Control of H-dimer formation of acridine orange using nano clay platelets

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Highlights
- Layer-by-layer (LbL) film of acridine orange.
- Dimeric band of acridine orange predominates in LbL film.
- Interaction of acridine orange with laponite clay in LbL film.
- Controlling of dimeric band of acridine orange in LbL film.

Abstract
Acridine orange (AO) forms dimer even in aqueous solution. In layer-by-layer (LbL) film of AO dimeric sites predominate over monomeric sites. This communication reports the control of H-dimer of AO in LbL film by incorporating nano clay platelets. This was studied by using UV–Vis absorption spectroscopy. Atomic force microscopic (AFM) image of the LbL film was taken to confirm the presence of nano clay platelets in the LbL film.

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Introduction
Acridine orange (AO) is a metachromic dye. AO belongs to the acridine family. AO absorbs the incoming radiation because of its ring structure. The excess energy effectively passes around the ring, being distributed between the various bonds that exist within the ring. AO remains in cationic form at pH below 10 and is completely deprotonated at pH greater than 10 [1]. The basic form of AO can penetrate into the membrane of some cells by accepting protons, whereas the cationic form cannot do so and therefore, remains within the cell and causes local ion concentration fluctuations [1]. Studies on the cationic behavior of acridine orange in solution and interaction with synthetic and biological systems were discussed by various researchers [2]. These planar heterocyclic aromatic compounds are used as fluorescence dyes in molecular biology, biochemistry, toxicology and supramolecular chemistry [3]. Aqueous solution of AO forms dimers even at low concentration [4]. Surface basicity of oxygen planes of expanding clay minerals are determined by AO [5]. AO is used as a probe for measuring pH gradients in membrane vesicles [6]. Systematic studies on the formation of superstructures of a planar cationic
earlier [19]. It was observed that the dye formed H-aggregates at the interface. Acridine orange has been widely used as a stainer for the characterization of biopolymers [8]. The aggregation feature of AO is of high importance concerning the use of AO as a molecular probe for intercalation in DNA [9–13]. The interaction of AO with anionic polyelectrolytes as models has vast technical applications [11,8]. In another work, two new water soluble gold nanoparticles (AO-TEG-Au and AO-PEG-Au NPs) were prepared and characterized. They were stabilized by thiolalkylated oligoethylene glycols and functionalized with fluororescent AO derivatives [14]. An electrochemical DNA biosensor was developed based on a gold electrode modified with a nanocomposite membrane made from an ionic liquid, ZnO nanoparticles and chitosan. A single stranded DNA probe was immobilized on this electrode. Jin et al. studied the Cytotoxicity of Titanium Dioxide Nanoparticles in Mouse Fibroblast Cells using AO as a stainer [15].

On the other hand, clay minerals, such as smectites are well known for their ability to adsorb organic materials and thus have shown a great promise for the construction of hybrid organic/inorganic nanomaterials due to their unique material properties, colloidal size, layered structure and nano-scale platelets shaped dimensions. They have high mechanical strength and mechanical stability and provide unique conducting, semiconducting and dielectric properties. In recent time organo-clay hybrid film is an important area of research and has applications in sensors, electrode modifiers, nonlinear optical devices and pyroelectric materials [16]. Smectite clay minerals exhibit a high affinity for metachromic cationic dyes because the negative charges of the smectites are compensated by the positive charges of cationic organic materials by cation exchange reaction. Cation exchange capacity (CEC) is an important property of clay minerals which depends on available surface area, crystal size, pH and the type of exchangeable cation. It is usually expressed in micro-equivalents per gram (μeq g⁻¹). The adsorption of different kinds of cationic dyes by the trioctahedral smectite clay laponite and its effects on the electronic absorption and fluorescence spectra of the clay dye suspension have been studied by several researchers [17]. Guocheng et al. reported the removal of acridine orange by low charge montmorillonite swelling clays and suggested that proportional desorption of exchangeable cations from the clays accompanying AO adsorption confirmed cation exchange as the most dominant mechanism for AO removal [18]. Relationship between dye molecular aggregation and layer charge of clay minerals has been studied earlier [19].

However the effect of nano clay platelets on acridine orange in the restricted geometry of ultra thin film was not studied before. Layer-by-layer (LbL) self assembled technique is a unique tool used for fabrication of mono and multilayered ultra-thin films of organic dyes onto solid substrates owing to its cost effectiveness and simplicity in fabrication. The LbL multilayer is formed by the alternate deposition of oppositely charged polyelectrolytes such that the charge oscillates between positive and negative with each layer deposition. In the recent times this method has been extended to a wide variety of interesting charged materials, metals and semiconductor nanoparticles [20]. The LbL method has also been used for the layering of biological components such as proteins, enzymes, cell membranes, Viruses and DNA [21–24].

In the present work detailed investigations have been done to study the effect of nano clay platelets on the spectral characteristics of acridine orange in layer-by-layer (LbL) self assembled film. Our results showed that formation of H-aggregate of acridine orange in the self assembled film can be controlled by the presence of nano clay platelets. This may have application in dye lasers, which require monomeric form of dye. It may be mentioned in this context that self assembled film of a derivative of acridine orange (d-AO) fabricated by Langmuir Blodgett technique showed prominent H-aggregates [25]. The formation of H-aggregate is enhanced in mixed film of d-AO and stearic acid (SA).

**Experimental**

Acridine orange (AO) (M.W. = 301.8), purity >99%, poly(acrylic acid) (PAA) purity >99% and poly(allylamine hydrochloride) (PAH), purity >99%, were purchased from Aldrich Chemical Co., and were used as received.

Electrolytic solution baths were prepared using triple distilled Milli-Q water (resistivity 18.2 MΩ-cm). Quartz substrates were first dipped into the anionic PAA solution for 15 min followed by rinsing in Milli-Q water baths for 2 min. The slides were then dipped into cationic bath solution of AO for 30 min followed by similar rinsing in a separate set of water bath to result one bi-layer of AO-PAA Lbl film. Whole sequence was repeated to reach the desired number of AO-PAA Lbl films. All the adsorption procedures were carried out at room temperature (25 °C).

The incorporation of clay laponite in the Lbl film was done with the help of polycationic PAH aqueous solution (0.5 mg/mL). The CEC of laponite is 0.74 meq g⁻¹ Aqueous solutions of AO with different CEC percentage of clay were prepared. The quartz slide was dipped in electrolytic polycation (PAH) solution for 15 min followed by same rinsing in water bath for 2 min and then dipped into the anionic clay dispersion which is again followed by rinsing action in water bath. The slide thus prepared was dipped into the cationic electrolytic solution of AO of different CEC percentage of clay. Sufficient time was given for deposition and drying of the slide.

The Lbl film of AO was also prepared in different pH solution. The same procedure mentioned above was repeated, but different pH aqueous solutions were used instead of normal aqueous solution (pH = 6.7).

UV–Vis absorption spectra were recorded using a spectrophotometer (Lambda-25, Perkin Elmer). Atomic force microscopy

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**Fig. 1a.** Normalized UV–Vis absorption spectra of aqueous solution of AO (10⁻⁵ M) and one bi-layer Lbl film of AO-PAA at 10⁻⁵ M AO concentration.
The AFM image of one bi-layer AO-clay-PAH LBL films was taken in air with commercial AFM system (Bruker Innova). The AFM image presented here was obtained in intermittent contact (tapping) mode. Typical scan area was $1 \times 1 \mu m^2$. The Si wafer substrate was used for the AFM measurement.

**Results and discussion**

**UV–Vis absorption spectroscopy**

The normalized UV–Vis absorption spectra of aqueous solution of AO ($10^{-5} \ M$), and one bi-layer AO-PAA LbL film of $10^{-4} \ M$ AO concentration are shown in Fig. 1a. AO solution absorption spectra shows distinct and prominent bands in the 450–540 nm region with a strong peak at 490 nm along with a weak shoulder at around 470 nm and are in well agreement with the reported results [26]. The 490 nm peak is due to monomer absorption of AO in solution and the weak shoulder at around 470 nm is due to presence of dimeric sites of AO in solution [26]. In the absorption spectrum of 1 bi-layer AO-PAA LbL film, the monomer peak was reduced to a weak hump and shifted to 500 nm whereas the dimeric band became strong with an intense peak at 470 nm. This dimer has been assigned as H-dimer [27]. It may be due to the fact that closer associations of AO molecules in the LbL film lead to an increase of dimeric sites.

Fig. 1b shows the normalized absorption spectra for different concentration of AO in aqueous solution. It is interesting to note that intensity of dimeric band increases with increasing solution concentration. At higher solution concentration dimeric sites of AO predominate. Inset of Fig. 1b shows the ratio of intensities of dimeric and monomeric bands as a function of concentration of AO in solution. It is observed that the ratio increases gradually indicating the predominance of dimeric sites.

Fig. 1c shows the normalized absorption spectra of AO-PAA LbL films prepared with varying concentration of AO. PAA being photophysically inactive, only the AO bands are observed. With increasing AO concentration, dimeric band increases in intensity and monomeric band reduces to a weak hump. The cationic part of AO molecule interacts with the anionic part of the PAA molecule and thus forms the complex species. AO molecules get closer side...
by side and closer association of AO molecules takes place resulting in a favorable condition for aggregation and consequent H-dimer formation of AO molecules.

**Adsorption kinetics**

Fig. 2 shows the absorption spectra of one bi-layer AO-PAA LbL films at different immersion times. Here in all the cases the immersion time in PAA was kept fixed at 15 min, but in the dye (AO) solution the immersion times were varied from 5 min to 60 min.

From the figure it is observed that the intensity of the absorption band increases for the films with dye immersion time up to 25 min and remains almost constant for the dye deposition time greater than 30 min. This is also evident from the plot of the intensity of the absorption maxima vs. time (inset of Fig. 2). This indicates that the interaction of AO molecules with the PAA layer was completed within 30 min and after 30 min no PAA molecule remained free within the film for further interaction with the AO molecules.

**Layer effect**

UV–Vis absorption spectra of different layered (1–20 bi-layer) AO-PAA self assembled LbL films is shown in Fig. 3. It is interesting to note that absorption spectra of different layered LbL films show almost similar band pattern irrespective of layer number except an increase in intensity. This indicates that AO and PAA molecules are successfully transferred in the LbL film during the process of fabrication. Inset shows the variation of maximum absorbance with the layer number. It also supports the thesis of successful transfer of molecules in the LbL film.

**pH effect**

To study the pH effect on the LbL film, different AO-PAA LbL films have been prepared by using aqueous solution of AO prepared at different pH values. Fig. 4 shows the absorption spectra of one bi-layer AO-PAA LbL films deposited by changing the pH value of the aqueous solution of AO (pH = 2.7, 4, 5.8, 6.6, 7.8, 8.5, 9.7, 12, 13). Concentration of the AO in aqueous solution and deposition time was kept fixed at $10^{-4} \text{M}$ and 30 min respectively for all the cases.

At lower pH (<10), the absorption spectra of one bi-layer AO-PAA LbL film shows the intense dimeric peak at 470 nm and the monomer peak is a weak hump at around 500 nm. But at higher pH (>10) the peak is shifted to shorter wavelength side. At pH 12 and 13, peak arises at around 435 nm which is blue shifted with respect to that at lower pH. It may be mentioned in this context that similar result was reported on the pH dependent study of the absorption spectra of aqueous solution of AO [1]. It was observed that at pH 12 of the AO aqueous solution ($10^{-6}$ to $10^{-4} \text{M}$) the absorption peak appeared at 435 nm [1]. This is due to the basic form of AO. AO is protonated at pH lower than 10 ($pK_a = 10.4$) due to which it can dimerize and at higher pH (>10) AO molecules get deprotonated [1]. From figure, it is also observed that the absorbance intensity decreases with increasing pH values. This may be due to the fact that at lower pH, AO molecule becomes dicaticionic, tricationic [26], for which it can interact electrostatically with more anions of PAA resulting in the intense absorption peak. Increasing the pH value, AO becomes mono-cationic (protonation of ring nitrogen atom) and thus interacts with less number of anions of PAA which results in less intensity of absorption peak. Absorption spectra of LbL film show strong similarity with the AO solution spectra. This indicates that AO molecules retain its identity in the restricted geometry of LbL film.

**Effect of nano clay**

Fig. 5 shows the normalized UV–Vis absorption spectra of AO-clay-PAH one bi-layer LbL film, the loading of AO is 10%, 20%, 30%, 40%, 50% of the CEC of clay laponite at 1 g/L clay in the suspension along with the spectra of LbL film without clay.
From the figure it is observed that LbL film of AO without clay gives intense absorption with dimeric peak at 470 nm and weak monomeric hump at 500 nm. After the inclusion of laponite nanoclay platelets into the LbL film of AO, the intensity of monomeric peak gradually increases and the intensity of dimeric peak at 470 nm decreases to a larger extent. The intensity of both bands can be controlled precisely by changing the loading of AO. It may be due to the fact that in the LbL film, AO molecules are adsorbed on the clay platelets by charge transfer types of interaction. Consequently AO molecules are arranged on the clay platelets. This reduces the possibility of dimer formation and increases monomer sites. As a result monomeric band increases in intensity.

Garfinkel-Shweky and Yariv [28] examined the adsorption of AO on laponite clay in aqueous suspension. It was observed that with increasing AO loading into laponite clay aqueous suspension, initially the monomeric band decreases with increasing of dimeric band. This was explained as due to flocculation of clay into aqueous dispersion. With further increase of AO loading, dimeric band again decreases with further increase of monomeric band. This is due to peptization and intercalation of AO into laponite clay.

Our result of spectroscopic investigation of the LbL film clearly indicates that AO molecules are most probably intercalated into the nanoclay platelets which reduce the possibility of dimer formation.

It is interesting to mention in this context that in order to form dimer, AO molecules should come closer to each other and distance between two neighbouring AO molecules should be of the order of 0.35 nm [29] or less. In absence of laponite the AO molecules are adsorbed onto the backbone of PAA electrostatically onto the LbL films. Thus AO molecules come closer to each other resulting in a favorable condition for dimerization in LbL films.

However, in AO-clay-PAH LbL films, initially laponite particles are attached onto the PAH backbone in the films following the adsorption of cationic AO onto the laponite particles. The clay particles laponite possess layer structure and have cationic exchange capacity [30]. Accordingly the AO molecules are adsorbed onto the clay layer through cationic exchange reaction and intercalation and thus fixed onto the clay particles in the AO-clay-PAH hybrid films. The adsorption of AO molecules onto the laponite particles decreases the probability of dimerization in the hybrid LbL films.

In one of our earlier work, it has been demonstrated that J-aggregate formation of a thiocyanine dye in Langmuir–Blodgett (LB) film can be controlled by incorporating clay particle laponite onto the LB films [16].

**AFM study**

The morphology and surface structure of the AO-clay-PAH LbL film on Silicon wafer was studied by atomic force microscope (AFM). The clay particles are clearly observed in the AFM image as shown in Fig. 6a, which confirms the formation of nano dimen-
sional organo-clay hybrid LbL film. From the height profile analysis, it has been observed that the thickness of the LbL films varies in between $-2$ and $+2$ nm. This height includes the height of clay laponite, PAH and the AO molecules onto LbL films. The AO and PAH molecules are not distinguishable in the AFM image since the dimension of these molecules are beyond the resolution of our AFM system. The clay particles are densely packed and cover almost the whole surface. It indicates the successful incorporation of nano clay platelets into the hybrid organo-clay LbL film. Fig. 6b shows the roughness profile analysis of AFM image of AO-clay-PAH LbL film. Roughness data was calculated by the software SPIP V.1.92. The roughness average calculated is 0.884773 nm and it is very low. R.M.S. roughness is 1.2474 nm and average height is 4.231 nm.

Conclusion

In conclusion our results show that even in aqueous solution of acridine orange (AO) the absorption intensity of H-dimeric band increases with increasing solution concentration and at higher solution concentration, dimeric band predominates. In the LbL film of AO dimeric band is highly intense and monomeric band reduces to a weak hump. H-dimer formation of acridine orange (AO) in the self assembled film can be controlled by incorporating nano clay platelets. This was evidenced from the decrease of dimeric band in the UV–Vis absorption spectra. AFM image of AO-clay-PAH LbL film clearly shows the presence of nano clay platelets in the hybrid LbL films.

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