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ARTICLE TYPE

'Aggregation Induced Phosphorescence' Active Iridium(III) Complexes for Integrated Sensing and Inhibition of Bacteria in Aqueous Solution

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The present study attempts to develop sensitive method to utilize 'aggregation induced emission (AIE)' active iridium(III) complexes as potential agents for "integrated" sensing and inhibition of bacteria in aqueous systems. The utilization of iridium(III) complexes for microbial detection in water bodies has been demonstrated using *Escherichia coli* (*E. coli*) as a representative bacterial strains. The tested iridium(III) complexes also exhibited antibacterial properties against representative Gram positive and Gram negative bacterial strains with the minimum inhibitory concentration (MIC) values of 4 and 8 μg/mL, respectively. Microscopic observations indicated that these complexes could penetrate into the bacterial cells and resulted in subsequent cell death. Preliminary mechanistic studies showed that the DNA binding ability of iridium(III) complexes is responsible for their antibacterial properties. The observed "dual" role in detection as well as inhibition of bacterial growth makes this study a highly promising and encourage to explore the applicability of other less expensive metal complexes in monitoring and controlling the bacterial levels in drinking and sea water systems at commercial level.

Introduction

Population explosion and the stress of modern life have attracted many people towards sea shore holiday offers from various sources. These have resulted in overcrowding and over use of sea beaches and thus require proper checking of water health quality of beaches and costal areas.[1a] Monitoring of recreational beaches for fecal indicator bacteria is mostly performed using culture-based technologies that require more than a day time for laboratory analysis and compelling swimmers at risk.[1b,c] In spite of development of quantitative polymerase chain reaction (qPCR) methods,[1d] classic microbiological indicators such as faecal coliforms *Escherichia coli* and *Enterococci* strains are most commonly used to evaluate the level of fecal contamination in water. These classical methods are also used to assess the efficiency of pathogen removal in water purification processes. Based upon the comparative success of transition metal complexes as anticancer agents,[2–5] there is growing interest in the use of metal complexes as antimicrobial agents.[6–12]The interaction properties of inert octahedral metal complexes with cell's deoxyribonucleic acid (DNA) has also become a hot topic of research in the field of biosensors in recent years.[13]

Till date different classes of luminophores, such as fluorescent proteins, quantum dots, up converted luminescent nanoparticles and organic dyes have been used as biological probes in cellular imaging.[14a-c] The essential properties of luminophores used in cell imaging include their ability to enter into the cells, localize in desired cell compartments, biocompatibility, resistant to photo bleaching, excitation and emission at non-damaging wavelengths (visible/NIR). In addition, the luminophores should have large stokes shift and large fluorescence lifetime which allows differentiation from auto-fluorescence. Most of the fluorescent probes used in the cell imaging have one or more limitations such as easy photobleaching, small stoke shifts, difficulty to filter the auto-fluorescence of certain organisms and poor quantum efficiency.[15]

Heavy-metal complexes having d^6, d^8 and d^{10} electron configuration show strong spin–orbit coupling (SOC)[16], leading to efficient inter system crossing from the singlet excited state to the triplet excited state which result high quantum yields at room-temperature. Iridium(III) complexes have tunable phosphorescence emission spectra that extend to the near-infrared region, large stokes shifts which avoid the excitation beam and inner filter effects, and long emission lifetimes (μs) that permit the implementation of time-gated imaging techniques.[17-18a-d] Additionally, the synthetic versatility for tailoring the molecular properties with exceptional photochemical, physicochemical stabilities, redox stability etc. has made them as a potential candidate for bioimaging. The luminescent materials including iridium(III) complexes has a common tendency to exhibit 'aggregation caused quenching (ACQ)' effect[19] which degrades the performances of many solid state devices. Tang and co-worker developed a novel molecule namely 1-methyl-1, 2, 3, 4, 5-pentaphenylsilole with a breakthrough phenomenon known as 'Aggregation Induced Emission (AIE)'.[20a] The molecule with AIE property, are non emissive or weakly emissive in solution state but strong emissions are observed in aggregated state. The aggregation induced phosphorescence (AIP) active iridium(III) complexes are getting immense research interest to the scientific community.[20b-d] Herein, we report the contemporaneous role of six AIP active iridium(III) complexes in sensing and inhibition of bacteria in aqueous solution.

Experimental Section

Agarose (molecular biology grade), Tris-(hydroxymethyl) methane-HCl (Tris-HCl) and ethidium bromide were purchased from Sigma Aldrich (USA). All the buffer solutions were prepared using deionisedMilli-Q water. Culture medium used for the growth and maintenance of bacteria was purchased from HiMedia Laboratories, India. The six iridium(III) complexes $[(C\hat{}N)Ir(PAr_x\text{-}_1R_y)_2(Cl(H)]$ were used (where C^N=2,4 difluorophenyl pyridine and $PAr_x\text{-}_1R_y$= tris(4-(trifluoromethyl)phenyl)phosphine for a; C^N=2 -phenyl pyridine and $PAr_x\text{-}_1R_y$= tris(4-(trifluoromethyl)phenyl)phosphinefor b; C^N=2- phenyl pyridine and $PAr_x\text{-}_1R_y$= methyldiphenylphosphine for c; C^N= 2-(naphthalen-2-yl)pyridine $PAr_x\text{-}_1R_y$= triphenylphosphine for d; C^N= 2-(naphthalen-1-yl)pyridine, $PAr_x\text{-}_1R_y$= triphenylphosphine for e; C^N= Dibenzo[f,h]quinolinebenzo[h]quinoline, $PAr_x\text{-}_1R_y$= triphenylphosphine for f). The details regarding the syntheses and characterization of these iridium(III) complexes were described in our previous report.[21]

Staining of bacterial cells and fluorescence microscopy

Axenic culture of *Escherichia coli* (representative water-borne microorganism) was maintained in Luria-Bertani broth medium (1 % tryptone; 0.5 % yeast extract, 1 % sodium chloride; pH 7.5). The bacterial cells (10^8 cfu/mL) were taken from an actively growing culture and separately exposed to all the iridium(III) complexes (100 µg/mL) for 30 min. The stained bacterial cells were thoroughly washed with water: ethanol (70:30; v/v) mixtures and subsequently separated by centrifugation (5000 rpm, 5 min, 4 °C). A loopful of stained bacterial cells was used to prepare a smear on a clean glass slide. The prepared slides were observed using Olympus BX41 upright fluorescence microscope (Olympus, Rungis, France) equipped with fluorescent isothiocyanate (FITC) and DAPI filter (band pass filter, 350–390 nm; long pass filter, 456 nm) at 800X magnification. The images of bacterial cells were captured using a digital camera (Olympus, Germany) and were processed with Image J v1.47 software.

***In vitro* antibacterial assays**

Two bacterial strains, Gram positive *Bacillus subtilis* MTCC 121 and Gram negative *Escherichia coli* MTCC 1652 were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. Standard broad spectrum antibiotic chloramphenicol (Sigma Aldrich) at a concentration of 30 µg/mL was used as a positive control for *in vitro* antibacterial studies.

Zone of inhibition assay was performed at 64 µg/mL concentration for all the iridium(III) complexes (a-f) using disk diffusion method.[22] For this purpose, Mueller-Hinton agar medium (HiMedia, India) was prepared and sterilized by autoclaving at 121°C at 15 psi for 15 min. The medium was poured into sterile Petri dishes under aseptic conditions using a laminar air flow chamber. After the solidification of medium, the suspension of the test organism (10^6 cfu/mL) was swabbed onto the individual media plates using a sterile glass spreader. A sterile disk (9 mm diameter) impregnated with compound was placed over media surface and the plates were incubated at 37°C for 24 h under dark conditions. The determination as to whether the organism is susceptible, intermediate, or resistant was made by measuring the diameter of zone of inhibition in comparison with standard antibiotic.

Minimum inhibitory concentration (MIC) assay was performed to determine the lowest concentration of compound necessary to inhibit a test organism. MIC values were evaluated for all the six iridium(III) complexes (a-f) in the concentration range of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 and 64.0 µg/mL using both micro-dilution method in a 96-well microtiter plate. The MIC assay was carried out by incubating the microorganisms in presence of iridium(III) complexes for 24 h at 37°C under aerobic conditions, as per the standard guidelines.[23] MIC values were defined as the lowest concentration of compound that prevented the visible growth of bacteria (no turbidity) after the incubation period. All the experiments were performed in three replicates.

DNA interaction studies

All the DNA interaction experiments were performed at room temperature unless otherwise stated. Stock solutions of DNA were prepared in 50 mmol Tris-HCl buffer (pH 7.2) and stored at 4 °C for a maximum of 4 days. The DNA concentration per nucleotide was determined by absorption spectroscopy using the standard molar extinction coefficient value of 6600 dm^3 mol^{-1} cm^{-1} at 260 nm prior to use.[24]

Electrophoretic mobility study

The electrophoretic mobility of the iridium(III) complexes (a-f) was studied using agarose gel electrophoresis. Briefly, 50 µ mol DNA (in house purified plasmid) and 50 µmol iridium(III) complex were mixed in 50 mmol Tris-HCl buffer (pH 7.2) and the mixture was incubated for 1 h at 37 °C under dark conditions. After the incubation period, 2 µL of sample buffer containing 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol was added and mixed by gradual pipetting after which it was immediately loaded on to 1 % agarose gel. Electrophoresis was performed for 1 h at 80 V in Tris-acetate-EDTA buffer (40 mmol Tris, 20 mmol acetic acid, and 1 mmol EDTA, pH 8.0) on a Sub-Cell® GT agarose gel electrophoresis system (Bio-Rad, USA). The gel was subsequently stained using 1 µg/mL ethidium bromide in the same buffer and photographed under UV light using a Molecular Imager® Gel Documentation XR system (Bio-Rad, USA). The proportion of DNA in each fraction was quantitatively estimated from the intensity of each band with the Bio-Rad Quantity One® software. The experiments were carried out in triplicate under similar conditions.

DNA cleavage studies

For the DNA cleavage experiments, 50 µmol super-coiled pUC19 DNA (in-house plasmid isolated from recombinant *E. coli*) was dissolved in 50 mmol Tris-HCl buffer (pH 7.2) containing 50 mmol NaCl and 100 µmol of the tested iridium(III) complexes and incubated at 37 °C for 24 h. After incubation the sample was mixed well with the loading buffer (2 µL) containing 25 % bromophenol blue, 0.25 % xylene cyanol, and 30 % glycerol. Each reaction mixture (5 µL) was loaded into 0.8 % w/v agarose gel and electrophoresis was performed under standard conditions. The experiments were carried out in triplicate under similar conditions. The cleavage efficiency was measured by comparing the ability of individual iridium(III) complex to convert the super-coiled DNA into nicked circular and/or linear form.

Ethidium bromide fluorescence displacement studies

For ethidium bromide fluorescence displacement study, the reaction mixture was prepared by keeping the constant concentration of nucleic acid and ethidium bromide (10×10^{-5} M and 3.3×10^{-5} M respectively). The concentration of individual iridium(III) complex used was varied in the range of 0 to 6×10^{-5} M and the reaction mixture was stirred at room temperature for 5 min. The fluorescence spectra of reaction mixture were recorded on a Horiba spectrofluorometer by keeping the excitation wavelength at 520 nm. The emission spectra were measured in the range of 530-700 nm using 5 nm slit width. The fluorescence quenching efficiency was evaluated using the classical Stern-Volmer equation:

$$I_0/I = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q]$$

where, I_0 and I represent the fluorescence intensities in the absence and presence of quencher, respectively. K_q is the quenching rate constant of biomolecule, K_{sv} is the dynamic quenching constant and τ_0 is the lifetime of the biomolecule.

Results and Discussion

Bacterial staining potential of iridium(III) complexes

The potential of iridium(III) complexes for the detection of bacteria in aqueous system was assessed using the traditional staining methodology. Out of the tested six iridium(III) complexes (a-f), three complexes (a, d and f) were found to stain the bacterial cells. As evident from Figure 2, these iridium complexes showed AIP phenomenon after binding to the bacterial cells in aqueous solution. The binding of compounds resulted in development of fluorescent colour as visualized by the fluorescence microscopy. It is important to note that luminescence was observed only in the stained bacterial cells which suggest that the iridium complexes could penetrate into the cells. Previous study on bioimaging of mammalian KB cells using poly(N-isopropylacrylamide) containing iridium(III) complexes demonstrated that probe molecules get readily internalized into KB cells.[25] In contrast, Tang and co-workers reported an AIE molecule namely 4,4′-(1,2-diphenylethene-1,2-diyl)bis(4,1-phenylene)diboronic acid (TPE-2BA) which can penetrate only in dead bacterial cells.[26] However, TPE-2BA has significantly lesser shelf life as compared to the triplet emitting iridium(III) complexes. In absence of bacterial cells, the iridium complexes showed negligible background fluorescence which further signifies the importance of AIP phenomenon. These observations clearly prospects the use of iridium(III) complexes (a, d and f) as potential fluorometric staining agents for bacterial cells. Earlier studies also demonstrated the use of similar biocompatible complexes as fluorometric staining agent for mammalian cells.[27a-b]

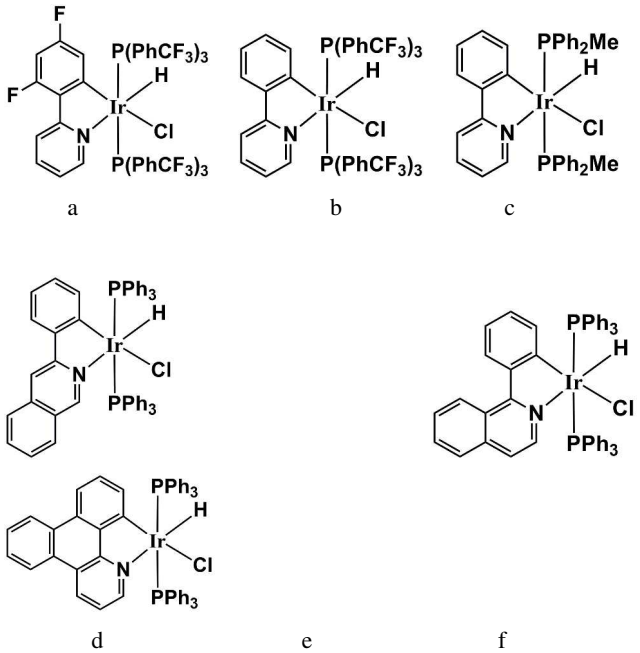

Figure 1 Molecular structures of the iridium(III) complexes used for biological applications.

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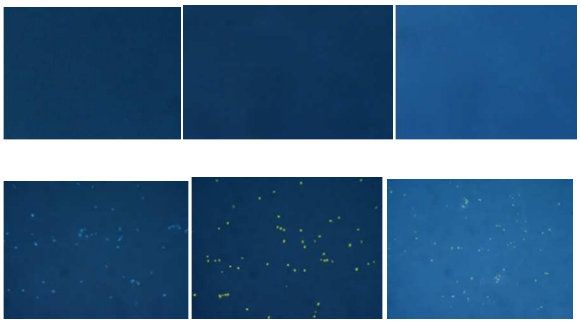

a **d** **f**

Figure 2 Fluorescence microscopic images of *E. coli* top*:* Probe; bottom:Probe + *E. coli*

Antibacterial properties

We further explored the antibacterial properties of the six iridium(III) complexes against the representative bacterial strains of both classes viz. Gram positive (*B. subtilis*) and Gram negative (*E. coli*). The zone of inhibition and minimum inhibitory concentration (MIC) values were measured to determine the magnitude of antibacterial efficiency of tested iridium(III) complexes. Conventional broad spectrum antibiotic chloramphenicol was used as positive reference to compare the antibacterial activity. All the tested iridium(III) complexes except complex **a** exhibited moderate to good antibacterial activity against both Gram positive and negative bacterial strains (Table 1). The highest bactericidal potential was observed for complex **d** followed by **f** and **c**. Moreover, it has been observed that these complexes were more effective against Gram positive bacteria (*B. subtilis*) as compared to Gram negative bacteria (*E. coli*). This may be due to the close interactions between the thick peptidogly can component of Gram positive cells and iridium(III) complexes which leads to the disruption of cell wall resulting in cell death. Previous study by Pandrala *et al*. (2013)[13] also showed potential of inert polypyridyl-iridium(III) complex and chlorido-bearing iridium(III) complexes as antibacterial agents against *E. coli*, *Staphyllococus aureus* and *Pseudomonas aeruginosa*. In contrast, no or less significant antibacterial activity was observed for cyclometalated iridium(III) complexes containing dithiocarbamate derivatives in their study.

Table 1 Zone of inhibition and minimum inhibitory concentration (MIC) values of iridium(III) complexes against Gram positive and Gram negative bacteria.

Compound	Zone of Inhibition (mm)		MIC (μg/mL)	
	B. subtilis	*E. coli*	*B. subtilis*	*E. coli*
a	9	9	>64	>64
b	11	12	64	64
c	11	11	32	32
d	14	13	4	4
e	10	11	>64	64
f	12	12	32	32
Chloramphenicol	22	20	16	16

DNA interaction studies

The understanding of interactions of iridium(III) complexes and biological entities is a pre-necessitate step to decipher the mechanism of bactericidal action. It has been well demonstrated that iridium(III) complexes possess a high DNA binding and cleavage properties.[28] In particular, the property to form stable DNA adducts through non-covalent intercalative binding has been utilized for antitumour properties.[29] In the present study, microscopic observations speculating internalization of iridium complexes in bacterial cells and subsequent cell death encouraged us to investigate the interactions of iridium(III) complexes with DNA molecules. Preliminary study was carried out by evaluating the ability of six iridium(III) complexes to bind DNA and modify its electrophoretic mobility. As illustrated in Figure 3, no DNA band was observed in the agarose gel in case of four iridium(III) complexes (a, b, c and e). The observation signifies the non-specific interactions between DNA molecules with these iridium complexes (a, b, c and e) which resulted in degradation of DNA. Interestingly, two complexes (d and f) showed intact DNA band signifying specific interactions between DNA bases and these iridium complexes which may be due to the involvement of photo-induced electron-transfer process. The most anticipated

mode of the interaction could involve intercalation or interaction with the major groove as reported in case of organometallic compounds.[30]

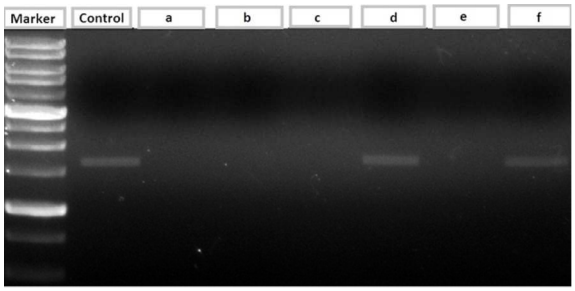

Figure 3 Agarose gel electrophoresis of DNA treated with tested iridium(III)complexes a-f. Lane 1 (marker), Lane 2 (control DNA), Lane 3-8 (complex a-f).

Further, DNA cleavage studies were performed to develop a deep understanding of the binding mode between the DNA and tested iridium(III) complexes. DNA cleavage studies are widely employed to determine the intercalation and nicking properties of ligands.[31] Cleavage of DNA molecules by ligands can be evaluated by observing the conversion of supercoiled circular conformation of plasmid DNA into nicked circular and/or linear conformations. During electrophoresis, circular plasmid DNA in closed circular conformations shows faster migration in the agarose gel as compared to nicked circular and/or linear conformation.[32]

In the present study, the incubation of iridium(III) complexes with plasmid DNA resulted in varying responses towards DNA cleavage (Figure 4). Two iridium(III) complexes (b and c) resulted in complete degradation of plasmid DNA at the tested concentration. In contrast, complex a, e and f showed minute cleavage activity, however, majority of the plasmid DNA got degraded which may be due to the non-specific interactions with nicked DNA. Interestingly, a controlled cleavage activity was observed in case of complex'd' which resulted information of all the three DNA forms. This observation further strengthens the possibility of specific interactions between the iridium(III) complex 'd' and DNA bases.

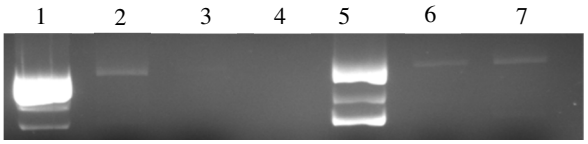

Figure 4 Electrophoretic mobility pattern of plasmid DNA incubated with tested iridium(III) complexes. Lane 1 (control DNA), Lane 2-7 (complex a-f).

The efficiency of the iridium(III) complex 'd' to bind DNA bases was further confirmed by the EB fluorescence displacement experiment. For this, a constant concentration of DNA (100 µM) and ethidium bromide (33 µM) was used with varying concentration of the complex 'd' in range of 0-60 µM. EB emits intense fluorescence at 605 nm in presence of DNA due to its strong intercalation between the adjacent DNA base pairs. Presence of another compound that is able to displace the EB molecules can quench the fluorescence of EB-DNA system.[33] The quenching degree of the EB-DNA system fluorescence is usually used to determine the binding strength. An appreciable decrease in the molar absorptivity and slight bathochromism (3-5 nm) was observed in the emission spectra of the EB-bound DNA with increasing concentrations of complex 'd' (Figure 5). It clearly demonstrate that complex 'd' competes with EB to bind with DNA and displaced some EB molecules by an intercalation mode of binding with DNA bases. The binding affinity of complex 'd' to DNA was calculated using Stern-Volmer equation by potting a graph between I_0/I and [I] values (Figure 4 Inset). The obtained linear plot allowed us to calculate the slope quenching constant (Kq) as 5.94×10^3 M^{-1}. The observed low quenching constant suggests that the interaction between the complex 'd'and DNA is of moderate intercalation which support the results obtained in the DNA cleavage experiment.

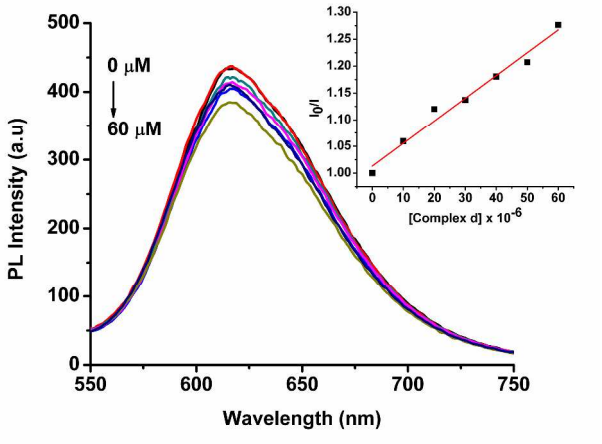

Figure 5 Changes in the fluorescence spectra of the EB-bound DNA in aqueous buffer in the absence and presence of increasing amounts of iridium(III) complex 'd'. λ_{ex} = 520 nm, [EB] = 0.33 μM, [DNA] = 10 μM, [iridium(III) complex] = 0-60 μM (in 10 μM increments). T = 298 K. Inset: Stern-Volmer plot.

Conclusion

The present study demonstrates the use of 'aggregation induced emission (AIE)' active iridium(III) complexes as potential fluorometric staining agents for bacterial cells in aqueous conditions. Microscopic observations indicated that these complexes could penetrate into the bacterial cells and resulted in subsequent cell death which suggested their dual role in bacterial sensing as well as inhibition. Out of the six tested iridium(III) complexes, two complexes (d and f) were found to inhibit the growth of both bacterial types viz. Gram positive and Gram negative. We further deciphered the mode of bactericidal action which indicated involvement of tested complexes in DNA binding and cleavage. EB fluorescence displacement studies revealed the moderate intercalation binding between iridium complex and DNA bases. The observed "dual" role in detection as well as inhibition of bacterial growth makes this study a highly promising and encourage to explore the applicability of other less expensive metal complexes in monitoring and controlling the bacterial levels in drinking and sea water systems at commercial level.

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