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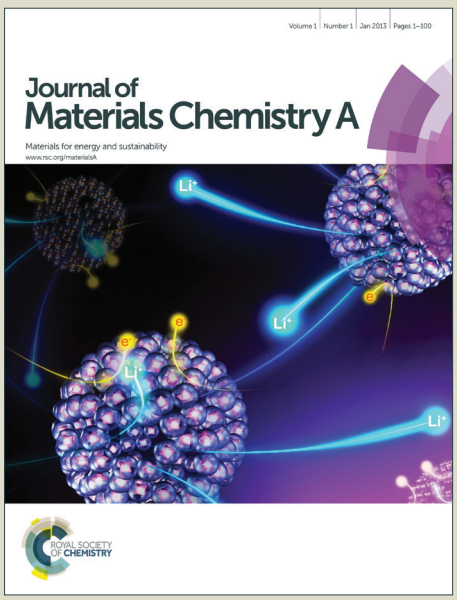

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ARTICLE TYPE

Cytochrome C Encapsulated Metal Organic Framework as Bio-material for Sulfate ion Recognition

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An ensemble of cytochrome c and 2, 6 naphthalene dicarboxylate based metal organic framework is synthesized. The bio-material selectively sense sulfate ions in 100 % aqueous solution and in solid phase with practical application.

Metal organic frameworks (MOFs) are organic-inorganic hybrid crystalline materials known for their wide applications in gas storage,[1] catalysis,[2] drug delivery[3] and sensing.[4] One of the least investigated areas related to MOFs is immobilization of bio-molecules in its pores.[5] Encapsulation of bio-molecules in the pores of MOFs[6] could generate new class of bio-materials for diverse applications. In this context, we aimed at preparation of biomaterial **4** (*vide infra*) in which protein cytochrome c is encapsulated in pores of MOF **3**.[7] Cytochrome c (cyt c) is a small heme protein found loosely associated with the inner membrane of the mitochondrion.[8] It is an essential component of the electron transport chain and is capable of undergoing oxidation and reduction. These properties of cyt c make it a suitable candidate for anion sensing applications. Therefore, we evaluated material **4** for its anion sensing application. Among all the ions tested the biomaterial **4** (*vide infra*) selectively senses sulfate ions. The sulfate ions plays a significant role in the environment[9] and is crucial for many biochemical processes.[10] Sulfate is also responsible for the permanent hardness of water,[11] hampers the vitrification process of nuclear waste sites[12] and thus is a known inorganic pollutant in the environment.[12] Therefore the detection of SO_4^{2-} ions is highly significant.

Among various techniques used for the detection of analytes, fluorescence is one of the most significant technique because of its simplicity and high sensitivity. Generally, the systems showing fluorescence enhancement on addition of analytes are preferred over systems showing fluorescence quenching. This is because fluorescence quenching is unfavorable for a high signal output upon recognition and hampers temporal separation of spectrally similar complexes with time resolved fluorometry.[13] On the other hand, chemical systems showing fluorescence enhancement permits a lower detection limit and high-speed spatial resolution.[14] In addition, responding to an external stimulus to achieve the conversion between two different states with "*on*" and "*off*" functions make use of such chemical systems for the construction of molecular switches and molecular electronic devices.[15]

Our research work focuses on development of new organic – inorganic hybrids for diverse applications.[16]Recently, we reported 2, 6 naphthalene dicarboxylate based co-ordination polymer which worked as synthetic blood plasma anticoagulant.[17] Naphthalene dicarboxylate ligands have also been used as an excellent linker for construction of MOFs.[18] The MOFs based on 2, 6 naphthalene dicarboxylate exhibit large surface area and are known for their adsorption properties.[18] Thus, we solvothermally synthesized our precursor Mn-2, 6 naphthalene dicarboxylate based MOF **3** ($MN_3(NDC)_3DMF_4$).[7] Before we evaluated the synthesized MOF for encapsulation of cyt c, we desolvated it for the loss of DMF molecules by the reported procedure.[7] We named desolvated **3** as **4**. The solvated crystals of **3** which were originally yellow in color (figure 1B) turned grey on desolvation (1C) indicating the removal of DMF. The desolvation was further confirmed by TGA, powder XRD and IR studies.

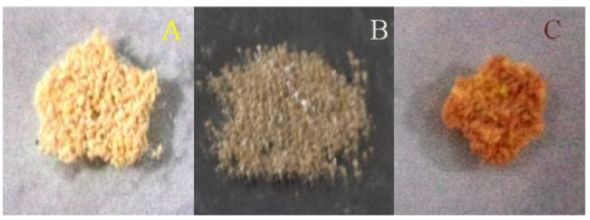

Figure 1. Photographs of (A) **3** (yellow) (B) **4** (grey color) (C) **5** (orange color).

The TGA analysis of **3** shows a degradation curve from 140 °C to 230 °C (see supporting information S6) corresponding to the loss of DMF molecules. The absence of this curve in the TGA of **4** (see supporting information, S6) confirms the removal of DMF molecules from **4**. The powder XRD exhibiting sharp (see supporting information S7) peaks of **3** shows its crystalline nature whereas the XRD of **4** shows broad peaks (see supporting information, figure S7) due to its amorphous nature after removal of DMF molecules. The IR spectrum of **3** shows stretching bands at 1664 and 2933 cm^{-1} corresponding to C=O and C-H aliphatic of DMF molecule (see supporting information S8). The absence of these bands in IR spectrum of **4** further confirms the desolvation of **3** (see supporting information S8). Thus, precursor **4** is ready for the loading of protein cyt c.

For immobilization of cyt c in **4**, it was incubated in saturated solution of bovine heart cyt c in HEPES buffer at 37 °C for 72 h. After incubation, upper orange colored layer (color of cyt c) solution converted into color less indicating immobilization of cyt c. The grey color of compound **4** became distinctly orange originally the color of cyt c (figure 1C). We numbered this **4**-cyt-c ensemble as **5**. The translocation of the protein in to the pores of **4** was further confirmed by optical, TGA and powder XRD studies.

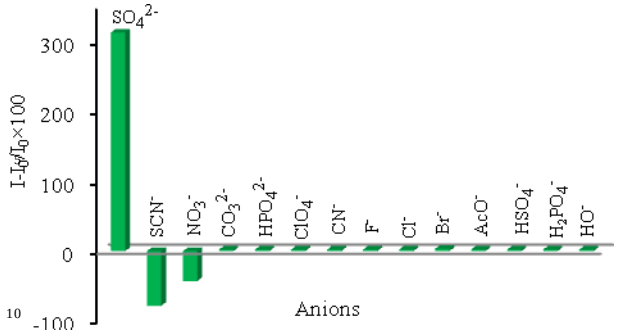

Figure 2: Fluorescence emission changes in **5** on addition of various anions; I = the final fluorescence intensity on addition of anions and I_0 is the initial fluorescence intensity of **5**.

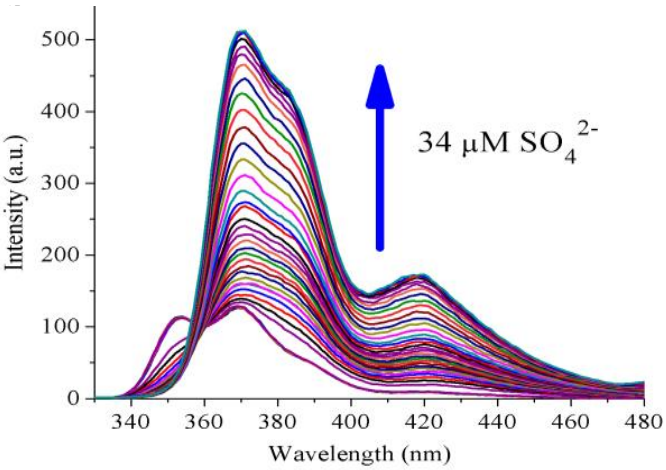

Figure 3: Fluorescence emission spectrum of **5** on addition of 34 μM of SO_4^{2-} ions.

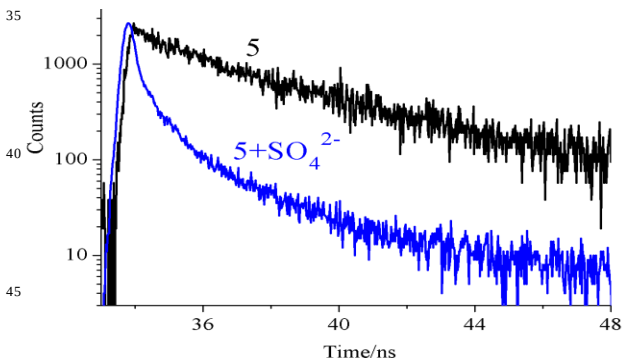

Figure 4: Fluorescence Life time measurements of **5** and **5**+SO_4^{2-} ions

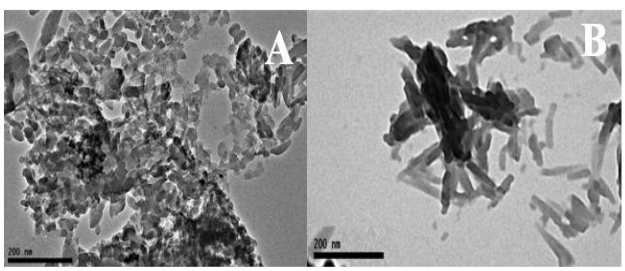

Figure 5: TEM images of (A). **5** (B) **5** + SO_4^{2-} ions.

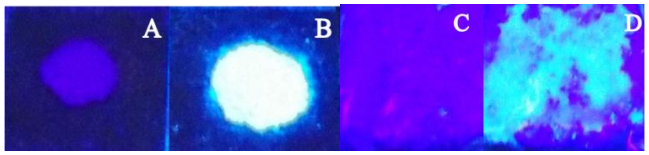

Figure 6: Solid state luminescence photographs of (A) **5** (B) **5** + SO_4^{2-} (C) SO_4^{2-} adsorbed on clay (D) after addition of **5** to SO_4^{2-} adsorbed on clay under luminescence (UV) emission of 365 nm.

The UV-vis spectrum of **4** in H_2O (buffered with HEPES 7.4) shows one single band at 243 nm with two small peaks and low intensity doublet type bands at 287 and 296 nm and very small bands at 326 and 340 nm (see supporting information S9). The UV-is spectrum of **5** under similar conditions shows three sharp and high intensity peaks at 240, 285 and 410 nm (see supporting information S9). The disappearance of peaks at 287, 296, 326 and 340 nm and appearance of peaks at 285 and 410 nm reveals the occurrence of change in the chemical nature of **4**. The characteristic peak of cyt c at 410 nm and a high intensity peak at 285 nm[8a] corresponding to tryptophan of the cyt c confirms the presence of cyt c in pores of **4**. The tryptophan moiety exhibits its absorption spectrum only when cyt c is in partially unfolded state.[8a,19]

The fluorescence emission spectrum of **4** in H_2O (buffered with HEPES 7.4) exhibits strong emission bands at 355 and 370 nm. The emission spectrum of **5** (ϕ = 0.043, for details supporting information S4)[20] shows less intense bands at similar wavelength in addition to a very weak band at 420 nm (see supporting information S10). The excitation wavelength for both the emission spectra was taken at 240 nm since λ_{max} for both **4** and **5** were observed at this wavelength. The additional band at 420 nm observed in the case of **5** is characteristic band for cyt c in partially unfolded state.[21]

TGA analysis of **5** shows (see supporting information, S6) a weight loss of 14 % from 40 °C to 168 °C corresponding to H_2O and organic moieties of cyt c[22] (both these curves were absent in the TGA analysis of **4** (see supporting information, S6) *vide supra*) and 61.9 % from 388 °C -558 °C with residual mass of 24.1 % due to presence of metal oxides. The residual mass in case of **4** (see supporting information, figure S6) was 27.7 % which showed a decrease of 3.6 % in case of **5**. This decrease in residual mass also confirms the presence of cyt c in the pores of **4**. The powder XRD of **5** showed sharp peaks in comparison to the XRD of **4** due to its revival of partial crystalline nature which might be due to the entrapment of cyt c.

It is well known that heme containing proteins are associated with photo induced redox processes[8] and prevalent of this phenomenon enables these proteins to be utilized for various applications. In the present context, we aimed at utilizing cyt c based material **5** as fluorogenic probe for anion recognition. Therefore, we evaluated the fluorescence recognition behavior of ensemble **5** towards various anions (SO_4^{2-}, SCN^-, NO_3^-, CO_3^{2-}, HPO_4^{2-},ClO_4^-, CN^-, F^-, Cl^-, Br^-, OAC^-, HSO_4^-, $H_2PO_4^-$, and OH^-) (figure 2). Among various anions tested, the maximum affinity was observed towards SO_4^{2-} ions. On addition of only 34 μM of SO_4^{2-} ions, the emission band of **5** at 420 nm showed 4.5 times enhancement (ϕ = 0.227, for details supporting information S4)[20] with formation of new band at 372 nm which increases on gradual addition of SO_4^{2-} ions (figure 3). No significant change was observed in presence of any other anion under similar conditions except SCN^- (see supporting information S11) and NO_3^- ions (see supporting information S12) which showed 79 % and 42 % quenching of fluorescence emission respectively.

To test the practical applicability of ensemble **5** as SO_4^{2-} selective fluorescent chemosensor, we carried out the

fluorescence titration experiments with SO_4^{2-} ions in presence of other anions under similar conditions. The SO_4^{2-} ions generated similar fluorescence response in presence of all the other anions (see supporting information S13). Only minor shift in the behavior was observed in cases of SCN^- and NO_3^- . This may be due to the quenching of fluorescence observed in these cases. However, full revival in fluorescence intensity was observed on addition of extra amounts of SO_4^{2-} ions in both the cases. The detection limit of **5** as a fluorescent sensor for the analysis of SO_4^{2-} ions was determined from a plot of change in fluorescence intensity as a function of the concentration of the added anion. It was found that **5** has a detection limit (supporting information S14) of 8.1 μM for SO_4^{2-} which is sufficiently low for the detection of submillimolar concentration range of SO_4^{2-} ions found in many chemical systems.[23]

The fluorescence back titration of **5**+ SO_4^{2-} ions with Ba^{2+} ions quenched the fluorescence emission (see supporting information S15) demonstrating the reversible nature of ensemble **5** as SO_4^{2-} ion sensor. We also compared our ensemble **5** with already reported chemosensors for sulfate ions (see supporting information S16) and observed that the ensemble **5** has most promising features as sulfate sensor considering all the sensing parameters like limit of detection, number of anions used for comparison, solvent system, techniques used and response time (see supporting information S17).

We also carried out the fluorescence life time measurements (see supporting information S18) of **5** in addition and absence of SO_4^{2-} ions. Time resolved fluorescence spectrum of ensemble **5** shows bi-exponential decay suggesting the presence of two emitting species τ_1 and τ_2 (figure 4) having average life time of 4.03 ns. Contrary, on addition of SO_4^{2-} ions to ensemble **5** tri-exponential decay was observed signifying the presence of three emitting species τ_1, τ_2 and τ_3 (figure 4) with average life time of 1.27 ns. After addition of SO_4^{2-} the life times of τ_1 and τ_2 decreased (figure 4) whereas the newly formed third exponential τ_3 shows high life time of 5.07 ns (17.55 %). The increase in the steady state fluorescence of ensemble **5** + SO_4^{2-} ions with decrease in the average life time is might be due to decrease in the HOMO-LUMO gap of ensemble **5** after addition of SO_4^{2-} leading to faster decay of the excited state.[24] The TEM analysis of **5** (figure 5A) shows a well packed rigid structure which completely changes to irregular mass on addition of SO_4^{2-} ions (figure 5B**).** The change in the morphology observed with the help of TEM analysis proves the interaction between **5** and SO_4^{2-} ions.

To have an insight in to the mechanism of SO_4^{2-} ion recognition with **5**, we performed UV-vis titration of **5** with SO_4^{2-} ions. The UV-vis spectrum of **5** showed a blue shift of 8 nm in the band at 410 nm (*vide supra*) with decrease in absorption band at 285 nm (see supporting information S19). The blue shift in the cyt c band at 410 nm is attributed to the breaking of Fe^{2+}-MET-80 bond [19,25] and decrease in the absorption intensity band of tryptophan at 285 nm could be ascribed to participation of tryptophan moiety in the binding of SO_4^{2-} ions. Eventually, further investigating the role of cyt c in overall binding process, we carried out fluorescence experiments and molecular docking studies[26] with native (*folded*) cyt c. The cyt c in the native state showed no fluorescence emission in absence and presence of SO_4^{2-} ions (see supporting information S20). This was also supported by molecular docking studies. The molecular docking studies of cyt c (pdb 2B4Z)[3b] showed negligible interaction of SO_4^{2-} ions with cyt c and showed its location far away from the Fe^{2+} ions of heme group (see supporting information S21). Based upon the above set of experiments and computational analysis we propose an overall following mechanism.

The cyt c gets unfolded after coming in contact with **4** due to surface adsorption and gets entrapped after partial unfolding.[6] Once the SO_4^{2-} ions comes in contact with **5** (ensemble of cyt c and **4**), in which cyt c is in partially unfolded state with exposed tryptophan, the SO_4^{2-} ions find their route to the Fe^{2+} ions located at the centre of heme group. The electron transfer process from SO_4^{2-} ions to the Fe^{2+} ions leads to breaking of Fe^{2+}-MET-80 bond as indicated by the UV-vis studies. In addition probably there also exists interaction between SO_4^{2-} ions and tryptophan moiety. All these binding phenomenon leads to the fluorescence enhancement in emission spectrum of **5** on addition of SO_4^{2-} ions.

The detection of SO_4^{2-} ions with **5** was also observed in solid state under illumination with UV lamp at 365 nm. The solid sample of **5** was mounted on a quartz plate (figure 6A) and under 365 nm illumination, no fluorescence was observed. On addition of same amount of SO_4^{2-} salt to the solid sample of **5** (figure 6B), it started emitting fluorescence which could be easily observed with naked eye. We also explored the possibility of practical application of **5** as bioprobe for SO_4^{2-} ions. As SO_4^{2-} ions are inorganic pollutant in environment, we planned to test the bio-probe in presence of soil sample. We took some moisture laden clay from the bank side of the river and mechanically adsorbed the similar amount of SO_4^{2-} ions on the clay sample (figure 6C). We dispersed that clay on the quartz plate and viewed it under the 365 nm illumination. Initially no fluorescence was observed but the moment we added few drops of stirred solution of **5** (figure 6D) in H_2O, it started emitting from the patches where the SO_4^{2-} ions had been adsorbed.

To conclude we have developed an ensemble of cytochrome c and a metal organic framework which can selectively sense SO_4^{2-} ions in 100 % aqueous medium with probability of practical application.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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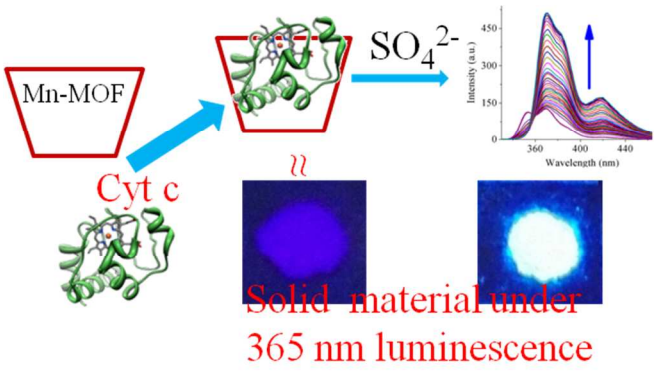

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