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Fluorometric sensing of Pb^{2+} and CrO_4^{2-} ions through host-guest inclusion for human lung cancer live cell imaging †

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Abstract

The formation of an inclusion complex between 1,5-dihydroxyanthraquinone (1,5-DHAQ;**1**) and β-cyclodextrin (β-CD) in aqueous media has been studied by UV-visible and fluorescence spectroscopy. A solid inclusion complex (β-CD:1,5-DHAQ;**2**) has been prepared and characterized by FT-IR, XRD, DSC and SEM analyses. The chemosensor probes **1** and **2** showed selective recognition and sensing ability towards the Pb^{2+} and CrO_4^{2-} ions. The association constants (K_a) of **2**•Pb^{2+} and Stern-Volmer quenching constant (K_{sv}) of **2**•CrO_4^{2-} were obtained to be $1.6x10^3$ M^{-1} and $1.9x10^6$ M^{-1} in water, and the corresponding detection limits were calculated to be $9.0\ x10^{-8}$ and $3.9x10^{-8}$ M according to fluorescence titration analysis. Theoretical studies on molecular docking and density functional theory (DFT) calculation has been performed to prove the binding of Pb^{2+} and CrO_4^{2-} ions with the chemosensor probes **1** and **2**.

Furthermore, bio-imaging indicated that these probes **1** and **2** have good cell permeable and suitable for monitoring intracellular uptake of Pb^{2+} and CrO_4^{2-} ions in living cells (human lung cancer A549) by confocal microscopy.

Keywords: 1,5-DHAQ, β-CD, Inclusion complex, Chemosensor, Live cell imaging, Computational studies.

Introduction

The harmful levels of lead (Pb^{2+}) have concerned researchers for a several years due to its debilitating effect on learning and memory. Lead exists in the air, drinking water, soil, dust, lead-based paints and several industrial products. Our physiologists understand that the human body is able to excrete about 2 milligrams of lead efficiently each day, but that measure in excess of that can cause serious health problems.[1,2] In addition the lead chromate can induce chromosomal damage. In cell experiments, high doses of lead chromate were weakly clastogenic; it induced a different spectrum of chromosomal aberrations. It is suggested that, the formation of lead inclusion bodies in normal human living cells exposed to lead chromate indicates that ionic lead is released from the particles and may then give to the cellular toxicity of lead chromate. The limit for Pb^{2+} and CrO_4^{2-} in drinking water, as set by the US Environmental Protection Agency (EPA) is 0.05 and 0.02 μM.[2] A variety of symptoms have been attributed to lead poisoning. Intake of even a very small amount of lead ion causes several health problems such as memory loss, anemia, and slow nerve conduction speed in children.[3] In particular; it can cause significant injury for children, even at low doses. Among the biologically important anions,[4] the chromium, found largely in the environment as chromate, (CrO_4^{2-}). Particularly, in humans and animals, chromate causes acute poisoning and cancer, even at low concentrations.[5]

However, standard techniques, such as atomic absorption spectrometry (AAS), mass spectrometry (MS), and square wave anodic stripping voltammetry (SWASV) measurement with Pb^{2+} ion[6] and extraction, ion exchange, chromatography and potentometric detection with CrO_4^{2-} ion,[7] often need generally time-consuming, and/or sample preparation. In addition, that the sophisticated instrumentation, less than the desired accuracy or are expensive and *in situ* analysis. As such, the invention of the suitable chemosensor probe is of great interest as they offer exciting opportunities for *in situ* and real-time detection without exposing the operator to the risk of cations and anions poisoning. Therefore, developing a new sensing technique is highly selective and sensitivity for the detection of Pb^{2+} and CrO_4^{2-} ions using inexpensive instruments one of the system of chemosensors. In the past few years, considerable efforts have been made to develop the fluorescent chemosensors for Pb^{2+} and CrO_4^{2-} ions have been published.[8] These studies suggest that the use of fluorescent chemosensors for the detection of some cations and anions can avoid expensive classical methodologies and can allow *in situ*, less than the desired accuracy and real-time detection. However, most of them are limited by interfering background fluorescence, nonspecific quenching from competing ions, and/or incompatibility with water. Hence, in the present work, the water solubility host-guest system properties of a new Pb^{2+} and CrO_4^{2-} ions specific fluorescent chemosensor. It has visible wavelength emission profiles, excellent selectivity and sensitivity for Pb^{2+} and CrO_4^{2-} ions over relevant other cations and anions in water.

The anthraquinones derivatives derived from hydroxyl terminals have been widely used in the chemistry application as a good chelating agent that they converted to complex form with metal ions.[9] Dihydroxyanthraquinone (1,5-DHAQ, probe **1**;Scheme 1a), an important class of organic compound that absorbs light in the visible region. They are used as an antioxidant in

medicine, have a wide range of biological and pharmaceutical applications. Hydroxyanthraquinones are well known colorimetric indicators for pH and metal ions.[10] In basicity of pH media, these substances exist in anionic (phenolate) form, which have absorption maxima in the visible region. It is attributed to an intramolecular charge transfer (ICT) transition from the donating phenolic groups to the π* orbitals of the quinone.[10] Cyclodextrins (CDs) are conically shaped, cyclic oligosaccharides in which the primary hydroxyl groups are situated on the narrow side of torus glucose residues and the secondary hydroxyl groups are located on the wider side. Among these CDs, β-cyclodextrin (β-CD), consisting of seven glucose units (Scheme 1b) having a moderate truncated cone-shaped hole, is commercially available and has been used in industry extensively. Aromatic organic compounds are particularly suitable guests due to the excellent docking abilities of the apolar aromatic ring, by establishing hydrophobic interactions with the host cavity of β-CD. β-CD has a hydrophobic inner cavity and hydrophilic outer surfaces. Supramolecular assembly formed by β-CD and probe **1** (probe **2**;Scheme 1b)complex are widely investigated to improve the water solubility of **1**. It is capable of interacting with a large variety of guest molecules to form inclusion complex are promising for biological and computational applications.[11]

The inclusion complex behaviors of β-CD molecules have been reported by our research group.[11] The complexation behavior has extensively verified in chemosensor application.[12,13] There was a little attempt to seek the application of the supramolecular function of β-CD to ions in chemosensor studies.[14] In this present paper, the absorption and fluorescence spectral properties of **1** and **2** were investigated. Based on this phenomenon, a chemosensor method was developed for the determination of Pb^{2+} and CrO_4^{2-} ions by **2** high selectivity and sensitivity. To

our best knowledge, there are no experimental or theoretical studies about the complexation **1** with β-CD as a chemosensor probes **1** and **2** (Scheme 1) to monitoring the Pb^{2+} and CrO_4^{2-} ions.

Experimental section

Materials and Instruments

β-Cyclodextrin (β-CD) were obtained from (HiMedia) and used without further purification before use. 1,5-Dihydroxyanthraquinone (1,5-DHAQ;**1**) (Sigma Aldrich) and other chemical reagents were of analytical reagent grade and used as commercial. Human lung cancer cell lines A549 were collected from National Center for Cell Science, Pune, India. All the metal salts and anions were purchased from the Sigma Aldrich. The stock solutions of the metal ions and anions ($1x10^{-4}$ M) were prepared in triply distilled water. From their chloride salts of Al^{3+}, Ba^{2+}, Bi^{3+}, Cu^{2+}, Cd^{2+}, Ce^{3+}, Co^{2+}, K^{+}, Fe^{3+}, Hg^{2+}, Sr^{2+}, Ti^{3+} and Sn^{2+}, nitrate salts of Pb^{2+}, Mg^{2+}, Mn^{2+}, Th^{4+}, Y^{3+} and Ln^{3+} and potassium salts of Br^{-}, Cl^{-}, OH^{-}, CO_3^{2-}, CH_3COO^{-}, CrO_4^{2-}, MnO_4^{-} and SO_4^{2-} anions, except $SnCl_2$ dissolved in 100 ml hot water solutions. In the solution pHs were prepared by adding the appropriate amount of NaOH and H_3PO_4. The solutions were prepared just before taking measurements. The stock solution of **1** ($1x10^{-4}$ M) were prepared using ethanol and β-CD solutions were prepared the following concentration 0.2, 0.4, 0.6, 0.8, 1.0 and $1.2x10^{-2}$ M respectively. All the studies were recorded at 30 ± 1°C.

The UV–Vis spectra (absorption spectral measurements) were carried out with Shimadzu UV-2401PC double-beam spectrophotometer and fluorescence spectra (an emission spectral measurement) were carried out with JASCO spectrofluorometer FP-8200. The pHs were measured on ELICO pH meter model LI-10T. FT-IR spectra were obtained with AVATAR-330 FT-IR spectroscopy using KBr pelleting, the range of spectra was from 500 to 4000 cm^{-1}. Powder X-ray diffraction spectra were taken by XPert PRO PANalytical diffractometer.

Differential Scanning Calorimetry is 200F3 Maia. The Scanning Electron Microscopic morphological measurements were taken by FEI Quanta 250 HR-SEM. Antibiofilm studies were measured by confocal laser scanning microscope CLSM-LSM 710 (objective 40;numerical apertures,0.6) confocal images were obtained by using zen 2009 image software (model 710; carl zeiss Imaging Germany) in the deconvolution mode. The computational analysis carried out using a single 1.0 GHz PC processor under the Linux operating system.

Preparation of solid inclusion complex of 1 with β-CD

Accurately weighed 1 g of β-CD was placed in to 50 ml conical flask and 30 ml triply distilled water added and then oscillated this solution enough. After that, 0.2116 g **1** was put in to a 50 ml beaker and 20 ml ethanol added and put over electromagnetic stirrer to stir until it was dissolved. Then slowly poured β-CD solutions in to **1** solution. The above mixed solution was continuously stirred for 48 h at room temperature. The reaction mixture was put into the refrigerator for 48 h. At this time, we observed that sandal powder precipitated. G4 sand filter funnels filtered the precipitate and it has washed with triply distilled water. After drying in oven at 50 ^{o}C for 12 h, the white powder product was obtained. This is solid inclusion complex of **1** with β-CD and it further analyses by FT-IR, XRD, DSC and SEM methods.

Biofilm formation and biofilm inhibitory concentration (BIC)

Biofilm inhibitory concentration was determined using the complexes with the different concentrations (1–5 μg/ml). Which are added into the bacterial suspension of 10^6 CFU/ml. BIC is determined as the lowest concentration of the complexes such as which showed the remarkable biofilm inhibition, disrupted biofilm architecture, and significant reduction in the test sample-treated wells when compared with the control (standard). For visualization, biofilm consisting glass pieces was immersed in the nutrient broth and the complexes such as (1–5 μg/ml) were

added to know the inhibitory activity. After 24-h incubation, the suspended cells and spent media are discarded and weakly adherent cells are removed by washing with deionized water and allowed to air dry before being stained. The stained biofilms were then air dried and visualized under a light microscopy (Nikon Eclipse Ti, Tokyo, Japan).

Confocal laser scanning microscopy (CLSM)

The effective biofilm inhibitions of complexes such as studied on glass slide surfaces. The tests culture grows for 24 h and dispensed in 24 well polystyrene plates are supplemented with complexes such as incubated for 24 h at 28 ºC. The biofilms are monitored under a CLSM (Model: LSM 710) (Carl Zeiss, Jena, Germany) after washing with PBS and staining with 0.02% acridine orange. The 488 nm Argon laser and 500–640 nm band pass an emission, filter are used to excite and detect the stained cells (human lung cancer A549). CLSM images are obtained from the 24-h old control, treated biofilms, and processed using Zen 2009 image software (Zen, Frickenhausen, Germany).

Computational study

The most probable structure of **1** and **2** (Pb^{2+} and $CrO4^{2-}$) complexes were determined by molecular docking studies using the PatchDock server (http://bioinfo3d.cs.tau.ac.il/PatchDock/). A docking protocol consisting of a global search by PatchDock and a refinement by FireDock was extensively tested (http://bioinfo3d.cs.tau.ac.il/FireDock/). The 3D structure of 1,5-DHAQ and β-CD was taken from the Cambridge structural database (CSD) Chemdraw ultra and Chem3D ultra (version 8.0, Cambridge.soft.com.,USA). The 3D structure of **1** and **2** (Pb^{2+} and $CrO4^{2-}$) complexes was generated by chimera 1.8.1 server (https://www.cgl.ucsf.edu/chimera). All geometries for **1** and **2** with ions (Pb^{2+} and $CrO4^{2-}$) were optimized by density functional theory (DFT) calculations using the B3LYP function was employed for calculations with a 6-

31G basis set on Gaussian 03W programmer except for Pb^{2+} and $CrO4^{2-}$, for which LANL2DZ was used.

Results and discussion

Influence of pH and effect of β-cyclodextrin

The absorption and fluorescence spectral properties of **1** have been verified through the effect of pH. The absorption and fluorescence maxima resemble the spectra observed in non-aqueous solvents. Thus, it has been assigned to be the neutral species (pH range 2.0 to 7.0).[12] But in pH 7.5 the absorption and fluorescence maxima of **1** is red shifted. It is due to the formation of monoanion by deprotonation of the hydroxyl group.[15] Here, probe **1** shows a strong π-π* absorption bands at 223.5 and 298.5 nm and weak n-π* band at 480 nm. The absorption maxima at 480 nm assigned to n-π* transitions of the hydroxyl group of **1** (λ_{ex} at 480 nm and λ_{em} at 591 nm). Hence, the effect of pH has been used for the optimization of the pH for the complexation of this molecule (anionic form) into β-CD.[16] The pH of solutions was adjusted at 7.5, in order to do better sensitivity for absorbance and fluorescence measurement and reduce the possible deprotonation of hydroxyl groups of **1**.[17]

Fig.1 depict the absorption and fluorescence spectra of **1** ($1x10^{-4}$ M) in pH 7.5 solution containing different concentrations of β-CD. At pH 7.5, the **1** exists as a monoanion form, hence the further β-CD inclusion complex is proceeding in the same pH.[18] The absorption and an emission spectral data of **1** in different concentration of β-CD are compiled in Table S1†. The absorption and fluorescence peaks of **1** appear in the visible range at 480 and 591 nm (Fig.1). There is an intramolecular hydrogen bond (IHB) formation between the hydroxyl (OH) and carbonyl (C=O) groups of **1** (Scheme 2). Upon addition of β-CD a sharp peak of **1** appears at 480 nm are slightly red shifted at 480.5 nm, indicating that the binding site of β-CD cavity. The

absorbance increases with increasing concentration of β-CD reaches at $1.2x10^{-2}$ M (Fig. 1a). Further investigation shows that the fluorescence intensity of **1** is dramatically enhanced upon addition of β-CD (Fig. 1b). However, the absorption and fluorescence spectra intensities in the pH 7.5 solution, **1** is regularly increasing with increasing concentration of β-CD.[11–13,15] The formation of an inclusion complex is responsible for the insertion of the hydrophobic group of the chosen **1** insight into the cavity of β-CD. The insertion of **1** inside the β-CD cavity largely depends on the number of releasing water molecules in the bulk water. There is probably that the inclusion complex may have 1:1 stoichiometric ratio of β-CD and **1**. The absorption and fluorescence intensity changes of **1** with β-CD is apparently due to the host-guest molecular interaction and consequent changes of the micro-environment of the guest molecule.[19]

The binding constant (*K)* and the stoichiometric ratio of the inclusion complex (**2)** can be determined according to the Benesi–Hildebrand (B.H) relation.[20] The B.H plot was plotted and gives a good linear correlation coefficient (R^2 = 0.9939 and R^2 = 0.9953) (Fig. S1†). It confirms the formation of a 1:1 inclusion complex, from the slope and intercept values of this plot the binding constant (*K)* was calculated to be 361.27 and 892.88 M^{-1} at 303 K (Table S1†). The large inclusion constant or binding constant values confirm a strong complexation between host and guest molecules.[21] These spectral change results essentially from the interaction between β-CD and **1** suggest the formation of an inclusion complex (Scheme 1c).

Characterization of solid inclusion complex

The solid inclusion complexation between β-CD and **1** is evidenced by FT-IR, XRD, DSC and SEM analysis shown in Fig. 2. In Fig.2a, the FTIR spectra of β-CD shows a broad O–H stretching vibration band at 3383cm^{-1}, a C–H stretching vibration band at 2926 cm^{-1}, a bending vibration peak of the O–H bond at 1240 cm^{-1} is due to O–H bending of physically adsorbed

water. The band at 1645 cm^{-1} corresponds to the H–O–H deformation band of water present in the cavity of β-CD. Bands at 1152, 1078 and 1020 cm^{-1} are assigned to stretching vibrations of C–O, C–O/C–C and C–O–C of glucose units, respectively. While bands at 995 and 943 cm^{-1} are attributed to absorption of C–O–C of the rings.[22] Furthermore, in the spectra of **1** the O–H group gives the characteristics stretching vibration bands at 3447 and 2857 cm^{-1}, because the stretching vibration bands of C–H group is at 2920 and 975 cm^{-1}. In addition, the bands at 1675 and 1625 cm^{-1} are absorbed of C=O group and 1599, 1569 and 1507 cm^{-1} of C=C in the benzene ring. The stretching vibration of the =C–O bond in the connected with benzene rings are observed at 1160, 1158 and 1153 cm^{-1}. The bands out of plane bend of the C–H in the benzene ring are registered at 850 - 700 cm^{-1}, respectively.[23] Compared with the spectra of β-CD, **1** and **2** are shown in Fig 2a. The bands of stretching vibration O–H group intensities are shifted and disappeared bands at 3396 and 2857 cm^{-1}, this is due to the hydroxyl group are included into the β-CD cavity. The characteristic stretching vibration of C=O at 1675 cm^{-1} and 1625 cm^{-1} intensities are reduced and shifted to 1653 and 1622 cm^{-1} this is due to the benzene ring entrapped into the cavity of β-CD.[21] The characteristic =C–O stretching vibration shifted to 1154 cm^{-1} and intensity also reduced. The above fact, the hydroxyl and carbonyl groups entrapped into the cavity of β-CD.[24] These suggest the possibility of formation of hydrogen bonds between the hydroxyl group of the host and the **1** carbonyl group.

The powder X-ray diffraction was performed to confirm the complexation between β-CD and **1**. The XRD patterns of β-CD, **1** and **2** are presented in Fig.2b. The strong and sharp diffraction peaks show that commercial β-CD is well-crystallized.[24] XRD pattern of **2** is characterized by diffraction peaks in which it is longer possible to distinguish the characteristic peaks of **1**. Encapsulation of **1** into the cavity of β-CD results in the shifted peaks at diffraction

angles 10.72, 24.27, 25.72, 28.77, 33.97 and 39.17 2θ, the disappearance of peaks 11.02, 17.42 2θ and new peaks are appearing at 10.72, 12.42 and 15.42 2θ to confirm the inclusion complex. Meanwhile, some peaks are disappearing, which is suggesting that, there is an interaction between β-CD and **1**, it may give to a loss of crystalline or amorphization of the samples.[25] The substantial increase of the inclusion complex crystalline in comparison with β-CD and **1.** The appearance of amorphous are the result of **1** molecule incorporation into the cavity of β-CD.[24,25] The result implies that there is an interaction between β-CD and **1**, and the inclusion complex could be formed.

Fig. 2c illustrates the DSC thermograms of β-CD, **1** and **2**, respectively. DSC analysis of the inclusion complex and initial compounds differ markedly. As seen from Fig. 2c, the typical endothermic peaks of β-CD at about 112 °C and 317 °C are observed, which is considered to be dehydration water molecules from the β-CD cavity.[25] The DSC analysis of **1** exhibited a sharp endothermic peak at about 282 °C, which can be due to the melting point of the **1**. When **1** is added, the bands of endothermic peaks are shifted to 97 °C and 272 °C, respectively (at 1:1 molar ratio of β-CD and **1**). Meanwhile, both of endothermic peaks become broader, which is probably due to the destruction of the β-cyclodextrin inclusion complex.[25] The morphological changes of the β-CD, **1** and **2** were observed by SEM (Fig. 2d). These pictures clearly elucidated the difference between β-CD, **1** and **2**. The SEM images of **1** are present in different forms from their inclusion complex. β-CD shows cake like structure and **1** shows the plate structure and the inclusion of **1** in the surface of **1** present in different morphological structure of **2** (Fig. 2d). The differences in the β-CD cavity observed morphology show that the formation of the inclusion complex between the β-CD and **1**.

Chemosensor

Response of chemosensor is often desirable analysis to detect silent substances such as various cations and anions by spectroscopic methods (absorption and fluorescence spectroscopy). The sensing chemosensor probes **1** and **2** show that the complex is sensitive to cations and anions. The addition of 1.0 equiv. of $Al^{3+,}$ Ba^{2+}, Bi^{3+}, Cu^{2+}, Cd^{2+}, Ce^{3+}, Co^{2+}, K^{+} , Fe^{3+} , Hg^{2+} , Sr^{2+}, Ti^{3+}, Sn^{2+}, Pb^{2+}, Mg^{2+}, Mn^{2+}, Th^{4+}, Y^{3+} and Ln^{3+} cations and Br^{-}, Cl^{-}, OH^{-}, CO_3^{2-}, CH_3COO^{-}, CrO_4^{2-}, MnO_4^{-} and SO_4^{2-} anions into the **1** and **2** leads to the change of absorption and fluorescence spectra (Figs. S2† and S3†). The chemosensor probes **1** and **2** with cations showed a highly selective and sensing chromogenic recognition behavior towards Pb^{2+} ion (**1**; 508.5 nm and **2**; 512 nm) and Fe^{3+} ion (**1**; 461 nm and **2**; 469 nm) and fluorogenic recognition behavior toward the Pb^{2+} ion only (**1** and **2**; 591.5 nm). Fig. S2† exhibited the absorption and an emission peak in aqueous pH 7.5 solution. In contrast, Pb^{2+} and Fe^{3+} ion in absorption intensity changes, now it is high selectively recognition behavior on Pb^{2+} ion chosen for the further cation sensitivity study. The probes **1** and **2** with anion recognition showed a sensing chromo-fluorogenic behavior toward only CrO_4^{2-} anion. The absorption (**1**; 208, 259.5, 362 nm and **2**; 221.5, 278, 370.5 nm) and fluorescence (557.5 nm) spectra in aqueous pH 7.5 solution is shown in Fig. S3†. Except Pb^{2+} and CrO_4^{2-} ions, stay all no detectable changes in the absorbance and fluorescence maxima and their intensities upon the addition of other cations and anions (Figs. S2† and S3†). In order to study the selection of **1** and **2** with cations and anions, the fluorescence results were performed well. It is evident that, Pb^{2+} ion induces the fluorescence intensity enhancement (591.5 nm) and CrO_4^{2-} ion induce the fluorescence quenching (557.5 nm), because other cations and anions showed negligible variations (Figs. S2† and S3†).[26] The histogram of absorption and fluorescence intensity changes shown in Figs. 3 and 4. As shown in the histogram, significant variation in the absorption and fluorescence intensities of probes **1** and **2**

shows highly selective behavior towards Pb^{2+} and CrO_4^{2-} ions were observed by comparison with the addition of other cations and anions. It is interesting to note that the absorption and fluorescence intensities of the probe **2** showed a significantly changing than probe **1** for the addition of Pb^{2+} and CrO_4^{2-} ions, it's represented in the Figs. 3 and 4.

Cation sensing

The binding activities of the sensor probe **1** with Pb^{2+} were studied by UV-visible spectroscopy (Fig. S2a†). Absorption spectrum of **1** shows an absorption maxima at 480 nm in the visible region. It is attributed to an ICT transition from the donating phenolic group to the π* orbital of the quinine[10,27] (Scheme 2). Upon the deprotonation of the phenolic hydroxyl substituent, the charge transfer donor strength is increased. And the absorption maxima shifted to longer wavelength.[28] When **1** with metal ions complexes are formed protons of the hydroxyl groups are replaced by metal ions and the absorption maxima also undergoes a red shift.[28] This absorption maxima is now about the intraligand charge transfer (ILCT) character. Upon addition of increasing amounts of Pb^{2+}, the longer wavelength absorption band was shifted to 508.5 nm could be assigned to O^- (phenolate)↔ Pb^{2+} (LMCT or MLCT) (Fig. S4†), indicating the formation of the **1**•Pb^{2+}complex. Metal complex to this **1** through the ionized 1-hydroxyl oxygen and the adjacent carbonyl oxygen, forming greater metal ion attraction, by means of which, **1**-metal complex are formed.[29] Formation of the chelate could increase the acceptor strength of the quinone moiety by withdrawing electron density to the metal cation. Accordingly facilitate the charge transfer (CT) in the ligand and the lowest energy visible absorption band of **1** red shift at 508.5 nm. The spectral responses can be attributed to the change in structural conformation of **1** an interaction with Pb^{2+}, which mostly reflects the coordinating interaction between the lone pair electrons of the carbonyl oxygen atom,[29] and then the charge transfer from the hydroxyl group.

In the **1** molecule, nonbonded lone-pair electrons in the carbonyl group will undergo a transition from the lowest energy an *n* orbital to a π* orbital and thus result in the absorption about at 508.5 nm.[30] Fig.S4†, the absorption intensity is increasing due to the addition of Pb^{2+} ion concentration at 508.5 nm (n–π^* electronic transition of carbonyl group). The stoichiometry of the complex formed between the probe and ion was determined by Job's plot.[31] In this method, the total molar concentration of **1** and Pb^{2+} ion are held constant, but their mole fractions are varied. A measurable factor that is proportional to complex formation (such as absorption signal) is plotted against the mole fractions of these components. The Job's plot showed that **1** formed a 1:1 complex with Pb^{2+} (Fig. S4a†). Fig.S4b†, the **1** dependence of Pb^{2+} ion absorbance can be analyzed by the Benesi–Hildebrand (B·H) equation.[20] The association constant ($K_{a)}$ of Pb^{2+} ion binding in the system was evaluated graphically the slope value and was found to be $2.0x10^4$ M^{-1}.

Noteworthy, that the absorption of the system is enhanced by the β-CD, reflecting on the sensitizing effect of β-CD for the determination of the cations (Fig. S2b†). The probe **1,** can dissolve easily in the organic solvents, but it is very difficult to dissolve in water. While in the presence of β-CD its soluble ability in water increases owing to the formation of the complex of **2**. Which could make **1** more easily to bind to Pb^{2+} ion (Fig. S4a†). To get insight into the binding properties as a sensitizer of β-CD with **1** toward different metal ions, we investigated the absorption changes upon addition of cations. The absorption intensity changes upon the addition of the cations are shown in Fig. S2b†. We showed that the intensity of **1** is slightly influenced by the presence of the cation. But, in the inclusion complex the absorption intensity of **2**•Pb^{2+} system increases more remarkably than **1**•Pb^{2+}, which could be attributed to supramolecular assembly after binding with metal ions in the presence of β-CD solution.[32] Because, it is

expected that produce novel functions that are different from those found in free molecules. Under the same experimental conditions, the **1**•Pb^{2+} absorption spectrum was recorded in the presence of β-CD marginally shift at 512 nm shown in Fig. 5a (**2**•Pb^{2+}). The suggesting the complex formation between **2** and Pb^{2+} ion there is a change in the absorption intensity by further addition of Pb^{2+} ion. The stoichiometry of the ternary complex **2**•Pb^{2+} was confirmed by Job's plot method, which exhibits a maximum at mole ratio 0.5 reflecting the formation of the complex by one mole of **2** with one mole of Pb^{2+} absorption intensity change at 512 nm (Fig.S5†). The association constant (K_a) was calculated using the linear Benesi–Hildebrand expression by plotting a graph between the measured absorbance $1/[A-A_0]$ at 512 nm as a function of $1/[Pb^{2+}]$ (inset fig. 5a). The association constant of $4.4x10^5$ M^{-1} was obtained by dividing the intercept with a slope. For Pb^{2+} ion binding, **2** has a five-fold higher association constant than **1**. The absorption intensity of **1**•Pb^{2+} increases slightly in aqueous solution, which could be ascribed to the formation of metal-chelate complex to increase the rigidity of C=O and –OH group reagent **1** bind with Pb^{2+} ion. However, the absorption intensity of **1**•Pb^{2+} increases more remarkably in the presence of β-CD. [12,13,33]

Sensitivity is a very important factor to evaluate the performance of a fluorescence chemosensor. In good agreement with the findings from absorption study, the probes **1** and **2** also exhibited a specific fluorescence response towards cations under similar conditions (Fig. S2c and S2d†). The enhanced fluorescence intensity upon the complexation of **1** with Pb^{2+} is mainly due to the ICT and chelation enhanced fluorescence (CHEF) effect shown in Fig. S6†.[33c] The chelation of Pb^{2+} ion with the isomerized **1** resulting in more rigidity of the molecule via inhibiting the free rotation of **1** the lone pair of electrons present in carbonyl oxygen. Consequently, carbonyl oxygen and the electron donating nature of deprotonated oxygen (anion)

would be greatly increased which makes the possible coordination of Pb^{2+} ion. Due to the rigid conjugation caused by the CHEF effect, an emission is taking place within the enhanced fluorescence intensity at 591.5 nm. Pb^{2+} ion was found to form both ground as well as excited state complex with **1,** thus giving a remarkable fluorescence enhancement. With the increasing concentration of 1 eq. of Pb^{2+} ion, gradual increase in an emission band at 591.5 nm was observed (Fig. S6†). As can be seen from inset Fig. S6†, the job's plot showed that 1:1 stoichiometry of the complex **1** with Pb^{2+} and good linearity proposed by the Benesi-Hildebrand plot. The free probe **2** an emitted at 591.5 nm, when excited at 512 nm shown in Fig. S2d†. Upon addition of Pb^{2+} ions, the fluorescence of **2** was selectively enhanced at 591.5 nm due to the electron transfer from **2** to **2**•Pb^{2+} complex. The fluorescence intensity of **2**•Pb^{2+} increases more remarkably, which could be attributed to supramolecular assembly than binding to **1**•Pb^{2+} in the presence of β-CD solution.

The suggesting the complex formation between further addition of Pb^{2+} ion and **2** there is a change in the fluorescence intensity (Fig. 5b).[12,13] Owing to its excellent optical properties, **2** is sensitive enough to detect environmentally relevant concentrations of Pb^{2+} ions in an aqueous solution. As shown in Fig. 5b†, an increase of fluorescence intensity could be observed with increasing Pb^{2+} concentration ($0.8x10^{-7}$ – $0.6x10^{-6}$ M). Binding analysis using the method of continuous variations (Job plot) established a 1:1 stoichiometry of the ternary complex for the **2**•Pb^{2+} complex. The association constant (K_a) was estimated graphically by plotting $1/[I–I_0]$ against $1/[Pb^{2+}]$ (inset fig. 5b). The data was linear (fitted according to the Benesi–Hilderbrand equation) and the K_a value was obtained from the slope and intercept with a good linear correlation coefficient (R^2 = 0.9987). The K_a value of the **2**•Pb^{2+} complex was found to be $1.6x10^3$ M^{-1}. The value suggested that the probe **2** has a high affinity towards Pb^{2+} ion. The limit

of detection (LOD) **2**•Pb^{2+} was also calculated from the fluorescence titration data. According to the IUPAC definition, the LOD were calculated using the relationship LOD = (3 x standard deviation)/slope.[34] To calculate the relative standard deviation, the absorption measurements of ten blank samples were taken. The calibration values between the minimum and the maximum intensity and then a linear regression curve was fitted to these normalized data to get the slope. With this approach, the LOD were found to be $9.0x10^{-8}$ M. As shown in Fig. 5b, incremental addition of Pb^{2+} ion concentration, the maximum US EPA limit for allowable levels of lead in drinking water,[2a] to a 0.05 μM solution of **2** affords a 55% emission increase. Comparison of proposed fluorescent chemosensor with previously reported sensor for Pb^{2+} ion are also listed in the Table 1, it is very easy to understand that still sensitivity, selectivity, linear response range and pH ranges of the chemosensors for the detection of a particular metal ion. In the Table 1, the main analytical characteristics (LR, LOD and pH) of the proposed chemosensor for the determination of Pb^{2+} ion were compared with those of some of the previously reported fluorescent chemosensor. Including N-[4(1-pyrene)-butyroyl]-l-glutamic acid,[8a] calix[4]arenes,[8b,c] polydiacetylenes, [8d] porphyrin, [8e] styrylcyanine,[8f] pyridine,[8g] chalcone,[8h] 5-(4-carboxy-phenylazo)-8-hydroxyquinoline,[28] and xanthenes is listed in this Table 1.[40c] As can be seen from Table 1, the proposed fluorescent chemosensor detection of Pb^{2+} is more sensitive than the previously reported chemosensor. Thus, probe **2** could monitor Pb^{2+} ion in water quantitatively with excellent sensitivity.

Anion sensing

The absorption spectra represented of the probes **1** and **2** in the presence of various anions in pH 7.5 solution (Fig. S3†). There was unique and selective intensity change in absorption spectra of probes **1** and **2** in the presence of anions. Interestingly, the absorption

spectra of probes **1** and **2** showed increased absorption intensity with CrO_4^{2-},[36] while here were no changes with any other tested anions. The absorption intensities of the electronic absorption bands can be predicted by the electronic transitions. The absorption spectra of probes **1** and **2** the effects of CrO_4^{2-} ions showed absorption peaks at **1**; 208, 259.5, 362 nm and **2**; 221.5, 278, 370.5 nm respectively, due to the IHB. The –OH group formed IHB with O (oxygens).[35] There is a change in the several unpaired electrons (**1**) in going from the ground state to the excited state the electronic transition is referred to as spin multiplicity. Hence, the **1** interact with CrO_4^{2-} ion, because in basic solutions above pH 7.

The absorption spectra represented of the probe **1** in the presence of various anions in pH 7.5 solutions (Fig. S3a†). The pH dependent chromate ion is quite labile, and in addition of **1** that form a complex. Hence, this transition corresponds to excitations of electrons from non-bonding (or) anti-bonding orbitals essentially located in the **1** to anti-bonding orbitals located on the CrO_4^{2-} ion. So, the charge transfer transitions occur in the UV region and are much more intense, the absorption maxima being in the range 200 – 400 nm.[36] The position of these bands (probe **1**, 208, 259.5 and 362 nm) depends on the nature of **1** and the CrO_4^{2-} ion. The n–π^* transitions between non-bonding atomic orbitals holding unshared pair electrons and anti-bonding pi-orbital are involved in the electronic transitions.[37] The non-bonding electrons are held more loosely than σ bonding electrons and so undergo transitions at comparatively longer (362 nm) wavelength. This transition occurs with **1** containing double bonds involving hetero-atoms bearing unshared pair of electrons (e.g., C=O).

The spectra are exhibiting three bands; more intense at 208 and 259.5 nm, corresponding to π–π^* transition and a low intensity (less probable) n–π^* transition at about 362 nm are observed.[37] Where, is in the n–π^* transitions are always less intense. Because the electrons in the

n-orbital are situated perpendicular to the plane of the π–bond and so the probability of the jump of an electron from n–π^* orbital's is very low energy. However, atoms brings about a partial overlap between the perpendicular planes and so n–π^* transition does occur, but only to a limited extent. The chemosensor probe **1** coordinates with CrO_4^{2-}, because the crystal field stabilization energy (CFSE) in tetrahedral stereoisomer. The electronic configuration of CrO_4^{2-} is d^0 in the weak and strong field (–Dq) for regular tetrahedral complexes and the CFSE is zero. In addition, this type of complex is intrinsically smaller than in an octahedral field because they have less direct effect on the d-orbitals.[38] The binding of chemosensor probe **1** with CrO_4^{2-} was further verified by titration with UV spectroscopy. The stepwise addition of CrO_4^{2-} ($2x10^{-5}$ – $32x10^{-5}$ M) to chemosensor probe **1** in pH 7.5 solution caused an increasing in absorbance (Fig. S8†). The stoichiometry between **1** and CrO_4^{2-} in the complex system was determined by Job's plot the changes in the absorbance response of **1** with varying concentrations of CrO_4^{2-}(inset fig. S8a†). The association constant (K_a) of **1** with CrO_4^{2-} was calculated using the linear Benesi–Hildebrand expression by plotting a graph between the measured intensity $1/[A-A_0]$ at 362 nm as a function of $1/[CrO_4^{2-}]$ (inset fig. S8b†).

The absorption spectra of **1**•CrO_4^{2-} remained undisturbed in the presence of the same equivalents of all other anions in pH 7.5 solution (Fig. S3b†), correlating its specific and selective binding. Moreover, in addition to β-CD chemosensor probe **2** was analyzed in the same pH 7.5 solution by UV spectroscopy. Here in the absorption intensity of **2**•CrO_4^{2-} complexes less enhancement when compared to absence of β-CD. The supramolecular assembly of CrO_4^{2-} anion with **1** in the presence of β-CD solution ($1.2x10^{-2}$ M) have been employed under the same experimental conditions. The absorption spectral maxima was observed at 362 nm in absence of β-CD. The addition of β-CD to the chemosensor probe **1** (**1**•CrO_4^{2-}) to shift the absorption

maxima to 370.5 nm (Fig. 6a).[37] Furthermore, binding analysis using the method of continuous variations (Job's plot) establishes that a 1:1 stoichiometry of the ternary complex (**2**•CrO_4^{2-}) complex is responsible for the observed absorption intensity increases (Fig. S9†). The association constant K_a was evaluated graphically by plotting $1/[A-A_0]$ against a $1/[CrO_4^{2-}]$ (inset fig. 6a). The data was linearly fit according to the Benesi–Hilderbrand equation, and the K_a value was obtained from the slope and intercept of the line. The K_a value of the **2**•CrO_4^{2-} complex was $2.5x10^3$ M^{-1}. The detection limit of **2** as a sensor for the analysis of CrO_4^{2-} was determined from the plot of fluorescence intensity as a function of the concentration of CrO_4^{2-}. **2** was found to have a detection limit of $1.3x10^{-6}$ M, which is reasonable for the detection of micromolar concentrations of CrO_4^{2-}.

The chemosensor probe **1** as a selective fluorescent moiety for CrO_4^{2-} anion in the selectivity experiments (Fig. S3c†). The coordination of chemosensor probe **1** with a number of anions was spectrofluorimetrically analyzed in an aqueous solution at room temperature. An aqueous pH 7.5 solutions of sensor probe spectrofluorimetrically analyzed with micro-litre amounts of $1x10^{-4}$ mol L^{-1} solution of anions at an excitation wavelength at 370.5 nm. In addition, it is important to note that the probe **1** are quenched by the CrO_4^{2-} anion at 557.5 nm.[39] The intensity of the fluorescence is decreased (quenching) by a coordinated process of CrO_4^{2-} anion.[39] Similarly, the fluorescence is quenching phenomena could also be observed. The detection of CrO_4^{2-} by fluorescence quenching of a nano-chemosensor recently reported.[39d] The observed fluorescence quenching may be attributed to an increase in electron density upon interaction of CrO_4^{2-} with an anionic form of deprotonated **1**. Otherwise, this coordination complex to a solution (pH 7.5; anionic form) of chemosensor probe **1** exhibited strong fluorescence quenching, suggesting the ICT or photoinduced electron transfer (PET) process due

to the deprotonation of the –OH group.[40] Moreover, quenching can occur by a CrO_4^{2-} ion. Because, the quenching occurs when the excited state fluorophore (**1**) is deactivated upon coordinate with CrO_4^{2-} ion in pH 7.5 solutions, which is called the CrO_4^{2-} is a quencher. The fluorophore (**1**) is returned to the ground state during a diffusive encounter with the CrO_4^{2-} ion. The **1** and CrO_4^{2-} molecules are not chemically altered in the fluorescence spectra (Fig. S10†). The electron deficient molecule like CrO_4^{2-} can act as quencher. For instance, quenching of **1** by CrO_4^{2-} is probably due to CrO_4^{2-}, which does not occur in the ground state (UV spectra). The response of the sensor to CrO_4^{2-} may be resulted from the oxidation of the primary alcohol by CrO_4^{2-} sources, which weakens the PET effect from the hydroxyl to the fluorophore. A commonly accepted mechanism for the quenching phenomenon involves an inversion between the strongest emission π–π^* and the poorly emission n–π^* states of this fluorophore. The quenching results are observed from a hydrogen bond interaction of phenolic OH with anions. It leads to the stabilization of the n–π^* with respect to the π–π^* states and a subsequent decrease of an emission intensities.

Protection against quenching is observed for **1** (chemosensor probe) bound to β-CD. In fact, binding of chemosensor probe **1** with anion to β-CD has been used to obtain room temperature fluorescence (Fig. S3d†). The β-CD provide protection from the pH 7.5 solution, but usually not complete protection from other than CrO_4^{2-} quenchers (Fig. 6b). Especially, the chemosensor based on PET in quenching has been used. In a typical PET sensor, an aromatic fluorophore such as **1** is linked to a –OH, typically by a short bond. If the –OH group is not deprotonated it not possible to quench the **1**. Nevertheless, in pH 7.5 solution, the **1** is deprotonated. Hence, deprotonated O^- increases its ability to donate an electron. Wherein, the CrO_4^{2-} anion is coordinated with **1** and the fluorescence intensity is decreased.[40] But, the basic

idea is that quenching by –OH requires the lone pair of electrons localized on the aromatic hydrocarbon of **1**.[41] When that the probe **1** is in the excited state, these lone pair electrons are in a Higher-energy orbital (HOMO, top). The excited electron leaves the energy of the vacancy. Hence, an electron from the CrO_4^{2-} enters this lower-energy orbital, effectively quenching the fluorescence. As shown in Fig. 6b, probe **2** decreases the fluorescence intensity could be observed with increasing CrO_4^{2-} concentration. Jobs plot of an emission intensity shows a maximum in the plot corresponds to ~0.5 mole fraction indicating 1:1 stoichiometry of the ternary complex formation of **2** with CrO_4^{2-} (Fig. S11†). Normally, quenching of fluorescence is described by the common Stern-Volmer equation.[42] From this equation F_0 is the total concentration of fluorophore (**2**), F is the Stern-Volmer constant and [Q] is the CrO_4^{2-} concentrations. Note that the dependence of F_0/F on CrO_4^{2-} anion concentration is linear, which is identical to that observed for the quenching, except that the quenching constant (K_{sv}). The corresponding Stern–Volmer plot is shown in the inset fig. 6b. The quenching of **2** by CrO_4^{2-}ion showed a good linear relation in the Stern–Volmer plot (F_0/F versus [CrO_4^{2-}]) with the K_{sv} value of 1.9×10^{6} M^{-1}. The K_{sv} suggested that CrO_4^{2-} exhibited a strong quenching ability toward the fluorescence of **2**. The high quenching efficiency imparts the assay high sensitivity. The detection limit of **2** as a fluorescent sensor for the analysis of CrO_4^{2-} was determined from the plot of fluorescence intensity as a function of the concentration of CrO_4^{2-} (0.1×10^{-7}–0.95×10^{-7}M). **2** was found to have a detection limit of 3.9×10^{-8} M, which is reasonable for the detection of micromolar concentrations of CrO_4^{2-} ion, the maximum US EPA limit for allowable level of chromium in drinking water.[2b] Comparison of proposed fluorescent chemosensor with previously reported sensor for CrO_4^{2-} ion are also listed in the Table 1. The main analytical characteristics (LR, LOD and pH) of the proposed chemosensor for the determination of CrO_4^{2-} ion is compared

with those of some of the previously reported chemosensor, including d-TPE/p(NIPAM-co-AAc),[8i] 1,8-Naphthalimide,[8i] perylene,[28] and benzimidazole.[28] As can be seen from Table 1, which indicates that **LC** has the best detection limit for CrO_4^{2-} ($3.9x10^{-8}$ M) indicating that these results are acceptable for the recognition of CrO_4^{2-} in water at micromolar concentrations.

Molecular modeling studies

The molecular model of **1** and **2** with Pb^{2+} and CrO_4^{2-} ions docking scores calculated by PatchDock and FireDock servers. The most probable structure based on the energetic parameters shown in Fig. 7 and Table S2†.[43] The docking studies revealed that the complex, highest geometric shape complementarity score, approximate interface area size of the complex and atomic contact energy by PatchDock server. The refinement by FireDock was extensively tested the highest global energy, attractive and repulsive van der Waals interaction energies and atomic contact energy was the highly probable and energetically favorable model. The metal center closely fits into the hydroxyl and carbonyl group of **1** molecule reveals that an excellent deal with the hydrophobic cavity of β-CD.[11a] These interactions probably suggested a process in which water molecules in the cavity were replaced by guest molecules by van der Waals forces between molecules. The docking results are in good correlation with results obtained through experimental methods.

To understand the binding structures as well as the reason behind the diversity of the receptor structures in ions recognition, we performed Density Functional Theory (DFT) calculation on the probes (**1** and **2**) with ions (Pb^{2+} and CrO_4^{2-}). The B3LYP function was employed for the calculations with a 6-31G basis set of Gaussian 03W programmer. For Pb^{2+} and CrO_4^{2-} for which LANL2DZ was used.[44] The probes **1** and **2** having three-dimensional structure. It is forming an oxygen, carbon and hydrogen of the carbonyl and hydroxyl groups, where the

B3LYP/6-31G basis set was employed for their optimization (Fig.8).[45] However, **1** and **2** (Pb^{2+} and CrO_4^{2-}) were optimized by using B3LYP/6-31G and LANL2DZ basis set for Pb^{2+} and CrO_4^{2-} (Fig.8). Optimized structures revealed the possible mode of complexation in which deprotonated hydroxyl group. It's due to its flexible nature and preeminent fit ions coordination sites between Pb^{2+} and CrO_4^{2-} and the probes (**1** and **2**).[46] In coordination with Pb^{2+} and $CrO4^{2-}$, there is an increase in the stability of the systems which was confirmed from the value of energy optimization (Fig.8). In contrast to the HOMO of **1** (Fig. 9a), the significant amplitude on the carbonyl groups, which means that the HOMO–LUMO transition of **1** has less hydroxyl to carbonyl charge transfer character. This may explain **1** has tendency to undergo intramolecular proton transfer.[45,47] Therefore, the energy of the LUMO (E_{LUMO}) indicates the ability of the molecule to accept electrons; hence these are the acceptor states. The lower the value of the E_{LUMO} is more probable and their molecules would accept the electrons.[45,47]

On the other hand, **1** with ions, the high value of the E_{HOMO} has to show a tendency of the molecule to donate electrons to the proper acceptor molecules. With low energy and empty molecular orbital, the value of E_{LUMO} indicates the ability of the molecule to accept the electrons.[48] However, noticeable ICT and the lowering of band gap was observed in the deprotonated forms. It shows in the Fig. 9a,[49] in the optimized structure of the probe **1** with CrO_4^{2-}, it was observed that one of the oxygen atom of CrO_4^{2-} abstract the proton from one hydroxyl hydrogen atom of **1.** While the other oxygen atom of the $CrO4^{2-}$ makes a covalent bond to the aromatic carbon atom.[50] This abnormality of CrO_4^{2-} may seem to be responsible for the quenching of the fluorescence for probe **1** compared to the potassium chromate (K_2CrO_4) case (Fig. 9a).

The gap between HOMO-LUMO is less (**1**•Pb^{2+} and **2**•Pb^{2+}) and (**1**•CrO_4^{2-} and **2**•CrO_4^{2-}) as compared to probes **1** and **2** alone, as depicted in Fig. 9. Further, the HOMO-LUMO (**1**•Pb^{2+} and **2**•Pb^{2+}) and (**1**•CrO_4^{2-} and **2**•CrO_4^{2-}) showed that LUMO is the main contributor in electronic transition. In an inclusion complex of **1** with β-CD, the HOMO and LUMO energies different from that in free **1**, indicating that the inclusion complexation exhibit negligible influence on the distribution of the frontier orbitals and the active site of **1**.[16] But the inclusion complexation show the remarkable influence of the energies of HOMO and LUMO. Compared with free **1**, inclusion in β-CD induces a slight decrease in HOMO and LUMO energies. This indicates that the electron donating capacity of **1** is enhanced by β-CD. Moreover, the cavity size of host molecules and the inclusion mode show remarkable influence on HOMO energies. Binding energies obtained from theoretical studies have proved those energy values of **1** and **2** probes decrease with the addition of Pb^{2+} and CrO_4^{2-}. Fig. 9, the energy gaps between HOMO and LUMO in the probes **1 (**Pb^{2+} and CrO_4^{2-}) 1.8461, 1.3552 and 1.1033 eV and **2 (**Pb^{2+} and CrO_4^{2-}) 0.7733, 0.1020 and 0.0825 eV, respectively. The energy gap between HOMO and LUMO in complex decreases shows a favorable complexation according to proposed coordination. Hence, leading to the formation of the stable complexes.[51] The energy, decrease in case of **2**•Pb^{2+} and $CrO4^{2-}$ is significantly more than that of **1**•Pb^{2+} and CrO_4^{2-} proving **2**•Pb^{2+} and $CrO_4^{2-}$to be more stable than **1**•Pb^{2+} and CrO_4^{2-}. The negative HOMO value of these stabilization energy showed that a stable inclusion complex was formed. Thus, the complex formed by **1** with ions prefers the encapsulation of β-CD cavity.[52] The calculations carried out by DFT methods shows that inclusion complex of the **1** with the coordination of ions in the presence of β-CD are stables and thus confirm experimental observations.

Bio-imaging and living cell Studies

As per the literature survey, there is limited reports are carried out on an antibiofilm activity from β-cyclodextrin and their chemosensor studies.[53] Fig. 10(A) clearly shows the inhibitory effect of complex system against *B.subtilis,* and *E.coli* microorganisms after 24h incubation was studied. The difference between the thicknesses of the biofilm formation was examined for all complex systems; it was compared with control system shown in Fig.10(A). In the microbial cells be likely to form aggregates in two different cultures easily visible in the biofilm generation.[11b,54] While following treatment with the complex system the biofilm formation appeared to be more diffuse and the extent of bacterial aggregation obviously reduced for all the two strains examined showed in Fig. 10(A). After conjugation with Pb^{2+}, the inclusion complex **2** with Pb^{2+} exhibits the enhanced antibacterial activity and biofilm inhibition; this is due to the involvement of metal ion and β-CD with the bacterial motility (on flagella) and biochemical metabolism, this was checked with bath assay. In addition, it also penetrates into the bacterial outer membrane and reduces the Exopolysaccharides (EPS) and slime level, due to the inert nature of bacterial cells, the confocal images showed the reduced level layer of bacterial cells on its surface when compared to the control one. The reason is due to the inhibition effect of **1** was increased rapidly when complex with Pb^{2+} and CrO_4^{2-} ions included by then β-CD (**2**•Pb^{2+} and **2**•CrO_4^{2-}),the potential ability of microbial inhibition was increased rapidly when compared to control. Owning to the encouraging selectivity and sensitivity of **1** and **2** towards Pb^{2+} and CrO_4^{2-} ions, bio-imaging experiments were conducted to prove the ability of **1** and **2** to detect Pb^{2+} and CrO_4^{2-} in living cells (human lung cancer cell A549).[24a,55] In this experiment complex **1** and **2** was allowed to uptake by the cells of interest and the images of the cells were recorded by the fluorescence microscopy following excitation at 508.5, 512, 362 and 370.5 nm by argon laser.

The A549 cells incubated with only probe **1** (a), β-CD (d) and probe **2** (e) exhibited no intracellular fluorescence was observed shown in Fig. 10(B). Again, the A549 cells were pre-treated with $2.0x10^{-5}$ M of $Pb(NO_3)_2$ and K_2CrO_4 in the growth medium for 30 min at 28 ^oC. The cells were then three times washed with PBS buffer to remove the remaining Pb^{2+} and CrO_4^{2-} and further incubated with probes **1** and **2** for 30 min at 28 ^oC. After incubation in the presence of probes **1** and **2**, A549 cells presented no intracellular fluorescence without the addition of Pb^{2+}, but exhibited a green fluorescence, as seen by the fluorescence microscope, upon the addition of Pb^{2+}. A549 cells incubated with probe **1** initially display a strong fluorescence image in the presence of CrO_4^{2-}. For the reason that the cellular uptake with CrO_4^{2-} species produce hydroxyl radicals, which can then react with molecular oxygen, leading to increases in cellular levels of reactive oxygen species and oxidative stress.[55e,f] Penetration of Cr (CrO_4^{2-}) into the nuclei of living cells is likely to involve the binding of CrO_4^{2-} to probe **1**. The Cr (CrO_4^{2-}) are less toxic, mobile and available for biological uptake, while Cr (CrO_4^{2-}) due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular probe molecules.[55g] Accordingly, Cr (CrO_4^{2-}) and its compounds can be easily absorbed by living cells.[55h] This event is followed by CrO_4^{2-} dissociation from the probe **1** (due to the high affinity of hydroxyl radicals of probe **1**) and its enhanced by β-CD in living cell activity (**2**•CrO_4^{2-}). Whereas a bright green fluorescence signal was observed in the cells stained both the probes **1** and **2** with Pb^{2+} and CrO_4^{2-} ($2.0x10^{-5}$ M). Moreover, bright green fluorescence cells obtained from the incubation of the chemosensor **1** and **2** with Pb^{2+} and CrO_4^{2-} became noticeable in fluorescence imaging in living cell activity. This bio analysis reveals the good cell permeability of **1** and **2,** which can be further explored as a biomaterial for the probing of bioactive analytes in the intracellular environment. With the results of fluorescence titrations and

bio-imaging analysis, the binding mechanism was proposed as 1:1 (**1** and **2**•Pb^{2+} and CrO_4^{2-}) binding mode was confirmed by Job's plot,[31] the association constant (K_a) values calculated using Benesi-Hildebrand (B-H) method[20] and detection limit values calculated using the relationship LOD = (3 x standard deviation)/slope.[34] These results demonstrated that **1** and **2** is cell-permeable and can respond to Pb^{2+} and CrO_4^{2-} ions within living cells.[55]

Conclusion

The inclusion complex of **1** with β-CD was prepared in solution and solid state. The inclusion complex between β-CD and **1** was formed an emission spectra confirm the 1:1 stoichiometric ratio and the association constant of 892.88 M^{-1}, while FT-IR, XRD, DSC and SEM analysis showed that the –OH group attached aromatic ring of the **1** encapsulated the β-CD cavity. Probes **1** and **2** showed highly selective and sensing behavior recognition towards Pb^{2+} and CrO_4^{2-} ions. The formation of a complex structure of **1**•Pb^{2+} and **1**•CrO_4^{2-} with increased solubility provided by β-CD. This approach can clearly be used as a simplistic and capable sensor for metal ion. The proposed sensor (**2**•Pb^{2+} and **2**•CrO_4^{2-}) with a limit of detection (LOD) $9.0x10^{-8}$ M and $3.9x10^{-8}$ M. Molecular docking studies and DFT calculation were used to correlate the experimental observation. The intracellular uptake of Pb^{2+} and CrO_4^{2-} ions by **1** with β-CD complex also successfully tested in the human lung cancer cell A549 with the support of confocal laser scanning microscopy.

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Table 1. Comparison of the proposed chemosensor with those of the previously reported fluorescent chemosensor for determination of Pb^{2+} and CrO_4^{2-} ions.

S.No	Reagent	Sample	Linear range (M)	LOD (M)	pH	Ref.
	Pb^{2+}					
1	pyrene	Water:DMSO	$0.6 – 8.0 \times 10^{-6}$	1.5×10^{-6}	5.5	8a
2	calix[4]arenes	$CH_3CN:CH_2Cl_2$	$0 – 1.0 \times 10^{-5}$	2.9×10^{-7}	–	8b
3	calix[4]arenes	CH_2Cl_2:MeOH	$1.0 – 6.0 \times 10^{-5}$	5.5×10^{-6}	–	8c
4	polydiacetylenes	HEPES buffer	$0 – 9.0 \times 10^{-6}$	0.8×10^{-6}	7.4	8d
5	porphyrin	CH_2Cl_2:MeOH	$0 – 1.0 \times 10^{-6}$	3.1×10^{-7}	–	8e
6	styrylcyanine	CH_3CN:water	$0.4 – 2.4 \times 10^{-5}$	3.4×10^{-6}	–	8f
7	pyridine	DMF:water	$0 – 1.6 \times 10^{-5}$	0.5×10^{-6}	–	8g
8	chalcone	HEPES buffer	$0.5 – 2.0 \times 10^{-7}$	2.0×10^{-7}	7.0	8h
9	CPA-8-HQL	HEPES buffer	$0 – 3.2 \times 10^{-6}$	4.9×10^{-7}	7.0	28
10	xanthenes	HEPES buffer	$1.0 – 5.0 \times 10^{-6}$	2.1×10^{-7}	7.0	40c
11	β-CD:1,5-DHAQ	Water	0.8×10^{-7}– 0.6×10^{-6}	9.0×10^{-8}	7.5	This work
	CrO_4^{2-}					
12	d-TPE/p(NIPAM-co-AAc)	Water:MeOH	$0 – 1.0 \times 10^{-6}$	0.5×10^{-6}	–	8i
13	1,8-Naphthalimide	DMF:water	$0 – 9.0 \times 10^{-6}$	3.6×10^{-7}	–	8j

14	perylene	Acetate buffer	$0.5 – 5.0 \times 10^{-6}$	0.8×10^{-7}	4.5	26b
15	benzimidazole	Water	$5\times10^{-6}–1.4\times10^{-3}$	8.9×10^{-7}	6.1	37c
16	β-CD:1,5-DHAQ	Water	0.1×10^{-7}– 9.5×10^{-8}	3.9×10^{-8}	7.5	This work

Graphical Abstract