand was prepared by literature method[33,34] and characterized by various spectroscopic techniques (**Fig S1**). The spectrophotometric titration for the interaction of DFC-DO (20 μM) with AsO_2^- (560 μM) and $H_2AsO_4^-$ (1200 μM) at 25 °C in pure aqueous medium at pH 7.24 (10 mM HEPES buffer, μ = 0.05 M, NaCl) reveals that there is small increase in absorption intensity of DFC-DO at 376 nm for AsO_2^-; additionally, there is a red shift with slight decrease in absorbance at 340 nm with 2 fold fluorescence enhancement. For $H_2AsO_4^-$ there are slight increase absorbance at 340 and 376 nm with the increase in the concentration (**Fig.S2**).

Photophysical properties of DFC-DO was studied in purely aqueous medium at pH 7.24, μ = 0.05 M, NaCl buffered with 10 mM HEPES. It showed a weak blue emission centred around 460 nm (λ_{ex} = 340 nm), may be attributed to twisted intra-molecular charge transfer (TICT) process.

Addition of AsO_2^- to DFC-DO afforded 5 fold enhancement of the emission intensity at 476 nm (Fig 1) while for $H_2AsO_4^-$ it appears at 460 nm (**Fig. S3**) on excitation at 340 nm. At room temperature quantum yield of the resultant systems were found to be: 0.031 (DFC-DO), 0.055 (DFC-DO–$H_2AsO_4^-$) and 0.078 (DFC-DO–AsO_2^-). The increase in quantum yield of free ligand in presence of $H_2AsO_4^-$ or AsO_2^- may be attributed to presence of intermolecular hydrogen bonding interactions.

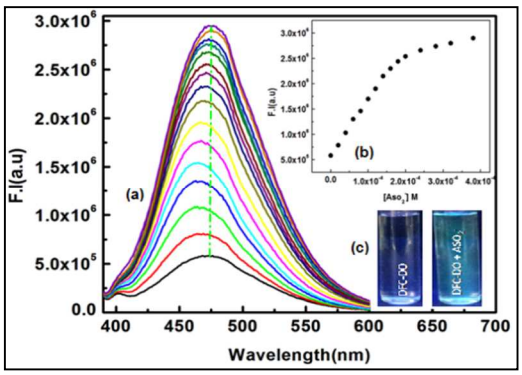

Fig.1.(a) Change in fluorescence spectra of DFC-DO (20 μM) upon addition of AsO_2^- in HEPES buffer at pH 7.24 in H_2O at 25 °C μ = 0.05 M, NaCl, [$As^{III}O_2^-$] = 0–560 μM. (b) plot of F.I (at 476 nm) vs. [AsO_2^-]; (c) UV-exposed emission image of DFC-DO and DFC-DO in presence of with $As^{III}O_2^-$.

The LODs of $H_2AsO_4^-$ and AsO_2^- were calculated by 3σ method and found to be 0.23 and 1.32 μM As^{III} and As^V respectively (**Fig S4**). When we plotted fluorescence intensity (FI) as a function of [As^{III}] or [As^V] non-linear curves of decreasing slope were obtained and can be easily solved by using a non-linear equation:

$$y = (a+b*c*x^n)/(1+c*x^n) \quad (1)^{35}$$

where, *a* and *b* are FI in the absence and presence of excess anions, respectively, *c* (= *K*) is the formation constant and *n* is the stoichiometry of the reactions. The non-linear least-squares curve-fit (Fig 2) of the titration data gives: $c = K = (2.80 \pm 0.58) \times 10^4\ M^{-1}$, $n = 1.15 \pm 0.02$ and $K = (2.03 \pm 0.97) \times 10^5\ M^{-1}$, $n = 1.33 \pm 0.58$ for AsO_2^- and $H_2AsO_4^-$, respectively at pH 7.2, in pure water, μ = 0.05 M, NaCl. The 1:1 compositions of the ensembles were further confirmed by Job's plot (**Fig S5**) and ESI-MS$^-$((m/z) (**Fig S6**) studies. The fluorescence enhancement was lower (~2 folds) for As^V than As^{III} (~5-fold) due to slight loss in planarity of the ligand in H_3L–$H_2AsO_4^-$ensemble as delineated by DFT calculations (Fig 3). Again, the formation constant was found to be higher for the former and may be explained by considering the fact that the saturation was achieved at lower concentration (560 times) of As^V than that of As^{III} (1200 times). DFC-DO was found to be selective towards AsO_2^- and $H_2AsO_4^-$ in presence of various cations and anions (**Fig S7**) including organic arsenic species like $(CH_3)_2AsO(OH)$ and $PhAsO(OH)_2$(**Fig. S7(c)**). The application of this probe under physiological conditions was confirmed by pH dependent fluorescence studies (**Fig S8**) which indicates that the free probe is only

weakly fluorescent at physiological pH but becomes highly fluorescent in presence of AsO_2^- and $H_2AsO_4^-$.

In order to strengthen the mechanism of H-bonding interactions, ^{1}H NMR titration was performed by concomitant addition of H_2AsO_4- to the D_2O + DMSO-d_6 solution of DFC-DO (**Fig. S9**). After addition of $H_2AsO_4^-$, ^{1}H- signal corresponding to oxime (11.46 ppm) and phenolic (10.57 ppm) H-atoms get diminished which completely vanish on adding 5 equivalent of $H_2AsO_4^-$ or AsO_2^-. All the other ^{1}H-NMR signals remain almost invariant. The H-bonding interaction of DFC-DO with $H_2AsO_4^-$or AsO_2^- (Fig. 3) and consequent change in absorption peaks (red shifts) are best described by the decrease in HOMO-LUMO energy gaps (Fig. 4) on H-bonding interactions.

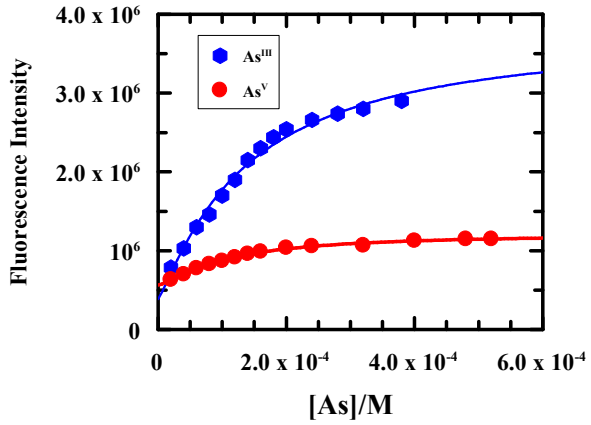

Fig 2. Non-linear least-squares curve-fit of the titration data for the host-guest reaction between DFC-DO (20 μM) and As^{III} (blue) and As^{V} (red) (0-560 μM). Other conditions are same as stated in Fig. 1.

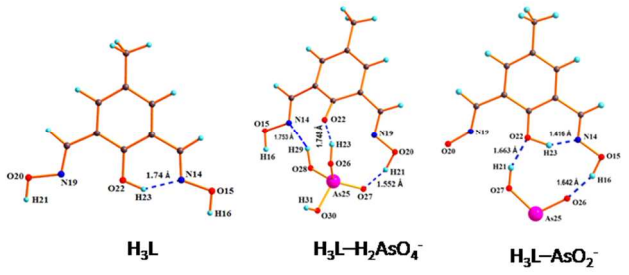

Fig. 3.Optimized geometries of DFC-DO and DFC-DO+$H_2AsO_4^-$ and DFC-DO+AsO_2^- ensembles showing the modes of H-bonding interactions.

In the present study, we found that the novel ligand **DFC-DO** has an excellent sensing capability for the detection of $H_2AsO_4^-$ and AsO_2^-. For this purpose we first inspect the effect of this ligand over cell viability by MMT assay on HepG2 cells, which clearly demonstrated that there was no severe cytotoxicity till 80 μm (<30% cytotoxicity) of **DFC-DO**.

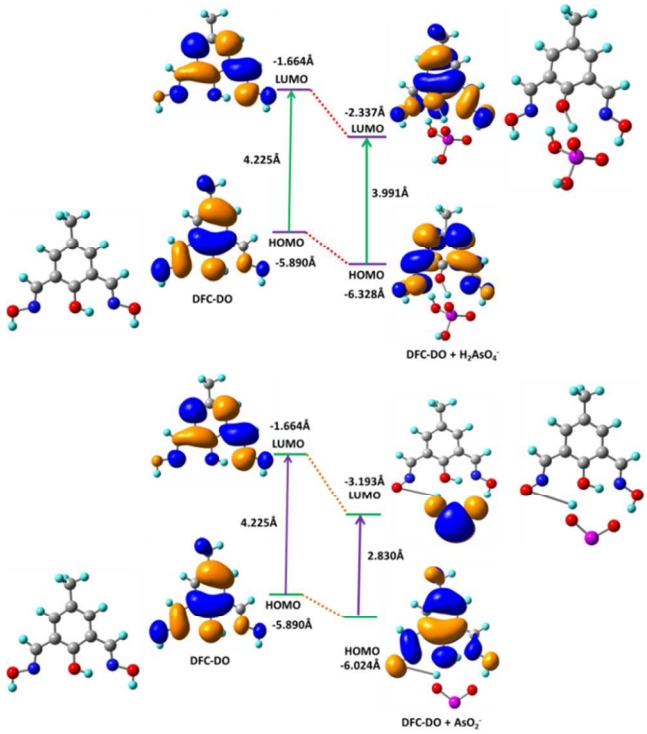

Fig 4. The energy differences between HOMO and LUMO in the ground state for free DFC-DO, DFC-DO–$H_2AsO_4^-$ and DFC-DO–AsO_2^-.

Along with this, more than 90% cell viability was observed for **DFC-DO** at 10 μM concentration for 12 h of exposure (**Fig. S10**). Hence, further experiments were carried out with safer 10μM dose of **DFC-DO** to capture the intracellular As (III) and As(V) efficiently.

The As(III), As(V) and ligand **DFC-DO** (10μM) when treated separately with HepG2 cells for 2 h , 4 h and 6 h at 37°C, it did not show any intracellular fluorescence (**Fig.5**). Interestingly, intracellular blue fluorescence was observed when **DFC-DO** forms complexes with intracellular AsO_2^- and $H_2AsO_4^-$. DFC-DO–AsO_2^- complex gives higher fluorescence intensity than DFC-DO–$H_2AsO_4^-$ complex **(Fig. S7)** as observed in extracellular conditions. Furthermore, to ensure the uptake of arsenic species by HepG2 cells and to monitor intracellular DFC-DO–AsO_2^- and DFC-DO–$H_2AsO_4^-$ complex formation, cells were pre-incubated with AsO_2^- and $H_2AsO_4^-$ for 2 h, 4 h and 6 h in individual culture dish followed washing with 1X PBS buffer for two times to remove the extraneous As species and then incubated with **DFC-DO** for 30 min at 37 ºC and then further washed with 1X PBS buffer for two times and invested under fluorescence microscope. Likely, the excellent blue fluorescence for As(III) was observed compared to As(V) which clearly demonstrate an easy diffusion of **DFC-DO** as well as As-species into the cell thereby enabling to monitor intracellular As-species through

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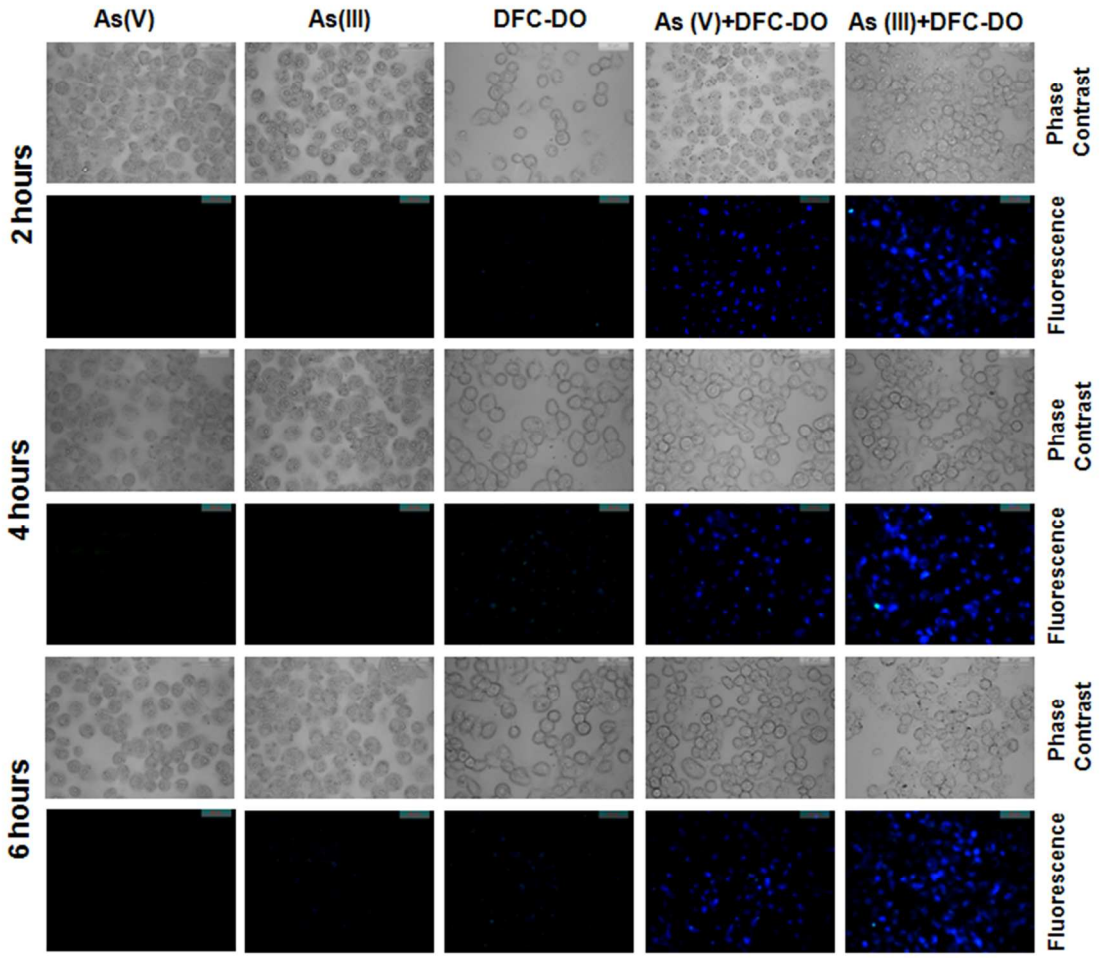

Experimental Set I

Fig.5. The phase contrast and fluorescence images (40X) of HepG2 cells were taken after incubation with 10 μM $H_2AsO_4^-$, 10 μM AsO_2^- and 10 μM DFC-DO for 2 h, 4 h and 6 h at 37ºC followed by washing with 1X PBS for two times to remove the extraneous $H_2AsO_4^-$, AsO_2^- and DFC-DO species (Column 1, 2 and 3). Concurrently, HepG2 cells were pre-incubated with 10 μM $H_2AsO_4^-$, 10 μM AsO_2^- for 2 h, 4 h and 6 h at 37° C followed by washing with 1X PBS for two times to remove the extraneous $H_2AsO_4^-$ and AsO_2^- specie and, then incubated with 10μM **DFC-DO** for 30 minutes at 37ºC, then further washed with 1X PBS for two times to remove the extraneous **DFC-DO** and observed under microscope (column 4 and 5) as mentioned previously. We have performed three parallel sets (Set I, Set II and Set III) (Please see Fig. S11).

DFC-DO–AsO_2^- and DFC-DO–$H_2AsO_4^-$ complex formation **(Fig. 5, Fig. S11)**. This clearly demonstrates that the present ligand would be sensitive probe to monitor low concentration (μM) intracellular AsO_2^- and $H_2AsO_4^-$.

The H-bonds assisted aggregation of the receptor (DFC-DO) in the absence and in presence of guest ions (AsO_2^- and $H_2AsO_4^-$) was confirmed by DLS, optical microscopic and FE-SEM studies (Fig. 6). The DLS studies reveal the diameter of free DFC-DO and DFC-DO- AsO_2^- and DFC-DO-$H_2AsO_4^-$ ensembles in 1:1 mole ratio as 220, 666 and 1620 nm respectively. The FE-SEM analysis of the same solution reveals that in aqueous solution free ligand gives oval shaped morphology while in presence of one equivalent of AsO_2^- or $H_2AsO_4^-$, DFC-DO undergoes further aggregation through intermolecular H-bonding to form well defined block-shaped microstructures (Fig. 6, middle panel) along with

the presence of oval-shaped microstructures; which clearly demonstrates that in 1:1 mixture of DFC-DO and guest molecules, there are sufficient number of free ligands. This is further demonstrated by optical fluorescence microscopic studies of the same slides (Fig. 6, lower panel). Astonishingly, in presence of excess guest molecules no microstructures are available, may be due to abstraction of all the protons on the ligands by the guest molecules which is convinced by ^{1}H-NMR studies in presences of excess guest molecules where all the signals corresponding to oxime protons vanishes.

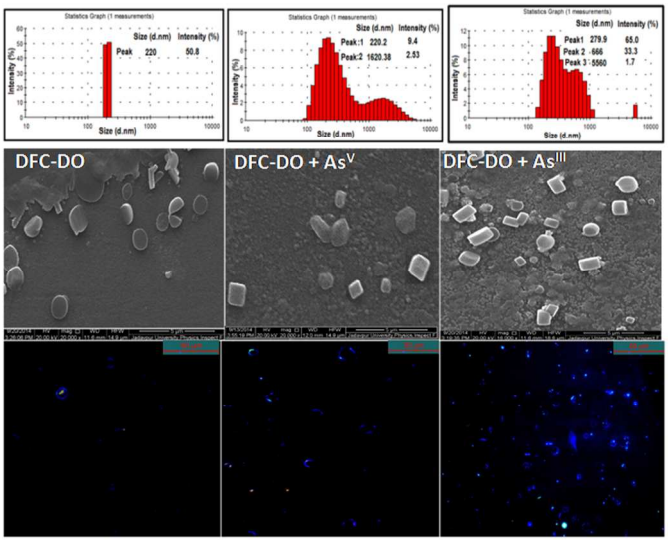

Fig. 6. DLS (upper panel), FE-SEM (middle panel) and Fluorescence microscopic (lower panel) images of microstructures formed in aqueous solution. (i) DFC-DO = 20 μM; (ii) DFC-DO-As^V = 20 μM each; and (iii) DFC-DO-As^{III}=20 μM each.

We attempted to determine the As content as $H_2AsO_4^-$ in different samples collected from different parts of Kolkata and suburban. The content of $H_2AsO_4^-$ was found to be below the detection limit of the probe. So we added $H_2AsO_4^-$ externally and quantified the arsenic content from the linear part of the FI vs. [$H_2AsO_4^-$] plot and results are listed in **Table S1**.

In summary, we have synthesized a novel Di-oxime based selective fluorescent probe for monitoring AsO_2^- or $H_2AsO_4^-$ in purely aqueous medium. Cell permeability and non-or negligible cytotoxic nature of the probe facilitate the intracellular monitoring of these species by blue fluorescence emission. DLS, FE-SEM and fluorescence microscopic studies reveal that H-bonding interactions among the probe molecules as well as with AsO_2^- or $H_2AsO_4^-$ ions leads to the formation of microstructure.

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Supporting Information Available: Electronic Supplementary Information (ESI) available: experimental details regarding the synthesis and characterization of the ligand including spectroscopic details. For ESI format see DOI: 10.1039/c000000x/

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