CHAPTER - 2

BEHAVIOR OF HALLOYSITE
NANOTUBES WITH DNA
INTERCALATOR ACRIDINE ORANGE
ABSTRACT

Acridine Orange, a DNA intercalating agent interrupts the normal function of cellular DNA, resulting into the inhibition of gene expressions. A large number of carcinogenic chemicals damage DNA either by intercalation or by cross-linkage with DNA base pairs. Numerous repair strategies are available to block the progressive DNA damage accelerated by DNA intercalators. These strategies are intended to prevent intercalation through scavenging the reactive moieties of the intercalators. In this study, it has been demonstrated that there is binding of Halloysite nanotubes (HNT) with a DNA intercalator, Acridine Orange (AO) through a hydrophobic interaction referred to as 'polar bonding'. HNTs are comprised of silica on its outer surface and alumina within its internal surface. This inner and outer surface of tubular walls confers a resultant negative charge which makes HNT polyanionic in nature, thus enhancing the potential for polar bonding between HNT and amino substituted AO. This interaction can lead to polar interactions between the aromatic entity of AO and the oxygen plane of the HNT. This shift was confirmed by Fourier transform infrared spectroscopy (FTIR) analysis whereby in presence of HNT, the intercalator AO, intercalated within DNA-AO complex showed affinity for HNT as observed by the shift in FTIR peaks of halloysite nanotubes from 3623, 1722 and 1038 cm\(^{-1}\) to 3612, 1706, 1083 and 963 cm\(^{-1}\).
2.1 INTRODUCTION

Nanomaterials have wide-range of implications in a variety of fields including chemistry, physics, electronics, optics, materials science and biomedical sciences. Many nanomaterials are carcinogenic in nature because of their intercalating nature. These nanomaterials disrupt the normal DNA function by interfering with the normal gene expressions. Various strategies are used to block the DNA damage e.g. the anti-mitogenic chemicals trap carcinogens either via scavenging or by detoxification.\(^1\)

Nanotubes because of their tubular geometry and surface features can efficiently allow uptake of different molecules both natural as well as synthetic. One such nanotubes, Halloysite nanotube \([\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_42\text{H}_2\text{O}]\) is a clay mineral that has similar structure to kaolinite \([\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4]\). The electron microscopic analysis has revealed that the Halloysite morphology is tubular in nature with a 1:1 layer-like structure. This tubular morphology is achieved by layer rolling and that water separates the 1:1 layers from each other. Hence unlike kaolinite and other mineral clays, Halloysite has a larger cation exchange capacity, surface area and catalytic activity. It can exist in both the dehydrated and hydrated form. The hydrated form has water molecules situated between the successive layers.\(^2-3\)

Despite the wide use of nano scale materials, very little knowledge regarding the interaction of nanoparticles with nucleic acid and their subsequent toxicity is available. Ultrafine particles have great impact on the resultant properties of DNA. This impact is largely due to their nano dimension, efficacious surface interactions and particle compositions that can elicit biological responses. Several studies indicate that manufactured nano-scale materials like dyes can spread in our environment and later on transfer into various organisms through diverse ways. On certain occasions, these nano scale materials have been found to accumulate in specialized organelles. Furthermore, the unique and diverse physicochemical properties of nanomaterials suggest that interaction of these materials with the human genetic material could be a probable cause for associated toxicities.\(^4\)
Diamino acridines are fluorescent dyes that bind strongly to nucleic acids in solution. When these dyes are used as fluorochromes in living organisms, they become localized within nucleic acid-containing structures. One of these dyes, Acridine Orange (Figure 2.1) is prone to intercalate within nucleic acid of the living cells and can lead to chromosomal abnormalities. The interaction of acridine derivatives with DNA is an interesting area of research as the associated mutagenicity following the use of these compounds and the subsequent carcinogenicity of the benzacridines are poorly understood. Accordingly, there is a possible hypothesis that benzacridine derivatives DNA interaction mode has strong electronic interaction, which favor flat, face-to-face binding of the acridine moiety to the bases of the nucleotides.

![Structure of Acridine Orange](image)

**Figure 2.1 : Structure of Acridine Orange**

In this study we have explored the interaction behavior of Acridine Orange and DNA for binding with HNT. Halloysite is typical clay minerals and has a chemical composition of Al₂Si₂O₅(OH)₄·H₂O (with 1:1 layer). Nanotubular structure contributes towards Halloysites nanoscale dimensions. It occurs mainly in two different polymorphs, the hydrated form and the dehydrated form. The hydrated form converts irreversibly into the dehydrated form when dried at temperatures below 100°C. Halloysite get easily dehydrates at atmospheric pressures and temperatures around 60°C or in vacuum at room temperature. Halloysite (hydrated) are separated by water layer by each other and occur in scroll-like morphology.

Studies concerning the formation of Halloysite nanotube and DNA supra molecular adduct has been reported earlier ¹¹ However interaction behavior of HNT with DNA-AO complex have not reported anywhere. Here it is indicated and
confirmed that the Halloysite nanotubes behave like a poly anion due to its resultant negative charge given by their surface elements. This property also potentiates the interaction of HNT and AO in proximity of DNA-AO complexes by enhance polar bonding between AO specifically HNT.
2.2 EXPERIMENTAL APPROACH

2.2.1 Materials and Methods

Halloysite (premium grade) were obtained from New Zealand China Clays Ltd (New Zealand). DNA (a sodium salt of double stranded DNA from Herring testes) was purchased from Sigma. Acidine Orange was obtained from Merck Chemicals. Caffeine (CAF; 1,3,7-trimethyl xanthine) and HEPES buffer (1.0 M solution) were procured from Sigma Aldrich. In all experiments, when required double deionized water was used. Prior to all experiments, the Halloysite were first sieved (125 μm mesh) to remove granules and 42% fine powder of HNT were used.

2.2.2 Allophane removal from Halloysite nanotubes

The bulk of Halloysite is usually associated with Allophane. To avoid the possibility of binding of DNA with HNT, removal of allophone is necessary. Halloysite nanotubes were heated with 0.5 M sodium hydroxide solution at 60 °C for 8 h. By this treatment allophone present in sample gets dissolved. After cooling, the alkali solution was filtered and the recovered Halloysite was repeatedly resuspended and washed in water to free it of residuals, prior to drying to constant weight in a forced circulation oven (Net wind) at 50 °C for 48 h. 12-14

2.2.3 Ultra structural and Chemical characterization of HNT

The characterization of Halloysite nanotubes were carried out with different approaches such as Scanning Electron Microscopy, to determine the ultra structure of the HNT and Energy-Dispersive-X-Ray-Fluorescence analysis (EDXRF) by Carl Zeiss EVO-18 was used to confirm the elemental content. The changes in metachromasy following interaction of HNT with Acridine Orange were determined by Jasco spectrophotometer (V-630) at 430 nm and 660 nm. Finally, Fourier transform infrared spectroscopy (FTIR) was performed using Jasco FT-IR 4100 in order to investigate the vibration spectra.
2.2.4 DNA sample preparation

In this study, a DNA from Herring testes (average size of 1000 bp) supplied by MP Biomad (Place) was used. The DNA solution, which was diluted to a suitable concentration with Ultra pure water, was used for the adsorption experiments. DNA solution was analyzed by spectrophotometry at 260 nm using a 1-cm quartz cell to determine the amount of DNA.15-16

2.2.5 Preparation of DNA-Acridine Orange (AO) complex

To determine the optimum concentration of Acridine Orange require for formation of DNA and Acridine Orange complex, an experiment was performed. AO at concentration 0.5 m mol/l was prepared in double distilled water. Complex of dye and DNA were formed by adding Acridine Orange drop wise to the DNA solution with constant stirring to give the desired drug/DNA molar ratios of 1/50 and 1/25 at a final DNA concentration of 0.25% w/w or 5 mM DNA in 50mL of 0.5 m mol/L AO. Intercalation of Acridine Orange within DNA was confirmed by spectrophotometry as well as by recording FTIR spectra after 3 hr of the initial mixing of dye and DNA solutions.13-14

2.2.6 Preparation of Halloysite nanotubes with DNA and Acridine Orange complex

The HNTs were prepared with DNA-AO complex by mixing 100 milligram of the Halloysite nanotubes (HNT) in a beaker in a total volume of 50 mL of DNA-AO complex solution, as described above. The suspension was mixed for 1 hr at room temperature using Maxi Prep. After 3hr of mixing the sediment and the supernatant were collected into fresh beakers. The sediment collected was subjected to FT-IR analysis and labelled as HNT-AO complex. The control sample was prepared by a similar procedure, however this sample contains 100mg of Halloysite nanotubes in 50mL of 0.5 mmol/L Acridine Orange solution (without DNA) as described in a previous report.17
2.3 RESULTS AND DISCUSSION

2.3.1 Ultra structural and Chemical Characterization of HNT

In order to characterize the halloysite nanotubes Scanning Electron Microscopy (SEM) was done to determine the nanotube’s ultrastructure while FT-IR and Energy Dispersive X-Ray Fluorescence analysis (EDXRF) was done to determine the chemical and elemental character of the nanotubes. SEM image of Halloysite nanotube samples were taken with Carl Zeiss EVO-18 (Figure 2.2). Scanning Electron Microscopy reveals that majority of samples consist of cylindrical tubes with 100-200nm in a poly dispersed length wise. The EDXRF spectra were determined for HNTs, which detected multiple elements simultaneously (Figure 2.3). Interesting features of HNTs were its unique chemical structures comprising of silica, SiO$_2$ and alumina, Al$_2$O$_3$ sheet. The EDXRF analysis confirmed the presence Al and Si with corresponding element peaks positions for Al at 1.75 keV and Si at 1.62 keV. The vibration spectra for HNT were recorded from the processed samples as describe above and it shows characteristic peak at 3623 and other peaks at 1722 and 1038 cm$^{-1}$ (Figure 2.4). The band at 1038 cm$^{-1}$ in HNT was assigned to be Si-O-Si plane vibrations.

![Figure 2.2](image)

Figure 2.2 : Ultrastructural morphology of the Halloysite nanotubes. Scanning Electron Microscopy (Carl Zeiss, Germany) was used to determine the morphology of Halloysite nanoparticles (shown in red arrow). This experiment was performed in triplicates.
Figure 2.3: EDXRF spectra of HNT. HNT was characterized by using Energy dispersive X-ray fluorescence analysis (Carl Zeiss EVO-18) and observed energy peaks for Al at 1.75 keV and Si at 1.62 keV confirming the presence of the elements Al and Si. All the experiments were performed in triplicates.

Figure 2.4: Vibration spectra of HNT. Processed HNTs sample were subjected to FTIR analysis using FTIR 4100 (Jasco, Japan). FTIR spectral lines were observed at 3623, 1722 and 1038 cm\(^{-1}\) that was distinct for Si-O-Si plane vibrations, which was specific for HNT.
2.3.2 Spectroscopy analyses of Acridine Orange

Absorbance of AO is recorded at various concentrations. AO dissolved in 5µM HEPES solution buffered at pH 7.0 has two spectral peaks, as can be seen in Figure 2.5. AO at a concentration of 2.5 µM exhibits a distinct spectrum with a λ max of 492 nm. As the concentration of AO increase, a secondary spectral peak at 468 nm became more distinct and increased in amplitude. When the ratio of the absorbance value at 492 nm over the absorbance values at 468 nm was plotted vs. AO concentrations ranging from 2.5µM, 5µM, 10µM, 15µM and 20µM, a significant linear correlation ($R^2=0.99$) resulted (Figure 2.6). These results conform to the absorbance maximums reported by Kapuscinski and Darzynkiewicz.18-19

![Absorbance Spectra of Acridine Orange](image)

Figure 2.5: The absorbance spectra of Acridine Orange (2.5µM, 5µM, 10µM, 15µM and 20µM) at different concentrations. Absorbance was scanned over a range of wavelengths between 400 and 700 nm in a solution of PBS buffer at pH 7.0.
Figure 2.6: Calibration Curve for Acridine Orange. Linear regression analysis depicting the ratio of absorbance at 492 nm to the absorbance at 468 nm as a function of AO concentration.

There is an absorption maximum at 492 nm, which corresponds to the monomeric state of AO at low concentration. The absorption spectra changes with increasing concentrations of AO. Along with peak 492 nm, an additional peak of 468 nm appears. This result may be due to the aggregation of the monomer caused by concentration as reported by Tan and Schnelder and Robinson et al.\textsuperscript{20-21}

Figure 2.7: Fluorescence spectra of DNA in the presence of AO (aqueous solution). Fixed concentrations of DNA (10 µM) were titrated against variable concentrations of AO (0.0 µM, 2.5 µM, 5 µM, 7.5 µM, 10 µM) respectively.
In the fluorescence spectra, the monomer AO has a fluorescence maximum at 530 nm (\(\lambda_{\text{max}}\)). With increasing concentration of AO in solution, an increasing of AO dimers results in the red-shift of this peak.\(^{18}\) DNA has a natural fluorescence, but the intensity is so weak that the direct use of the fluorescence emission of DNA is limited to study its properties.\(^{22-24}\)

The experimental results confirm again that AO intercalates into the DNA. As one can see from Figure 2.7, the fluorescence intensity of DNA at 525 nm is weak, but after the addition of AO into the solution containing the DNA, the fluorescence intensity is dramatically enhanced. The AO intercalates into the base pairs in a helix of DNA and the complex formed is stabilized by the \(\pi-\pi\) stacking interaction between AO and the DNA bases.\(^{25-26}\)

### 2.3.3. Caffeine acts as interceptor with Acridine Orange

Caffeine (1,3,7-trimethylxanthine) is a class of methylxanthines, with conjugated planar ring systems. It is present in various dietary sources including coffee, tea, cola beverages, and chocolate. Regular consumption and relatively high daily intake of caffeine (CAF) in various dietary sources has stimulated question of their toxicity.

Studies suggest that CAF can alter the absorption spectrum of DNA intercalator, acridine orange by forming \(\pi-\pi\) complex with the dye in aqueous solution, confirm the role of caffeine as an interceptor molecule.\(^{27}\)
Figure 2.8: Absorption spectra for AO titrated against with caffeine. Intercalator’s concentration was 10 µM in 5 mM HEPES buffer, pH 7.0. Caffeine is titrated in increments of 1 mM from a 50 mM stock solution. Spectra of the caffeine-intercalator complexes contain 5 mM caffeine.

The optical spectrum of AO is shown in Figure 2.8. The spectrum of Acridine orange exhibits an absorption band in the visible region with absorption maxima at 493 nm. Upon addition of caffeine, the absorption maxima of AO shift to 499 nm. The red-shift in the absorption maxima of AO in the presence of caffeine provides a convenient method for determining the equilibrium constants of complex formation. This method is demonstrated in Figure 2.8 Which exhibit optical difference of titration between AO and CAF. The optical difference of spectrum between caffeine-AO complexes displays a single isosbestic point characteristic of only two absorbing species in solution. 28-29
Figure 2.9: Absorption spectra of AO titrated with CAF, HNT and HNT-CAF. Intercalator concentration is 10 µM in 5 mM HEPES buffer, pH 7.0. Caffeine is titrated in increments of 1 mM from a 50 mM stock solution. Spectra of the caffeine-intercalator complexes contain 5 mM caffeine. 100mg of HNT were used as interceptor.

In presence of HNT with AO, Absorption maxima also shift towards 499 nm as in case of Caffeine. While in presence of HNT and Caffeine both, it shows more inhibition of AO fluorescence compare to HNT and CAF alone (Figure 2.9).

2.3.4 Spectrophotometric analysis AO-DNA complexes

The formation of AO-DNA complexes was investigated by UV-Vis spectroscopic analysis. An experiment was performed to determine the optimum proportion of DNA and AO required forming AO-DNA complex. Acridine Orange intercalates within DNA and the concentration of resultant solution was determined by spectrophotometrically. The absorption maxima of AO was found to be at 492 nm and while on subsequent addition of DNA at concentration of 2.5mM and 5 mM caused a shift in the absorption maxima to 502 nm. From Table 2.1, it can be seen that the relative absorption of AO and 5mM DNA were comparable to that of relative absorption of AO and 2.5mM DNA concentration. The intercalation of AO to DNA results in a shift of the λ_{max} of AO from 492 nm to 502 nm, which shows significant
intercalation of AO to DNA. It is noteworthy that as the concentration of ds-DNA increases from 2.5mM to 5mM, absorption of DNA-AO complex decreases, which confirms the enhanced intercalation as well.\textsuperscript{14-17,29}

**Table 2.1**: Changes in UV-Visible absorbance maxima of Acridine Orange at different concentration of DNA

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{\text{max}}) nm</th>
<th>(#\text{Abs} \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>492</td>
<td>6.80 ± 0.12</td>
</tr>
<tr>
<td>AO+dsDNA(2.5mM)</td>
<td>502</td>
<td>6.90 ± 0.19</td>
</tr>
<tr>
<td>AO+dsDNA (5mM)</td>
<td>502</td>
<td>5.78 ± 0.08*</td>
</tr>
</tbody>
</table>

\# According to Beer’s law

Mean ± SD, \(n = 3\)

\*p value = 0.0003 considered extremely significant, Student’s test.

**Figure 2.10**: Absorption spectra of AO-DNA complexes at 2.5 mM and 5 mM DNA concentration (AO stands for dye without DNA). The total dye concentration of 0.5 mmol/L was used in each sample. We observed a change in the peak \(\lambda_{\text{max}}\) from 492 nm to 502 nm between pure AO and DNA-AO complexes. All the experiments were performed in triplicates.
2.3.5 Vibrational FTIR spectroscopic analysis of HNT (suggest HNT can act as an interceptor for DNA intercalator AO)

To study the interaction behaviour of AO and DNA in the presence of HNT, a vibrational spectroscopic analysis was performed using DNA-AO and HNT-AO samples. The HNT interaction with Acridine Orange was used as control and HNT interaction with DNA-AO complex was used as Test. Halloysite nanotubes were placed in Acridine Orange solution, due to elemental content of Halloysite nanotubes (dominant alumina or silica); Acridine Orange shows less binding affinity to Halloysite nanotubes. So Halloysite nanotubes cannot absorb Acridine Orange directly. Thus, there is less probability of interaction between HNT and AO and hence there is no binding. This has been confirmed by the FTIR spectra of HNT, which was recorded after interaction of HNT with AO (as describe above). The obtained vibration spectra do not show the presence of any additional peaks and thus there is no interaction between HNT and AO.

Intercalation of Acridine Orange into DNA bases show characteristic change in vibrations of DNA from 1717(G), 1088 and 968 cm\(^{-1}\) (PO\(_2\) stretching) , (Figure: 2.11), after complexation with Acridine Orange dye 1713(G) ,1221,1085 and 966 cm\(^{-1}\) (Figure: 2.12). This shifting of band at 1717 cm\(^{-1}\) was associated with increase in intensity of this band towards lower frequency at 1713 cm\(^{-1}\). The spectral changes (intensity and shifting) of guanine band at 1717 cm\(^{-1}\) was indicative of intercalation of Acridine Orange with G-C base pairs. Beside these intensity shift at 1221 was major intensity shift because of phosphate band and can be due to AO-PO\(_2\).
Figure 2.11: Vibrational spectra of DNA. FTIR spectra for DNA shows peak at 1717(G), 1088 and 968 cm\(^{-1}\) (PO\(_2\) stretching).

Figure 2.12: FTIR spectral map shows the interactions of AO between DNA and HNT. The FTIR spectra for the DNA-AO interaction shows a peaks at 1713(G), 1221, 1085 and 966 cm\(^{-1}\) suggesting shift at 1713 cm\(^{-1}\) corresponding to intercalation of Acridine Orange to DNA.
Amino acridines have very strong mutagenic potential. It usually intercalates well when binding with free DNA. The interaction between AO and DNA is highly dependent on dye/base pair ratio. At low of dye/bp ratio, AO intercalate into double stranded DNA by hydrophobic forces. If this ratio get disturb there will be chances of DNA damage. In DNA-AO complexes, the DNA interacts with the cationic dye by intercalation. Halloysite nanotubes can be used as an adsorbent to remove dyes in aqueous solution. Acridine Orange has pKa of 10.4 and should have been protonated as a cation before being adsorbed directly by HNT. When Halloysite nanotubes were placed in DNA-AO complex solution, both DNA and AO complex showed affinity for the HNT. In HNT, the external surface is composed of siloxane (Si-O-Si) groups, whereas the internal surface consists of a gibbsite-like array of aluminol (Al-OH) groups. Thus the addition of HNT can cause a generation of poly anionic electron density in the DNA-AO complex solutions.
The release of dye molecules from the DNA-AO complex in the presence of HNT can cause the HNT to interact with dye electrostatically. This interaction was confirmed by the FTIR analysis of the sediment obtained after this interaction. The interaction of DNA-AO complex with HNT shows changes in characteristic vibrations of HNT at 3623, 1722 and 1038 cm\(^{-1}\) and after interaction of Acridine Orange with halloysite shows peaks shift 3612, 1706, 1083 and 963 cm\(^{-1}\) (Figure 2.13). The peak at 3612, 1706, 1083 cm\(^{-1}\) confirm the interaction of HNT with AO and suggests the formation of planar bonding with Al/Si of HNT and amino group (-NH\(_2\)). These shifts in FTIR peaks clearly confirm the interaction of AO with HNT and the AO shows a tendency to undergo metachromasy.

This is possible, if the AO interacts with HNT through \(\pi\) interactions between the aromatic entity and the oxygen plane of the HNT. The oxygen from surface oxygen planes can donate electrons to the \(\pi\) system of AO cations. The addition of nucleic acid (DNA) can induce metachromatic effect and presumably through the association of the dye molecules with polyanion containing HNT which may show both electrostatic and hydrophobic interactions. Thus the induction of metachromatic effect may be occurring due to addition of nucleic acid.
2.4 Conclusion

In this study, we have characterized HNT and demonstrated that Acridine Orange, a DNA intercalator and carcinogen can bind to HNT. This binding of AO to HNT can inhibit or reduce the carcinogen’s DNA binding capability. The potential mechanism responsible for this is through formation of polarization bonding complexes between HNT and the Acridine Orange. The potency of the stacking interaction between HNT and Acridine Orange is through their electrostatic and π interactions. This interaction also renders Acridine Orange less available for DNA intercalation. From the evidence presented here, it is now clear that when Acridine Orange binds to DNA, the extent of intercalation is reduced in the presence of HNT environment. Through this study, it has been indirectly demonstrated that halloysite nanotubes material based matrices may offer a platform to study the biomolecular interactions for diverse biological applications, specifically in biomolecular adduct recognition. The surface and core properties of these nanomaterial based systems can be engineered for single or multimodal applications, such as biosensor development and biomolecular recognition. Recently, HNTs has gained recognition for their potential versatility in wide range in vitro and in vivo applications such as drug delivery, polymerization, solid phase separation etc. In order to fully utilize HNTs potential, it is necessary to address a number of issues such as developing reproducible manufacturing methods, its manufacturing scalability, as well as characterization of reliable support matrices. It is proposed that in future by exploration of the various facets of HNTs, further possibilities of utilizing this nanomaterial for developing biosensors for a broad range of biological and non biological applications will open up.
2.5 References


