CHAPTER-4

DNA LABELED GOLD BASED OPTICAL NANOBIOSENSOR FOR MONITORING TOXICOLOGICAL BEHAVIOR OF MITOXANTRONE
4.1 ABSTRACT

The present study focuses on the construction of optical nanobiosensor to determine toxicological behavior of Mitoxantrone (MTX), an anti-tumor drug. For this purpose gold nanoparticles (AuNPs) were labeled with DNA, respectively combining the physicochemical detector component with the biological analytical component. FRET (Fluorescence Resonance Energy Transfer) phenomenon works as the basic principle for the determination of MTX-DNA interaction by spectrofluorometry. Mitoxantrone intercalates with the DNA and produces MTX-DNA adduct which restricts protein synthesis and causes excessive production of free radicals in the myocardium that could eventually lead to cardiac arrest. Mapping the adverse reaction of MTX with DNA at molecular level, a conformational change within the nanobiosensor complex was evident that increased the distance between the fluorescent/quencher molecules. The consequent changes in the fluorescence spectrum of the sensor due to FRET modulation by varying concentrations of MTX proved the basis of the tox-screen. Paracetamol, an analgesic agent was used as controlled drug in this study. Results have demonstrated that the optical nanobiosensor is rapid and sensitive with a detection limit up to 1 µg of MTX interaction illustrating how it is a feasible technique for surveilling Drug-DNA interaction in molecular toxicology.
4.2 INTRODUCTION

Since the advent of nanotechnology, a lot of novel nanomaterials are being fabricated, their novel properties are being gradually worked on, and the applications of Nanomaterials in biosensors have also advanced greatly in recent times. Nanomaterials-based biosensors represent the integration of material science, molecular engineering, chemistry and biotechnology, which can markedly improve the sensitivity and specificity of biomolecule detection, hold the capability of detecting or manipulating atoms and molecules, and possess promises in applications such as biomolecule recognition, pathogenic diagnosis and environment monitoring. Inspired by the nanoscience we decided to work on DNA labeled Gold Nanoparticles Based Optical Nanobiosensor. AuNPs show a strong absorption band in the visible region due to the collective oscillations of metal conduction band electrons in strong resonance with visible frequencies of light, which is called surface plasmon resonance (SPR). Dipole resonance is a type of local surface plasmon resonance which when excited transmits non-radiative form of energy besides fluorescent energy. This energy can be transferred to a quencher (acceptor) molecule if proximal to the reporter (resonating excited donor) molecule. This phenomenon is popularly known by the acronym, FRET.

FRET assays are often used to identify the interaction between two different molecules. FRET is a two-step photo-physical process that occurs simultaneously as illustrated in the Fig. 1. In this, radiative excitation and relaxation of the donor molecule from D-to-D’-to-D takes place, followed by non-radiative energy transfer from D’ to A via D i.e. as a consequence of dipole-dipole coupling between the two ground states (D and A) happens. In 1948, Theodor Förster observed that when the resonating dipole moments of two molecules are identical, which in this case are D and A, an energy coupling between the two could occur resulting in photon-less transfer of energy. In addition, the excited acceptor molecule (A’) returns to the ground state (A) by losing its energy via photon emission (in case, acceptor is a fluorophore), i.e., fluorescence (Figure 4.1). Irrespective of the photo-physical characteristic of the acceptor, i.e., whether it is a chromophore or fluorophore, the energy transfer process is called as Förster resonance energy transfer colloquially referred to as fluorescence resonance energy transfer.
the acceptor molecule is non-emitting then the fluorescence intensity is solely due to the donor’s fluorescence, as is the case of this experiment. \(^ {10,11}\)

**Figure 4.1:** Schematic representation of biosensor fabrication process and FRET phenomena. Binding of DNA to MTX, results in increase fluorescence intensity due to FRET. Solid and wavy arrows indicate the radiative and nonradiative processes, respectively. Hydrogen bonding in unreacted DNA is shown by dotted lines on lateral sides of the DNA.

Mitoxantrone is an anti-cancer drug which is prescribed for chronic treatment. Such chemotherapy could produce fatal toxicity with incremental bioavailable dose built-up. Since it’s a drug which controls the cell differentiation with low specificity, it has a potential to cross nuclear membrane to interact with DNA thus produce toxicity. Specific molecular recognition is a fundamental prerequisite, based on affinity between complementary structures such as enzyme-substrate, antibody-antigen, receptor-hormone, drug-target and this property in biosensor is used for the production of concentration-proportional signals. \(^ {3}\) Many research papers have been reported on optical detection of DNA, proteins and virus by using gold nanoparticles and quantum dots. \(^ {12-17, 23-27}\) But
until now, a DNA labeled AuNPs based optical biosensor has never been developed which can monitor or detect toxicity. Thus, we have designed DNA labeled AuNPs optical sensor for monitoring DNA-MTX interaction.
4.3 EXPERIMENTAL

4.3.1 Chemicals

Highly polymerized calf thymus DNA (MP Biomedicals, US) was used in this study. DNA dilutions were prepared in Phosphate buffer pH 7. Phosphate buffer was prepared by dissolving 0.1M disodium hydrogen phosphate in water followed by the pH adjustment by adding 0.1M HCl. HAuCl_4 and Tri-sodium citrate were used to prepare AuNPs. All chemicals were purchased from E-Merck (Mumbai, India), SRL (Mumbai, India) and were all of analytical reagent grade. Mitoxantrone was obtained from Cipla Ltd. and used as received. Paracetamol was obtained from sun pharmaceuticals. All aqueous solutions were prepared in Milli-Q water from a Millipore purification system and all experiments were done at room temperature.

4.3.2 Characterization and Measurement

Fluorescence intensity was measured by Jasco FP-6500 spectrofluorimeter (Jasco, Japan) at a scan rate of 200 nm/min. Excitation and emission bandwidths of 20 nm and 10 nm were set respectively. UV spectra were obtained on a JASCO V-670 spectrophotometer (Jasco, Japan). Particle size of AuNPs was determined by Malvern Zeta-sizer (Model-The Zetasizer Nano ZS, UK). The morphology of AuNPs were carried by Scanning Electron Microscope (SEM) EVO-18, special edition, Carl-Zeiss.

4.3.3 Citrate-Capped AuNPs synthesis

AuNPs were prepared by citrate reduction of HAuCl_4 according to documented methods with mere modifications. A 25ml aqueous solution containing of 1.0×10^{-3} mol L^{-1} HAuCl_4 was brought to vigorously boil with continuous stirring in a round bottom flask; a 2.5 mL, 38.8×10^{-3} mol L^{-1} trisodium citrate solution was then added rapidly to the above solution. The mixture solution was heated for another 15 min approximately until the color of the solution changed from pale yellow to deep red. Subsequently, the solution was cooled to room temperature under the continual stir. Finally, a deep red,
monodispersed “naked” AuNPs obtained were then used as stock solution. The sizes of the NPs were measured by Malvern Zeta-sizer.

4.3.4 DNA labeled AuNPs

Citrate capped AuNPs were labeled with dsDNA (double stranded DNA). 20 mg/ml DNA solution was prepared in phosphate buffer 7. AuNPs solution priorly prepared was diluted (by double distilled water) to 2x. 2 ml of diluted AuNPs were added to 2 ml of DNA solution and the solution was stirred at 150 rpm for 3min which was then used for monitoring MTX-DNA interaction.
4.4 RESULTS AND DISCUSSION

4.4.1 Characterization of AuNPs

The morphology of AuNPs were obtained by SEM. Figure 4.2 illustrates that the particles are predominantly spherical in shape with diameter ranging from 15 to 25 nm. Larger and uneven shaped particles with diameter 30–50 nm were also obtained.

![SEM image of AuNPs](image)

**Figure 4.2:** SEM image of AuNPs

Distribution of particle was measured by Malvern zeta-sizer. Figure 4.3 shows the particle size distribution of AuNPs, with an average of 16 nm. The concentration of the AuNPs was determined by UV/vis spectroscopy from the characteristic absorbance peak at 527 nm for 16 nm particle.
**Figure 4.3:** (A) Average particle size distribution of AuNPs; (B) UV-visible spectra of AuNPs obtained at 527 nm, while the intensity peak decreased and shifted to 530 nm on binding with DNA. Also, peak of pure DNA is shifted from 260 to 256 on binding.
4.4.2 Evaluation of DNA labeled AuNPs

dsDNA has a stable double-helix geometry that presents a negatively charged phosphate backbone for interaction. \(^{19}\) ssDNA (single stranded DNA) presents a positively charged Nucleobases surface. So they have different chemistries to be adsorbed on the negatively charged surface of AuNPs in solution. Recently, Par Sandstrom et al has reported the mechanism for adsorption of DNA on AuNPs. They suggested that the adsorption is due to an ion-induced dipole interaction. The charge of the phosphate groups of DNA may induce a dipole in the highly polarizable gold particles. This mechanism is quite short ranged. Initially, the Coulombic repulsion keeps the species apart at longer distances. One incubation scenario is thus that the ionic strength of the solution needs to be raised in order to screen the long-range repulsion so that DNA and AuNPs are allowed to approach each other. At a certain distance, the ion-induced dipole interaction takes over, resulting in a net attractive force. \(^{21-22}\) Figure 4.4 is illustrative of DNA adsorbed on to the AuNPs. The pure DNA gives absorbance peak at 260 nm, while AuNPs give peak at 527 nm. The peaks of DNA labeled AuNPs were 256 nm and 530 nm, respectively. Now the peaks of DNA and AuNPs observed, shows hypsochromic and bathochromic shift, respectively. Also, the peak intensity happened to decrease in case of AuNPs, while in case of DNA it elevated. This confirms the adsorption of DNA to the citrate-capped AuNPs.

4.4.3 MTX-DNA interaction by optical nanobiosensor

Three series of different concentrations of MTX were prepared (100 µg/ml, 50 µg/ml, 10 µg/ml) and named series 100, series 50 and series 10, respectively. From each series 10 to 50 µL MTX was added to DNA labeled AuNPs solution and fluorescence measurement was performed by spectrofluorometer.

Bathochromic shift of the absorption of the AuNPs from Figure 4.3(B) in the presence of Nucleobases viz., adenine, guanine, thiamine and cytosine bound in the DNA is an obvious consequence of their ground state complexation \(^{28}\) with AuNPs (Figure 4.1) with a static quenching efficiency. Nucleobases are non-radiating in nature when excited at the AuNPs’ wavelength. Here, we have realized the Nucleobases as acceptor molecules and AuNPs as donor molecules as per the FRET theory. The experiment has been designed to
map the DNA toxicity proportional to the concentration of MTX. As the MTX concentration was incrementally increased (by 10 µL), the interaction of the same with the DNA increased. The first spectrum a shown in Figure 4.4 is of bare AuNPs and b spectrum is for DNA labeled AuNPs. On adsorption of DNA to AuNPs shows increase in fluorescence intensity. Spectrum from c to g is of increasing concentrations of MTX added to DNA-AuNPs solution.

![Fluorescence emission of DNA labeled AuNPs](image)

**Figure 4.4:** Fluorescence emission of DNA labeled AuNPs. (series 100 i.e., 100 µg/mL) Excitation(Ex) at 450 nm yield a fluorescence emission(Em) peak at 531 nm. [a: bare AuNPs, b: DNA-AuNPs, after addition of increasing amount of MTX to DNA-AuNPs, i.e., c:10µL, d:20µL, e:30µL, f:40µL, g:50µL]

The interaction of the MTX with DNA is seen as conformational change in the DNA structure as the former intercalates with the DNA by breaking the latter’s Hydrogen bonds laterally attached. Furthermore, the hydrogen bonds which are responsible for the compactness of the DNA helix, on breaking causes expansion of the DNA structure. The resultant expansion indicates an increase in distance between the Nucleobases and the
AuNP molecule. Therefore, FRET from AuNP to the Nucleobases decreases with reduction in the quenching effect of the colloidal gold which becomes evident from the increase in the fluorescence peak intensity with respect to MTX concentration in all the three concentration series.

**Figure 4.5:** Fluorescence emission of DNA labeled AuNPs. (Paracetamol at 100 µg/mL) Excitation(Ex) at 450 nm yield a fluorescence emission(Em) peak at 531 nm. [a: bare AuNPs, b: DNA-AuNPs, after addition of increasing amount of Paracetamol to DNA-AuNPs, i.e., c:10µL, d:20µL, e:30µL, f:40µL, g:50µL]

In case of Paracetamol, 100 µg/mL concentrations were prepared. Aliquots of 10 to 50 µL Paracetamol were added to DNA labeled AuNPs solution and fluorescence measurement was performed by spectrofluorometer. Figure 4.5 shows the increase in fluorescence intensity of DNA labeled AuNPs due to interaction between DNA and Paracetamol. But, as compare to MTX, the increase in fluorescence intensity was lower. The reason behind this was interacting behavior of drug. MTX is strong intercalating
drug, while Paracetamol shows very weak interaction.\textsuperscript{29-30} So, this is evident that proposed method was perfect for monitoring drug-DNA interaction. No significant changes in fluorescence intensity were observed at 50 and 10 µg/mL of Paracetamol concentration.

\textbf{Figure 4.6:} Fluorescence emission of DNA labeled AuNPs. (series 50) Excitation(Ex) at 450 nm yield a fluorescence emission(Em) peak at 531 nm. [a: bare AuNPs, b: DNA-AuNPs, after addition of increasing amount of MTX to DNA-AuNPs, i.e., c:10µL, d:20µL, e:30µL, f:40µL, g:50µL]

Figure 4.6 and Figure 4.7 shows the interaction of MTX with DNA at concentration 50 and 10 µg/mL respectively. In both, series 50 and series 10, the fluorescence intensity was increased due to conformational change in DNA-AuNPs conjugate on interaction. While, in case of Paracetamol, no significant changes in fluorescence intensity were observed, indicates that very weak interaction was occurring between Paracetamol and DNA.
Figure 4.7: Fluorescence emission of DNA labeled AuNPs. (series 10) Excitation(Ex) at 450 nm yield a fluorescence emission(Em) peak at 531 nm. [a: bare AuNPs, b: DNA-AuNPs, after addition of increasing amount of MTX to DNA-AuNPs, i.e., c:10µL, d:20µL, e:30µL, f:40µL, g:50µL]
4.5 SENSITIVITY AND SELECTIVITY OF NANOBIOSENSOR

To evaluate the sensitivity and selectivity of this optical nanobiosensor, 100 µg/mL, 50 µg/mL and 10 µg/mL drug concentrations was used and ranging from 10 to 50 µL. Figure 4.8 shows the increase in fluorescence intensity on interaction between MTX-DNA and Paracetamol-DNA, but in case of MTX, the increased fluorescence intensity was much higher than Paracetamol. (Figure 4.9) At concentration 50 µg/mL and 10 µg/mL of Paracetamol, no changes were obtained fluorescence spectra.

![Bar Graph](image)

**Figure 4.8:** Comparative study of MTX-DNA and Paracetamol-DNA interaction by optical nanobiosensor at 100 µg/mL

In case of MTX, fluorescent intensity rose steadily in both series with respect to increasing MTX concentration, but the increase in fluorescence intensity in series 50 appeared to be higher than that of series 10. (Figure 4.10) This confirms that the binding behavior of MTX with DNA of series 50 is more as compared to series 10 due to the reasons of difference in the MTX concentrations between both the series.
Figure 4.9: Comparison of interacting behaviour of MTX and Paracetamol with DNA

Figure 4.10: Comparative study of MTX-DNA interaction by optical nanobiosensor at 50 and 10 µg/mL
On addition of incremental concentrations of MTX to DNA-AuNPs, fluorescence intensity increases in all series. It is evident from the results that interacting behavior of DNA labeled AuNPs with the stock concentrations of MTX are as follows:

series 100 > series 50 > series 10.
4.6 ANALYTICAL PERFORMANCE OF NANOBIOSENSOR

The comparison of analytical performances for determining MTX-DNA and Paracetamol-DNA interaction are given in Table 4.1. Figure 4.12 shows the values of correlation coefficient ($R^2$), slope and intercept for MTX as 0.9929, 1.7602 and 516.96, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were found as 849 (ng/mL) and 2.572 (µg/mL) respectively.

\[
\begin{align*}
\text{y} &= 1.7602x + 516.96 \\
R^2 &= 0.9929
\end{align*}
\]

**Figure 4.12:** Plots of the concentration of MTX versus the intensity of MTX-DNA interaction (100µg/mL)

For Paracetamol, the values of correlation coefficient ($R^2$), slope and intercept were found as 0.9957, 0.9047 and 461.32, respectively. (Figure 4.13) LOD and LOQ were found as 1.225 (µg/mL) and 3.71(µg/mL) respectively.
Figure 4.13: Plots of the concentration of Paracetamol versus the intensity of Paracetamol-DNA interaction (100µg/mL)

Table 4.1: Comparison of the analytical performance for determination of MTX-DNA and Paracetamol-DNA interaction for series 100

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fluorescence intensity of MTX</th>
<th>Fluorescence intensity of Paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation (Y)</td>
<td>1.7602</td>
<td>0.9047</td>
</tr>
<tr>
<td>Slope (b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>516.96</td>
<td>461.32</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9929</td>
<td>0.9957</td>
</tr>
<tr>
<td>LOD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>849 (ng/mL)</td>
<td>1.225 (µg/mL)</td>
</tr>
<tr>
<td>LOQ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.572 (µg/mL)</td>
<td>3.71 (µg/mL)</td>
</tr>
</tbody>
</table>

<sup>a</sup>LOD = 3.3×SD/slope  
<sup>b</sup>LOQ = 10×SD/slope
Figure 4.14 shows the concentration of MTX versus the intensity of MTX-DNA interaction. There is clear correlation between them. The correlation coefficient, R, was calculated to be 0.9943 in case of 50 µg/mL of MTX concentration.

![Graph showing correlation]

**Figure 4.14:** Plots of the concentration of MTX versus the intensity of MTX-DNA interaction (50µg/mL)

Figure 4.15 shows the concentration of MTX versus the intensity of MTX-DNA interaction. The correlation coefficient, R, was calculated to be 0.9913 in case of 10 µg/mL of MTX concentration.
Figure 4.15: Plots of the concentration of MTX versus the intensity of MTX-DNA interaction (10µg/mL)
4.7 CONCLUSIONS

In conclusion, we have constructed an optical nanobiosensor for monitoring DNA-MTX interaction/toxicity based on its modulation of fluorescence changes due to FRET phenomenon between DNA and AuNPs in an aqueous solution on addition of varying concentrations of MTX. In fact, we can observe DNA interaction occurring with increased amount of MTX resulting in FRET decrease from AuNPs to Nucleobases of DNA, witnessing the underlying reason that MTX intercalates with DNA with breaking of its hydrogen bonds. In case of Paracetamol, a very weak interaction was observed. In addition, the present method features the briskness, simplicity, low cost and above all, an insight to the tox-screening of current and more importantly the new chemical entities (NCEs) in a tech-savvy way never done before.
4.8 REFERENCE


