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Colorimetric Sensor for Triphosphates and Their Application as a Viable Staining Agent for Prokaryotes and Eukaryotes

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The chromogenic complex 1·Zn (where 1 is *(E)*-4-(4-dimethylamino-phenylazo)-*N*,*N*-bispyridin-2-ylmethyl-benzenesulfonamide) showed high affinity toward the phosphate ion in tetrabutylammonium phosphate in acetonitrile solution and could preferentially bind to adenosine triphosphate (ATP) in aqueous solution at physiological pH. This binding caused a visual change in color, whereas no such change was noticed with other related anions (adenosine monophosphate, adenosine diphosphate, pyrophosphate, and phosphate) of biological significance. Thus, 1·Zn could be used as a staining agent for different biological cells through binding to the ATP, generated in situ by the mitochondria (in eukaryotes). For prokaryotes (bacteria) the cell membrane takes care of the cells' energy conversion, since they lack mitochondria. ATP is produced in their unique cell structure on the cell membrane, which is not found in any eukaryotes. These stained cells could be viewed with normal light microscopy. This reagent could even be used for distinguishing the Gram-positive and the Gram-negative bacteria (prokaryotes). This dye was found to be nonlipophilic in nature and nontoxic to living microbes (eukaryotes and prokaryotes). Further, stained cells were found to grow in their respective media, and this confirmed the maintenance of viability of the microbes even after staining, unlike with many other dyes available commercially.

The design of a chemosensor for selective recognition and sensing of an anion is an area of significant importance.[1,2] In this context, sensors that can detect and sense biologically important anions in aqueous environment and under physiological pH are of special significance owing to their application potential as biological markers.[3] In our earlier communication[4] we have shown that a Zn(II)-based coordination complex with a pendant azo functionality as the reporter group could be used for staining yeast cells (*Saccharomyces cerevisiae)* through preferential binding to the adenosine triphosphate (ATP) produced in situ. *S. cerevisiae*, a eukaryotic microbe, derives its energy in the form of ATP, and the surface of these eukaryotic cells is exposed with negatively charged ATP ions. These cells were found to be stained when exposed to the receptor molecule **1·Zn** for approximately 5 min. These preliminary results motivated us to explore the possibility of using this receptor molecule as a colorimetric staining agent for prokaryotic microbes, as well as for studying the growth kinetics. In this context, a viable staining agent has more relevance for practical applications.

ATP is a universal energy carrier in biological systems and contributes to biochemical reactions, active transport, nucleic acid synthesis, muscle activity, and the movements of cells.[5,6] A rise in the cytosolic ATP concentration is a key event in the functioning of membrane depolarization of ATP-dependent K^+ channels. Accordingly, measurement of cellular ATP levels in living cells with time is crucial as this would lead us to assess their metabolic state. In this regard, the nature of the staining reagent is important as it should not be toxic to the living system and should be able to sustain the cell proliferation and evaluate its rate. Further, it is well-known that prokaryotes and eukaryotes have different cell structures. Prokaryotes do not have any membrane-bound or-

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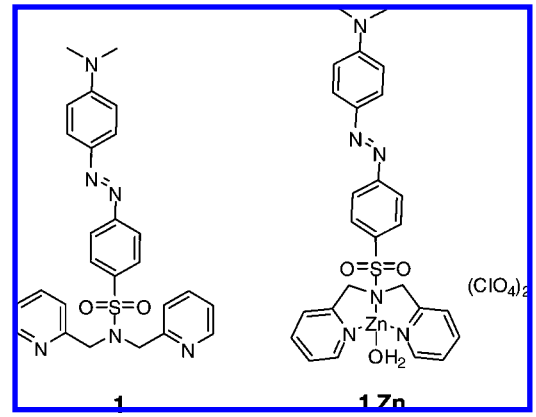
ganelle, which is present in eukaryotes; e.g., eukaryotes have mitochondria. Among the prokaryotes, Gram-positive (Gram +ve) and Gram-negative (Gram −ve) have different cell wall structures and chemical compositions.[7,8] Therefore, it would be even more important for the staining agent if it could effectively delineate the membrane of the smaller bacteria. Thus, to check its wider applicability, we have tested this reagent (**1·Zn**) with two different living cells, c.a., *Bacillus megaterium* and *Pseudomonas fluorescence*. Fresh water is essential for life, and methods to monitor bacteria in various sources of fresh water for human consumption, food preparation, and industries such as pharmaceutical manufacturing are required for microbiological quality assurance. Among various possibilities, the most popular method is the culture method, where over 24 h is usually required to obtain results and the bacterial number is sometimes underestimated because some viable bacteria are difficult to culture. Further, quantification and yeast viability are critical for fermentation,[9,10] medical examination,[11] wastewater treatment,[12] dental biofilm,[13] and bioengineering systems.[14] This necessitates the development of a more rapid and reliable method based on the viable staining of the cells. Though fluorescence staining reagents are more popular, use of colorimetric staining agents has its own significance owing to the simplicity in the detection technique. One can view the stained cells using a simple light microscope. In many instances, cells stained with certain fluorescent markers (e.g., 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)) are nonviable.[15] Moreover, one of the major aspects which has received relatively less attention is the time profile action of energy-dependent processes in yeast, which would enable us to monitor the bioprocess in a rapid, easy, and accurate manner through ATP binding with the growing microbes, i.e., their correlation to growth kinetics response.

Here, we have shown that **1·Zn** could be used as a viable staining agent for both prokaryotes and eukaryotes through binding to ATP, produced in situ during metabolic processes and, thereby, could be used for probing the growth profile of the respective microbes and cells.

EXPERIMENTAL SECTION

Materials and Methods. The chemicals such as bis(2-picolyl)amine, 4-(4-dimethylamino-phenylazo)-benzenesulfonyl chloride, tetrabutylammonium salts of anions, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and ATP were received from Aldrich and used as such. Synthesis of **1** and **1·Zn** was achieved in reasonably high yield following a procedure mentioned in our earlier communication.[4] Various analytical and spectroscopic data obtained for **1** and **1·Zn** (Scheme 1) provided necessary supports for the proposed formulation and required purity (Supporting Information). All other chemicals were of reagent grade and available locally. These were used without any further purification. Solvents such as THF and acetonitrile were dried before use, following standard procedures. Microanalysis (C, H, N) was performed using a Perkin-Elmer 4100 elemental analyzer. FT-IR spectra were recorded in KBr pellets using a Perkin-Elmer Spectra GX 2000 spectrometer. ^{1}H and ^{31}P NMR spectra were recorded on Bruker 200 MHz (Avance-DPX 200)/500 MHz (Bruker Avance II 500) FT-NMR spectrometers. Electronic spectra were recorded with a Shimadzu UV-3101 PC spectrophotometer.

Scheme 1. Structures of Compounds 1 and 1·Zn



1 1·Zn

Sample Preparation. *S. cerevisiae Cells.* Yeast (*S. cerevisiae*)[14p] was cultured in the glucose yeast extract agar (GYE) medium (glucose 1.0 g, yeast extract 10.0 g, distilled water 1000 mL, pH 7.4). The cells were harvested and vortexed for making the homogeneous suspension in sterile distilled water.

Gram-positive *Bacillus* sp. Gram +ve bacteria was isolated from the Okha sea coast (Gujarat, India) and cultured in nutrient broth (peptic digest of animal tissue 5.0 g, yeast extract 1.5 g, beef extract 1.5 g, sodium chloride 5.0 g in 1000 mL of distilled water, pH 7.4). The cells were harvested and vortexed for making the homogeneous suspension in sterile distilled water.

Gram-negative *Pseudomonas* sp. Gram –ve bacteria was isolated from the Okha sea coast (Gujarat state, India) and cultured in King's B medium (protease peptone 20.0 g, dipotassium hydrogen phosphate 1.5 g, magnesium sulfate 1.5 g, glycerol 15 mL in 1000 mL of distilled water, pH 7.4). The cells were harvested and vortexed for making the homogeneous suspension in sterile distilled water.

Staining of the Prokaryotes (Gram-Positive and Gram-Negative Bacteria). *Sample Preparation for Light Microscopy Images.* The bacterial cells were exposed to the receptor molecule **1·Zn** for 20 min, followed by subsequent washing with water/ethanol (70:30; v/v) mixtures, and then viewed under a light microscope (Carl Zeiss Axiom imager).

Sample Preparation for Scanning Electron Microscopy Images. The isolates of *Saccharomyces* sp. were cultured in GYE media, *Bacillus* sp. in nutrient broth, and *Pseudomonas* sp. in King's B medium for 24 h at 37 °C. An amount of 100 μL of this was taken

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(14) (a) Narvhus, J. A.; Gadaga, T. H. *Int. J. Food Microbiol.* **2003**, *86*, 51*S. cerevisiae* is the most commonly used species in the brewing and production of ethanol in the industry where quality control is required for consistent production and where such chemical sensors could be utilized to monitor the biological growth and also check the viability of the cells therein. The biological processes involved therein are unpredictable, so they need to be monitored at regular intervals.
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and allowed to incubate at room temperature along with 100 μL of 100 μM **1·Zn** solution (CH_3CN/C_2H_5OH; 1:9; v/v). The samples were fixed overnight at room temperature with 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer at pH 7.5. The samples were then washed with 0.1 M phosphate buffer (pH 7.5) at room temperature for 1 h. Postfixation was carried out in 2% (w/v) osmium tetroxide (OsO_4) in the same buffer, washing once with 0.1 M phosphate buffer for 20 min. Then, the water was removed by a graded water−ethanol series: 25% ethanol, 15 min; 50% ethanol, 15 min; 75% ethanol, 30 min; 90% ethanol, 60 min; absolute alcohol, 30 min. The specimens were rinsed in buffer and coated with gold in a sputter coater (Polaron SC7620) prior to microscopy. The material was examined in a scanning electron microscope (SEM) LEO 1430 VP at an accelerating voltage of 15 kV.

Viability Assay of the Bacteria after Staining with the 1·Zn Complex. Viability of the bacteria with dye was checked by mixing the bacterial culture with dye; then the culture was observed under the light microscope with a hanging drop prepared on a concavity slide for 3 h; then the mixture was streaked on nutrient agar and kept for further incubation of bacterial growth for 24−48 h at 37 °C (in duplicate). Natural growth for the stained cells using **1·Zn** as staining agent was observed.

Nonlipophilic Nature of the Dye. In order to check the nature of the dye (lipophilic or nonlipophilic) we checked its solubility in various oils and nonpolar organic solvents like hexane and toluene. Further, the dye complex solution was filtered using a biofilm (pore size 1.1 μm) made from polyhydroxybutyrate lipid (microbially synthesized) by a natural isolate of a bacterium.[16] We filtered the solution of **1·Zn** (1.0×10^{-3} M, 5 mL solution in ethanol−water; 1:9, v/v, filtering time was ~1.5 h) through this biofilm. The thickness of that porous film was found to be 0.22 nm, whereas the pore diameter was determined to be 500 nm. SEM images and EDX data of the biofilm before and after filtration of the solution were recorded (Supporting Information).

Spectrophotometric Titrations. A solution of 1.0×10^{-4} M **1·Zn** in acetonitrile was prepared and stored in the dark. This solution was used for all spectroscopic studies after appropriate dilution. The 1.0×10^{-3} M solutions of tetrabutylammonium (TBA) salts of the respective anions were prepared in dried and distilled acetonitrile and stored under an inert atmosphere. All titration experiments were performed using 2.0×10^{-5} M solutions of complex **1·Zn** and various concentrations of anions ($2.0-100.0 \times 10^{-6}$ M) in the same solvent. Affinity constants were evaluated after calculating the concentrations of the respective species, free **1·Zn**, A^- (A^- is the anionic analyte), and associated complexes, e.g., **1·Zn·A**$^-$ (1:1 complex of receptor **1·Zn** and A^-). The effect of the ionic strength on the affinity constant was also examined by repeating the studies at various (0−0.1 M [*n*-Bu_4N]ClO_4) supporting electrolyte concentrations. Affinity constants were evaluated from the collected absorbance data of the titration curve using 540 nm as the probe wavelength, and the equation $K_a = [\mathbf{1{\cdot}Zn{\cdot}A}^-]/\{[\mathbf{1{\cdot}Zn}]_{free}[A^-]_{free}\}$ was used for all calculations. For measurements in aqueous solutions, ultrapure water was used for the studies. The concentrations of the respective anions and the receptor molecule used for studies were kept unaltered. The pH for studies in aqueous solution was maintained with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer.

For growth kinetic studies, isolates of *Saccharomyces* sp. (eukaryote) were cultured in GYE media, *Bacillus* sp. (Gram +ve) in nutrient broth, and *Pseudomona*s sp. (Gram −ve) in King's B medium for 24 h at 37 °C. UV−vis spectral analysis was done by taking 1.0 mL of a sample of each culture at a regular interval of 2 h for 24 h to monitor the metabolic status of the culture. **1·Zn** was expected to bind the ATP, being released as the gradual progress of the growth occurs, and this was monitored by adding 100 μL of **1·Zn** to 1 mL of culture followed by incubation for 5 min at 25 ± 0.2 °C; subsequently absorbance was measured. Similarly, control experiments were performed with respective uninoculated media (blank).

To check the reversible binding of the **1·Zn** to ATP, sodium citrate solution (20% w/v in distilled water) was added to the adduct **1·Zn·ATP**, and electronic spectral changes were studied.

RESULTS AND DISCUSSION

Synthesis and Sensing Properties. **1** was synthesized following a conventional N-alkylation reaction and was isolated in the pure form. A methanolic solution of **1** was reacted with an aqueous solution of $Zn(ClO_4)_2$ to yield the receptor molecule **1·Zn** in good yield at room temperature.[4] Both **1** and **1·Zn** were characterized by standard analytical and spectroscopic techniques. Electronic spectra for a 50 μM solution of **1·Zn** in the absence and presence of various anions were recorded in acetonitrile solutions. The absorption spectrum recorded for **1·Zn**, showed an intense absorption band at 439 nm (ε = 15 720 M^{-1} cm^{-1}). On addition of excess tetrabutylammonium phosphate (TBAP) a new absorption band appeared at 510 nm (ε = 19 880 M^{-1} cm^{-1}).[4] However, no such spectral change could be observed on addition of an excess of other anions such as F^-, Br^-, Cl^-, I^-, CH_3COO^-, HSO_4^-, and $H_2PO_4^-$. We further checked the selectivity of the receptor molecule, **1·Zn**, toward different biologically important anions like ATP, ADP, AMP, and PPi (pyrophosphate) in aqueous media. The ground-state absorption spectra of **1·Zn** (25 μM) was recorded in a buffer solution [10 mM HEPES, pH ~ 7.2] at 25 °C (Figure 1). An intense absorption band centered at 463 nm was observed. On addition of an aqueous solution of ATP, the absorption maximum was found to be shifted at 484 nm. The observed shift of 24 nm for **1·Zn** on moving from acetonitrile to water as solvent is the result of the solvatochromic effect of the charge-transfer spectra. An associated color change from pale yellow to light pink was observed (Figure 1). The lower shift in the absorption band maximums for **1·Zn** on binding to ATP, as compared to that observed in acetonitrile solution, could be rationalized based on the higher solvation of the Zn^{2+} ion in aqueous solution. However, upon addition of ADP to a **1·Zn** solution, a much smaller red shift (8 nm) in λ_{max} occurred and no distinct change in color could be seen by the naked eye. Furthermore, no change in absorption spectra was observed on addition of AMP, PPi, or $H_2PO_4^-$ (Figure 1A). For experiments with excess CTP, similar a spectral shift was detected as was observed for ATP. Respective binding constants for ATP, CTP, and ADP were evaluated from spectrophotometric titration profiles and were found to be 1130 ± 6, 772 ± 5, and 250 ± 6 M^{-1}, respectively, in aqueous solution (pH ~7.2) at 25 °C. However, this was not possible for AMP and other anions, as the associated spectral changes were negligible due to a much weaker binding.

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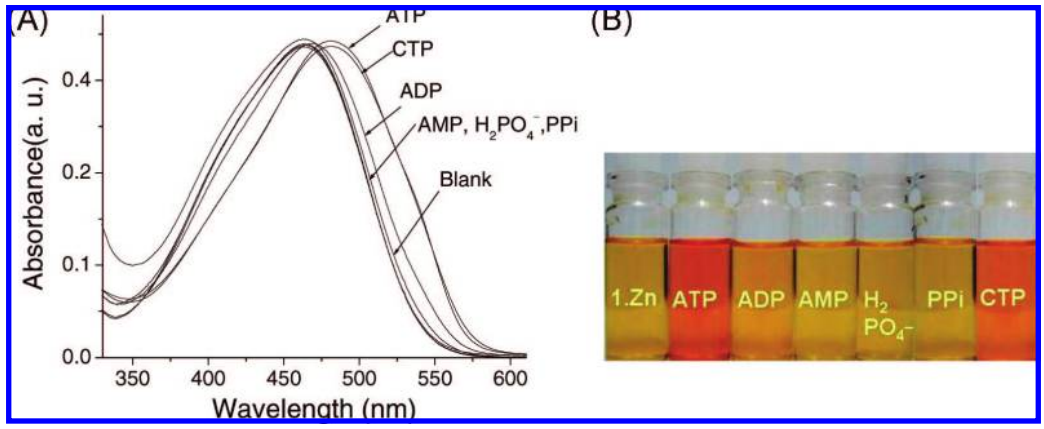

Figure 1. (A) Absorbance spectra of **1·Zn** (25 μM) in HEPES buffer solution (pH ~7.2) at 25 °C in the presence of various anions (250 μM) and (B) change in color of **1·Zn** in aqueous solution; from left to right: blank, with ATP, ADP, AMP, $H_2PO_4^-$ PPi, (anion concentration 100 μM), and CTP (125 μM).

Receptor molecule **1·Zn** showed a weak luminescence on excitation at 463 nm. The weak emission was found to be partially quenched on addition of ATP and CTP, whereas no such decrease in emission intensity was observed when excess ADP, AMP, PPi, or $H_2PO_4^-$ was added to HEPES buffer solution. This study further confirmed the efficient binding of ATP and CTP to **1·Zn**. Two factors might have contributed to this observed quenching of fluorescence. First, efficient binding of ATP and CTP to **1·Zn** could cause a more effective solvation of **1·Zn·**ATP as compared to **1·Zn** and cause a more efficient decay of the excited state through a nonradiative pathway. Second, the binding-induced blue shift of the charge-transfer spectra (Figure 1) reveals a narrowing of the HOMO−LUMO energy gap, and this could also contribute to a faster decay of the excited state and thereby the observed fluorescence quenching, following the energy gap law.

Binding of ATP and CTP was also confirmed by ^{31}P NMR spectroscopy (Supporting Information Figure 3). Downfield shifts for the ^{31}P signals for the γ- and β-phosphorus atoms of the ATP/CTP bound to **1·Zn** were observed. Similar shifts have been reported earlier by others.[3g] Downfield shifts for the ^{31}P signals for γ- and β-phosphorus atoms signify the binding to the Zn atom of **1·Zn** through the respective phosphate units. Thus, as expected the shift for the α-phosphorus atom was less. A very insignificant shift in ^{31}P signals was observed when similar experiments were repeated for ADP, and no such shift was observed when identical experiments were performed with AMP. Thus, the ^{31}P NMR spectral data also confirms the observed trend in binding affinity (ATP > CTP ≫ ADP ≫ AMP). Presumably, the enhanced electrostatic interaction between ATP/CTP and **1·Zn** is crucial for efficient **1·Zn**−O (phosphate) binding. Hence, the observed binding preference of **1·Zn**, ATP or CTP > ADP ≫ AMP, could be attributed to the difference in the number of the anionic charges of the phosphate species. Among ATP and CTP, presumably the presence of the pyrimidone functionality in CTP has made it a weaker electron donor, and this perhaps is reflected in the lower affinity constant. However, being a triphosphate, it binds more efficiently to **1·Zn**, as compared to ADP and AMP. Further, accessibility of the two adjacent β- and γ-phosphate units to the Zn center in **1·Zn** for the formation of a stable six-membered ring structure is expected to be more favorable for triphosphates for steric reasons as compared to diphosphates. This could also contribute to the higher interaction of **1·Zn** with triphosphates. Our experimental results further reveal that, among ATP/CTP and PPi, interaction of PPi with **1·Zn** is very weak in nature and cannot induce any detectable spectral change. Earlier studies reveal that PPi prefers to act as a bridging ligand in related binuclear complexes rather than forming a stable six-membered chelate complex.[3d,g] Further, solvation of the PPi in water is much higher as compared to that of ATP.[3i] For PPi solvation energy is unusually high and may account for the weaker binding of the effectively solvated PPi to the Zn(II) center in **1·Zn**. Though it is difficult to ascertain the exact reason, it is presumed that either of these two or both are crucial for the observed difference in binding and hence weaker perturbation of the energy levels and no change in spectral pattern.

Reversible binding of **1·Zn** to ATP could be demonstrated by adding a solution of sodium citrate (20% (w/v) in distilled water) to **1·Zn·ATP**. The original spectrum of **1·Zn** was restored when excess aqueous solution of sodium citrate was added to the solution of **1·Zn·ATP** (Supporting Information Figure 2).[18] This indicated the cleavage of the ATP−Zn covalent bond in **1·Zn·ATP** and, thereby, the reversibility of the binding process. Binding of citrate ion to the Zn center in **1·Zn**, on addition of aqueous solution of excess citrate ion to **1·Zn**, caused a detectable increase in absorption intensity without any change in the position of absorption maxima. Reversible staining of the yeast cell through binding of ATP, produced in situ, to **1·Zn** could also be viewed under a light microscope and will be discussed in the following section.

The nonlipophilic nature of the dye, **1·Zn**, was established, and details are provided in the Supporting Information.

Staining Studies of the Prokaryotes (Gram-Positive and Gram-Negative Bacteria). *Light Microscopy Image.* In our earlier study we could demonstrate that the **1·Zn** molecule can be used as a colorimetric staining agent for yeast cells and the staining nature could be viewed under a simple light microscope.[4] Binding of the ATP ions, produced in situ, on the cell surface to **1·Zn** was accounted as the plausible reason for the observed staining.

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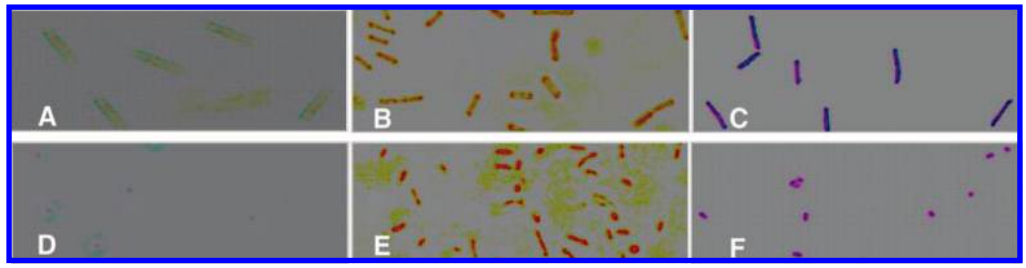

Figure 2. Gram +ve *Bacillus* sp. (A) without any staining agent, (B) when treated with **1·Zn**, (C) when treated with gentian violet dye; Gram −ve *Pseudomonas* sp. (D) without any staining agents, (E) when treated with **1·Zn**, (F) when treated with safranin dye.

Further light microscopic images revealed that the stained cells became colorless on addition of excess citrate ion to the stained yeast cell, i.e., mixture of yeast cells and **1·Zn** (Supporting Information). This observation could be explained on the basis of the reversible binding of ATP, produced in situ in the yeast cells. We also explored the possibility of using **1·Zn** as the staining agent for prokaryotes (Gram +ve and Gram −ve bacterial cells). Figure 2, parts A and D, reveals the images of the two bacteria, *B. megaterium* (Gram +ve) and *P. fluorescence* (Gram −ve), respectively, when viewed under a light microscope. Figure 2, parts B and E, reveals images of the same bacteria when viewed in the presence of **1·Zn**. These figures clearly show a distinct change in color of the bacterial stains in the presence of **1·Zn**, whereas Figure 2, parts C and F, shows the images of the stained bacteria in the presence of standard Gram staining agent, c.a., gentian violet and safranin, which are most commonly used for the preliminary identification of bacteria. For gentian violet dye, staining is based on the ability of the bacterial cell wall to retain the dye by Gram +ve bacteria during alcohol treatment, and the stained cells appeared violet when viewed through the optical microscope. This was dark pink when safranin (secondary dye) was used for Gram −ve bacteria. Figure 2, parts B and E, taken with identical magnification, revealed that the present dye **1·Zn** could also distinguish Gram +ve and Gram −ve bacteria through distinctly different color intensities and shapes of the stained cells. As expected, stained cells for Gram +ve bacteria appeared longer as compared to those of the stained Gram −ve bacteria. The difference in the staining intensity for two different bacteria could be better understood if one considers the difference in cell structure and cell wall composition of the respective bacteria.

The amount of extracellular ATP being released by Gram −ve bacteria is reported to be more than that of the Gram +ve bacteria.[5] The thinner hydrophilic and more porous cell walls of the Gram −ve bacteria are expected to allow higher excretion of ATP to the cell surface, where it gets bound to the present dye **1·Zn** and thereby causes the efficient staining. Thus, both these reasons are expected to contribute to the observed difference in the intensity of color in the two stained bacterial cells. Existing reports on the recognition of ATP are mostly based on the changes in either fluorescence or electrochemical properties,[19] and examples for the colorimetric detection of ATP in aqueous solution are rather limited.[20] Further, except a few examples, most reports are restricted to study in the recognition of the ATP or other biological phosphates in aqueous pH under physiological condition.[19o,p]

Scanning Electron Microscopy. SEM images of blank and stained eukaryote (yeast), Gram +ve, and Gram −ve cells have been recorded (Figure 3) to reveal the change(s) in the morphology of the outer surface of the cells on staining, as the maximum concentration of ATP is released through mitochondria in the periplasmic space and then gets excreted to the cell surface.[6]

Several ATP binding cassette proteins are also reported to accumulate in the plasma membrane.[7,8] SEM images of yeast cells without dye were found to be smooth in contrast to the images of the yeast cells with dye, where stained cell surfaces were found to be rough. This revealed the presence of the extraneous material, i.e., **1·Zn**, adhering to the cell. Changes on the cell surfaces on binding of the dye **1·Zn** were also evident from the SEM images recorded for Gram −ve and Gram +ve bacteria, in the absence and presence of staining agent (Figure 3C−F). The difference in the contrasts in the SEM images of the sample surfaces also signifies the nonhomogeneous chemical nature of the sample surface. The relatively brighter cell exterior for Gram −ve bacteria, as compared to the Gram +ve one, indicates the accumulation of higher negative charge on the extracellular surface. This observation correlates well with the earlier reports which state that extracellular ATP release is higher for Gram −ve bacteria and its more porous surface with a protein called porin.[17,22]

Higher selectivity, reversible binding of ATP to **1·Zn**, and the visual change in color led us to consider the possible use of this reagent for checking the viability of this staining agent.

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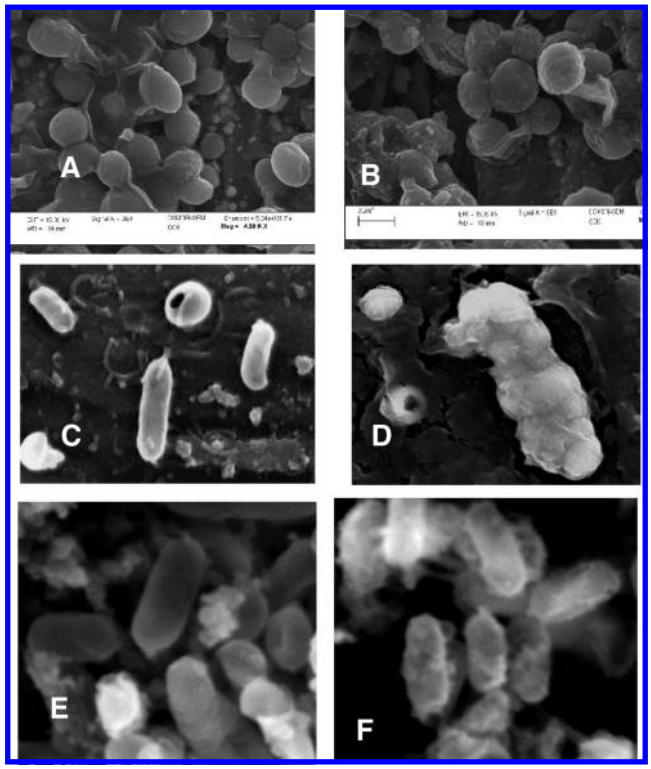

Figure 3. SEM images of (A) blank yeast cells, (B) yeast cells treated with **1·Zn** complex, (C) blank Gram −ve bacterial cells, (D) Gram −ve bacterial cells treated with **1·Zn** complex, (E) Gram +ve bacterial cells, and (F) Gram +ve bacterial cells treated with **1·Zn** complex.

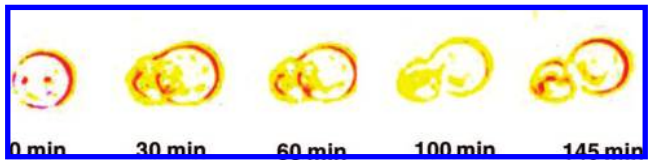

Figure 4. Light microscopy images of a yeast cell stained with **1·Zn** and monitored at different time intervals.

Viability Assay of the Cells after Staining with 1·Zn Complex. The viability of the cells was observed before and after staining with the **1·Zn** under the light microscope. The cell growth, cell division, as well as their motility could be observed when the stained cell suspension was taken as a hanging drop prepared in a concavity slide until 3 h (Figure 4).

Unaffected cell proliferation and growth confirmed that the staining agent (**1·Zn**) was nontoxic and kept cells viable after staining.[21] Thus, **1·Zn** could be used as an efficient in situ chemosensor for bioactive molecules like ATP and also as a viable staining agent. The growth profiles of eukaryotic yeast (*S. cerevisiae)* cells and prokaryotic bacteria (Gram +ve and Gram −ve) in an aqueous culture medium in the absence and presence of **1·Zn** are shown in Figure 5. The change in absorbance (at λ_{max} = 600 nm) versus time (t) was plotted up to 30 h, as no appreciable change in growth was observed thereafter. Figure 5a−f revealed a nice correlation between the cell growth and the ATP generation with progressive growth of the respective cells

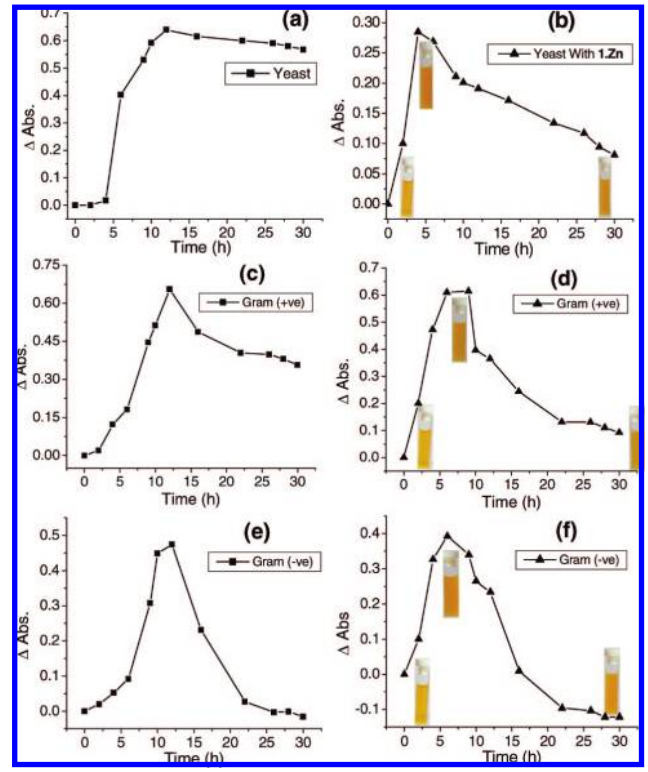

Figure 5. Growth curve of (a) *S. cerevisiae*, (c) *P. fluorescence*, and (e) *B. megaterium* obtained from change in cell count with time. Relative change in absorbance at 600 nm for aqueous culture medium of (b) *S. cerevisiae*, (d) *P. fluorescence*, and (f) *B. megaterium* in the presence of **1·Zn** (50 μM).

during the metabolic processes.[24] These kinetic studies were carried out to monitor the different phases of growth, wherein the increase in the ATP concentration as the batch culture grows from lag phase to log phase and then stationary phase and ultimately a decline in the growth curve was observed. Parts a, c, and e of Figure 5 show the actual growth curves of *S. cerevisiae*, *P. fluorescence*, and *B. megaterium*, respectively, with time, whereas parts b, d, and f of Figure 5 show relative buildup of ATP in cytoplasm with time for *S. cerevisiae*, *P. fluorescence*, and *B. megaterium*, respectively. Figure 5 reveals that the actual cell growth and buildup in ATP concentration in the cultures under identical experimental conditions matched nicely. The relative decrease observed in actual cell growth and ATP concentration (Figure 5) can be explained if one considers the catabolic processes, which consume ATP, become more important after certain time.[22]

Neither ATP nor ADP/AMP/PPi can be stored at high concentration within the cells of the living system. Their relative concentrations in cytoplasm are generally regulated over a narrow concentration limit by various biological processes.[24] Actually, bacteria impose regulatory mechanisms on metabolic processes to ensure that the needs of the cell are met but not exceeded.[24] Thus, the physiological consequences of this regulation are the same, though the mechanisms in Gram +ve and Gram −ve

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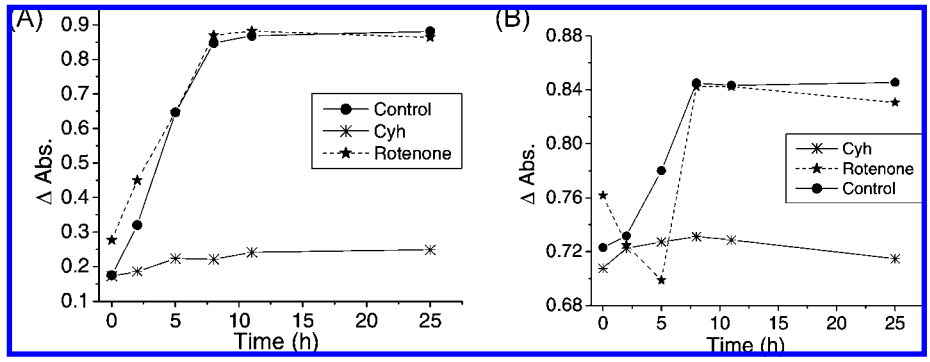

Figure 6. Growth curve of *S. cerevisiae* and relative change in [ATP] with time: (A) relative change in absorbance at 600 nm for aqueous culture medium of *S. cerevisiae* in the presence of **1·Zn** (50 μM) complex (control), 50 μL of 300 μg/mL rotenone, and 1 μL of 2 μg/mL cycloheximide (Cyh); (B) relative changes in ATP concentration in the presence (control) of **1·Zn** (50 μM)/**1·Zn** (50 μM) + 50 μL of 300 μg/mL rotenone (rotenone)/**1·Zn** (50 μM) + 1 μL of 2 μg/mL Cyh.

bacteria are entirely different.[25] However, kinetic studies revealed that cell growth and release of ATP during the metabolic process could be correlated using **1·Zn** as the staining agent. The extent of absorbance changes and the higher slope observed in the case of Gram −ve bacteria revealed a higher binding of **1·Zn** to ATP released during the cell growth, as compared to Gram +ve.

To ascertain that **1·Zn** binds to extracellular ATP produced during metabolic processes, we have studied the growth profile for the eukaryotic yeast cells and ATP produced during metabolic processes in an aqueous culture medium in the absence and presence of two different respiratory inhibitors, namely, rotenone (50 μL of 300 μg/mL)[26a] and cycloheximide (1 μL of 2 μg/mL).[26b] Among these, rotenone is known to inhibit the proton pump of yeast cells, and this causes the inhibition of the plasma membrane ATPase activity and does not interfere with the growth of the cells. For the control experiment when this was added to the culture media of the yeast cell, growth curves remain almost unaffected (Figure 6A). However, initially ATP generation with progressive growth of the respective cells during the metabolic processes was found to be appreciably affected on addition of rotenone to the yeast cell culture stained with **1·Zn**.

This observation agrees well with the previous reports which state that rotenone inhibits the generation of ATP.[26a] A small increase in ATP concentration was detected after initial decrease (~5 h) before it gets into the stationary phase. Due to an appreciable increase in the cell numbers after about 5 h (Figure 6B), the concentration of rotenone was not sufficient enough to completely inhibit the plasma membrane ATPase activity, and an increase in ATP concentration was registered until ~8 h, after which a stationary phase was attained. To develop a better insight we determined the cell growth of the yeast cells in the absence and presence of cycloheximide. This is known to inhibit the complete growth of yeast cells, as well as the ATP production.[26b] As seen in the growth curve with cycloheximide (Figure 6B), both cell growth and ATP generation were completely arrested. Thus, these results obtained in the presence of different respiratory inhibitors tend to confirm that the increase in absorbance and the detectable color change of the stained yeast cells are arising from the binding of ATP to **1·Zn.**

Thus, we could demonstrate that the present dye could be used as a viable staining agent for eukaryotic and prokaryotic cells. This could even be used for distinguishing the Gram −ve and Gram +ve bacteria and for studying the growth profile for the release of ATP during metabolic processes. Unlike many commercially available dyes/staining agents, namely, Gram stains (i.e., crystal violet, iodine, safranin, and neutral red), most azo dyes (e.g., Janus green, fast blue, etc.), and certain redox dyes (CTC, methylene blue, potassium ferricyanide, etc.), which are toxic,[27] **1·Zn** is nontoxic and maintains the viability of the living cells.

CONCLUSION

In summary, we have developed a sensitive chemosensor through coordination of ATP to the **1·Zn** complex in prokaryotes as well as eukaryotes. This dye is nonlipophilic in nature and was found to be nontoxic to living microbes (eukaryotes and prokaryotes). This reagent could even be used for distinguishing the Gram +ve, Gram −ve bacteria (prokaryotes), and yeast (eukaryotes) when viewed under a light microscope. Further, stained cells were found to grow in their respective media, and this confirmed the maintenance of viability of the microbes even after staining. The binding affinity of this complex is maximum in ATP > CTP ≫ ADP ≫ AMP. The growth kinetics of the cells in a batch process of culture studied showed a typical sigmoid curve, so the metabolic status of the culture and the process involved therein could be well-monitored. Studies with different respiratory inhibitors tend to confirm that the staining of the yeast cells occurs owing to the binding of extracellular ATP, produced in situ, with **1·Zn**. For prokaryotes, this could be well-utilized in the area of medical examination, the environment, and food industries. Thus, this

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colorimetric sensor molecule may find application in the fermentation industry, medical areas, environmental waste management, etc.

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SUPPORTING INFORMATION AVAILABLE

Details of the analytical data for **1** and **1·Zn**, change in emission spectra of **1·Zn** in aqueous solution (pH ~ 7.2) upon addition of various anions, absorption spectra of **1·Zn** and **1·Zn·ATP** in the absence and presence of citrate ion, partial ^{31}P NMR spectra of ATP, CTP alone, and ATP + **1·Zn**, CTP + **1·Zn**, recorded in D_2O at room temperature, light microscopy images of yeast cells stained with **1·Zn** and stained yeast cells after treatment with citrate ion, and growth profile for ATP (yeast cell culture in presence of **1·Zn** (50 μM), **1·Zn** (50 μM) + rotenone, **1·Zn** (50 μM) + Cyh. This material is available free of charge via the Internet at http://pubs.acs.org.

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