3.3. Chitosan–iron oxide nanocomposite platform for *N. gonorrhoeae* detection causing sexually transmitted infection

To obtain a sensitive, compact and stable DNA biosensor platform, desired probe DNA should be immobilized in suitable orientation on the electrode surface. Besides this, the desired DNA should be immobilized onto an appropriate matrix (Kaushik et al., 2009c & 2008b). In this context, many matrices including non-conducting polymers, conducting polymers, self-assembled monolayers, nanomaterials, nanocomposites, sol-gel derived nanostructures etc. have been proposed for the immobilization of biomolecules. Among these, organic (non-conducting polymer)-inorganic (metal oxide nanoparticles) hybrid nanocomposites have gained much interest for the development of electrochemical biosensor (Kaushik et al., 2009c & 2008b; Solanki et al., 2009c).

Among the various non-conducting polymer, CHIT has been found to be an interesting polymer for the immobilization of desired biomolecule (Ren et al., 2005). Several attempts have been made to improve the biocompatibility and chemical activity of CHIT via structural modification, adjustment of molecular factors and incorporation of metal oxide nanoparticles to fabricate organic-inorganic nanocomposites (Groboillot et al., 1993). CHIT has been reported as an interesting immobilization matrix for various pharmaceutical, environmental and biotechnological applications (Groboillot et al., 1993) due to its excellent film-forming ability, good adhesion, biocompatibility, high mechanical strength, and susceptibility to chemical modification due to the presence of reactive hydroxyl and amino functional groups.

Metal oxide nanoparticles due to large surface-to-volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability have been
used for a number of biosensor applications (Ansari et al., 2008). Among these, nanostructured Fe₃O₄ has recently been used for the immobilization of DNA due to low temperature processing, tunability in physical parameters, optical transparency, chemical inertness, thermal stability and negligible swelling in aqueous and non-aqueous solutions. The problem of aggregation has led to limited applications of Fe₃O₄ nanoparticles to biosensing. This problem can perhaps be addressed by modifying Fe₃O₄ nanoparticles with CHIT (Yang et al., 2009a) to prepare a nanobiocomposite. Efforts have recently been made to improve optical and electrical properties of CHIT for biosensor application by dispersing superparamagnetic Fe₃O₄ nanoparticles (Liang et al., 2007; Wang et al., 2007). However, CHIT can be chemically modified by covalently attaching biomolecules to its amino/hydroxyl groups. The main advantage of using CHIT lies in the formation of a complex with a given biomolecule including probe DNA to obtain desired stability.

The present section describes the construction of electrochemically deposited CHIT-nFe₃O₄ film onto ITO surface. Inspired by fascinating results, this CHIT-nFe₃O₄/ITO film has been utilized for the immobilization of single stranded biotinylated probe DNA (BDNA) specific for *N. gonorrhoeae* to reveal its application to biosensor for DNA detection using electrochemical as transducer.
3.3.1. Experimental

3.3.1.1. Preparation of CHIT–nFe$_3$O$_4$ composite film

Chitosan (0.50%) solution was prepared by dissolving 50 mg in 10 mL of acetate buffer (0.05 M, pH 4.2) and was kept for 4 days to obtain a clear solution at 25°C. 5 mg of Fe$_3$O$_4$ nanoparticles were dispersed in CHIT solution by stirring at room temperature after which it is sonicated for about 10 h. Finally, a highly viscous solution of CHIT with uniformly dispersed Fe$_3$O$_4$ nanoparticles was obtained and used for electrochemical deposition. It has been found that optimized ratio of CHIT and Fe$_3$O$_4$ nanoparticles taken as 10:1 to prepare CHIT-nFe$_3$O$_4$/ITO nanocomposite film exhibits maximum electrochemical current response and that 15 µL of CHIT–nFe$_3$O$_4$/ITO nanocomposite solution standardized for deposition onto ITO surface. CHIT–nFe$_3$O$_4$/ITO nanocomposite films were prepared onto ITO plate (0.25 cm$^2$) using electrochemical deposition of quiescent solution of CHIT-nFe$_3$O$_4$. ITO substrates were chosen because it was known that an oxide surface can interact strongly with -OH and -NH$_2$ of CHIT. The ITO plates were pre-cleaned with acetone, ethanol, and a copious amount of deionized water. To obtain uniform distribution of OH group on ITO surface, ITO plates were immersed in a solution of H$_2$O$_2$/NH$_4$OH/H$_2$O (1:1:5, v/v) for 30 min at 80°C for hydrolysis, after which they were rinsed thoroughly with deionized water and dried. The deposition was performed chrono-amperometrically at -1.0 V for about 360 s (via intermittent washing using autoclaved Millipore water after every 120 s of deposition). Then, the electrode was rinsed with autoclaved Millipore water three times to remove any
unbound particles and then air-dried. In this way, a robust and highly ordered CHIT-nFe₃O₄/ITO nanocomposite was obtained.

3.3.1.2. Functionalization of CHIT-nFe₃O₄ film using probe DNA

DNA solutions were prepared in tris-EDTA buffer. After electrodeposition, biotin labeled DNA was immobilized onto CHIT-nFe₃O₄/ITO nanocomposite electrode using avidin under optimized conditions with gentle shaking. 10 μl of avidin (1 mg/mL) (activated using 15 mM EDC and 30 mM NHS for 2 h at 25°C) was immobilized onto CHIT-nFe₃O₄/ITO nanocomposite surface. Avi-CHIT-nFe₃O₄/ITO electrode was then washed with autoclaved Millipore water and was subjected to 5 min incubation with 10 μl of 20-mer biotinylated oligonucleotide probes (1.0 μM) in a humid chamber at 25°C. The sequences of DNA probes used for the electrochemical DNA detection are as follows (Table 3.2):

Probe: BDNA or biotin- (GenBank accession no: PUID 9716119 snum 2705)

Table 3.2: Oligonucleotide sequences used for immobilization and hybridization

<table>
<thead>
<tr>
<th>SL.No.</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Probe DNA: Biotin (BDNA)</td>
<td>5'-CCGGTGCTTCATCACCTTAG-3'</td>
</tr>
<tr>
<td>2.</td>
<td>Complementary target DNA (cDNA)</td>
<td>5'-CTAAGGTGATGAAGCACCAGG-3'</td>
</tr>
<tr>
<td>3.</td>
<td>One-base mismatch DNA (OBM1)</td>
<td>5'-CTAAGGTGATGAAGCACCAGG-3'</td>
</tr>
<tr>
<td>4.</td>
<td>One-base mismatch DNA (OBM2)</td>
<td>5'-CTAAGGTGACGAAGCACCAGG-3'</td>
</tr>
<tr>
<td>5.</td>
<td>Two-base mismatch DNA (TBM1)</td>
<td>5'-CTAAGGTGATGAAGAACCAGG-3'</td>
</tr>
<tr>
<td>6.</td>
<td>Two-base mismatch DNA (TBM2)</td>
<td>5'-CTAAGGTGACGAAGCACCAGG-3'</td>
</tr>
<tr>
<td>7.</td>
<td>Two-base mismatch DNA (TBM3)</td>
<td>5'-CTAAGGTGACGAAGCACCAGG-3'</td>
</tr>
</tbody>
</table>
Table 3.3: Matched and mismatched duplex DNA used for hybridization onto the electrode

<table>
<thead>
<tr>
<th></th>
<th>1+2</th>
<th>1+3</th>
<th>1+4</th>
<th>1+5</th>
<th>1+6</th>
<th>1+7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matched</td>
<td>(C-T) 6</td>
<td>(A-C) 10</td>
<td>(C-T, C-A) 6, 14</td>
<td>(T-G, A-C) 4, 10</td>
<td>(C-T, A-C) 6, 10</td>
<td></td>
</tr>
</tbody>
</table>

3.3.1.3. Hybridization of BDNA–Avi–CHIT–nFe₃O₄/ITO bioelectrode

The BDNA-Avi-CHIT–nFe₃O₄/ITO bioelectrode was hybridized with complementary target oligonucleotide for about 60 s at 25°C. Fig 3.10 shows the schematic for the fabrication of CHIT-nFe₃O₄/ITO based DNA biosensor.

![Schematic diagram of CHIT-nFe₃O₄/ITO biosensor](image)

Fig 3.10: Schematic representation of CHIT–nFe₃O₄/ITO nanoelectrode based DNA biosensor for *N. gonorrhoeae* detection
3.3.2. Results and discussion

3.3.2.1. X-ray Diffraction (XRD) studies

Fig 3.11 shows the X-ray Diffraction pattern of Fe₃O₄ nanoparticles (JCPDS-International Center Diffraction Data, PDF cards 3-864 and 22-1086). The reflection peak at 2θ = 35.42° corresponds to the hexagonal spinel phase of nFe₃O₄. The diffraction peaks of the magnetic nanoparticles are found to be 2θ = 30.10°, 35.42°, 43.20°, 53.48° and 56.94°. The data is in good agreement with that of the Fe₃O₄ nanoparticles. The observed broad reflection planes may perhaps be due to small particle size. The average particle size of Fe₃O₄ nanoparticles has been estimated ~18 nm using Debye-Scherrer equation.

![Fig 3.11: XRD pattern of Fe₃O₄ nanoparticles](image)

3.3.2.2. Vibrating Sample Magnetometric (VSM) studies

Fig 3.12 shows the VSM curve obtained for Fe₃O₄ nanoparticles. The applied magnetic field H dependence of the magnetization M can be described by the Langevin equation. The VSM curve reveals that there is a hysteresis for the Fe₃O₄ nanoparticles. The coercive force is small (Hc = 100.5 Oe), in comparison to the rare
reported coercive force (500–800 Oe) for the bulk magnetic particles. However, it does not approach to zero, thereby exhibiting a super paramagnetic behavior. On increasing the applied field from 0 to 5000 Oe, the magnetization increases sharply; \( M \) is nearly saturated at about 2000 Oe. The \( \text{Fe}_3\text{O}_4 \) nanoparticles exhibit retentivity \( \sim 7.77 \text{ emu/g} \) and magnetism of \( \sim 52.64 \text{ emu/g} \). The measured magnetization of nanoparticles is found to be lower than that of bulk magnetite due to superparamagnetism.

![Graph showing VSM curve of \( \text{Fe}_3\text{O}_4 \) nanoparticles at room temperature](image)

**Fig 3.12:** VSM curve of \( \text{Fe}_3\text{O}_4 \) nanoparticles at room temperature

### 3.3.2.3. Fourier Transform Infrared (FT-IR) Spectroscopic studies

The FTIR spectra (Fig 3.13) of pure CHIT/ITO (Spectra A) displays all the IR bands corresponding to functional groups present in CHIT. The IR bands corresponding to \(-\text{NH}\) and \(\text{OH}\) stretching modes are shifted to the lower wave number in the IR spectra of CHIT–n\(\text{Fe}_3\text{O}_4\)/ITO nanocomposite film (Spectra B), corresponding to pure CHIT. This indicates that amine group of CHIT is involved in assembling of the \(\text{Fe}_3\text{O}_4\) nanoparticles.
Fig 3.13: FT-IR spectra of (A) CHIT/ITO, (B) CHIT-nFe₃O₄/ITO, (C) Avi-CHIT-nFe₃O₄/ITO and (D) BDNA-Avi-CHIT-nFe₃O₄/ITO electrodes

The absorption band of Fe₃O₄ nanoparticles appear at 582 cm⁻¹ arising due to both the stretching vibration mode and tensional vibration mode of Fe-O bond in the tetrahedral and in the octahedral sites. The FTIR spectra of Avi-CHIT–nFe₃O₄/ITO bioelectrode (Spectra C) shows characteristic peaks at 1565 and 1658 cm⁻¹ that are attributed to primary and secondary amide linkages of avidin molecules. In the FTIR spectrum of BDNA-Avi-CHIT–nFe₃O₄/ITO bioelectrode, vibration bands observed at 1067 and 1243 cm⁻¹ are due to the asymmetric stretching of P-O-C vibration and stretching vibration of P=O of the phosphoric acid group, respectively. Peaks seen at 1492 and 1606 cm⁻¹ are associated with carbonyl stretching vibration and C=C bonds in the purine and pyrimidine rings (Spectra D).

3.3.2.4. Scanning Electron Micoscopic (SEM) studies

The SEM images of an electrochemically deposited CHIT–nFe₃O₄/ITO nanocomposite (A) and BDNA-Avi-CHIT–nFe₃O₄/ITO bioelectrode (B) are given in Fig 3.14.
Fig 3.14: SEM Studies of (A) CHIT-nFe$_3$O$_4$/ITO and (B) BDNA-Avi-CHIT-nFe$_3$O$_4$/ITO electrodes

Fig 3.14 (A) shows that Fe$_3$O$_4$ nanoparticles are uniformly embedded in CHIT and are stable with least aggregation. The porous surface of the CHIT–nFe$_3$O$_4$/ITO nanocomposite film is suitable for the immobilization of biomolecules.

3.3.2.5. AFM Studies of electrochemically deposited CHIT–nFe$_3$O$_4$ film

Fig 3.15 presents the typical AFM images of an electrochemically deposited CHIT–nFe$_3$O$_4$/ITO nanocomposite (A) and BDNA-Avi-CHIT–nFe$_3$O$_4$/ITO bioelectrode (B). The AFM studies reveal that Fe$_3$O$_4$ nanoparticles are interconnected by dipole-dipole interactions in the film. In the presence of nFe$_3$O$_4$, CHIT–nFe$_3$O$_4$ film shows globular nanoporous rough morphology (Fig 3.15 (A)). It appears that Fe$_3$O$_4$ nanoparticles are regularly dispersed in the CHIT film and strongly adhere onto the substrate with porous morphology. The surface roughness of CHIT-nFe$_3$O$_4$ nanobiocomposite increases to ~25.4 nm.
Fig 3.15: AFM images of (A) CHIT-nFe$_3$O$_4$/ITO and (B) BDNA-Avi-CHIT-nFe$_3$O$_4$/ITO electrodes

Such nanoporous rough morphology results in increased effective surface area of the substrate electrode to load large amount of DNA, and could perhaps prevent leaching of the DNA molecules from the biosensor surface. The surface roughness of BDNA-Avi-CHIT-nFe$_3$O$_4$/ITO bioelectrode decreases to ~5.44 nm indicating immobilization of the biotinylated DNA. When DNA is entrapped in this network like film, aggregates of the DNA molecules exhibit island-like structures (Fig 3.15 (B)) that may facilitate specific reaction between substrate and DNA, resulting in enhanced amperometric response of the biosensor.

3.3.2.6. Optimization of deposition potential

CHIT-nFe$_3$O$_4$/ITO nanocomposite films have been prepared electrochemically in the potential range, -0.5 V to -2.5 V. In the case of CHIT-nFe$_3$O$_4$/ITO (Fig 3.16 (A)) electrode, there is a negligible shift in the peak of CHIT-nFe$_3$O$_4$/ITO CV at various applied potentials. On inclusion of the Fe$_3$O$_4$ nanoparticles the film is found to be stable.
Fig 3.16: (A) CV of CHIT-nFe$_3$O$_4$/ITO at different potential (i) 0.5 V, (ii) 1.0 V, (iii) 1.5 V and (iv) 2.0 V; (B) chronoamperometric curves (i) first deposition curve, (ii) second deposition curve and (iii) third deposition curve

It can be concluded that -1.0 V is the appropriate potential for the fabrication of CHIT–nFe$_3$O$_4$ film onto ITO plate since it yields higher peak current. The Fig 3.16 (B) show the various chronoamperometric curves obtained for CHIT–nFe$_3$O$_4$/ITO nanocomposite films.

3.3.2.7. Cyclic Voltammetric (CV) studies

Fig 3.17 (A) shows results of the CV measurements carried out on an electrochemically deposited CHIT/ITO film (i), CHIT–nFe$_3$O$_4$/ITO nanocomposite (ii), an Avi-CHIT–nFe$_3$O$_4$/ITO (iii), and BDNA-Avi-CHIT–nFe$_3$O$_4$/ITO bioelectrode (iv) in 0.05 M PBS of pH 7.0 containing 5 mM[Fe(CN)$_6$]$^{3-/4+}$ at a scan rate of 50 mVs$^{-1}$, respectively. The CV of pure CHIT electrode (Curve i) shows well-defined cathodic peak at -0.0137 V and an anodic peak at 0.260 V in the potential range of 0.6 to -0.30 V having peak current of 151 $\mu$A. However, significant change (Curve ii) in peak potential [cathodic peak potential ($E_{pc}$) to -0.0346 V and anodic peak potential ($E_{pa}$) to 0.295] with increased peak current of 238 $\mu$A is observed on inclusion of
Fe$_3$O$_4$ nanoparticles. This may be attributed to the presence of Fe$_3$O$_4$ nanoparticles with increased mobility resulting in enhanced adsorption of the redox moieties on cationic CHIT–nFe$_3$O$_4$/ITO nanocomposite surface with improvement in electron transfer and electrical conductivity. It can be seen that there is considerable decrease in the intensity of these redox peaks after the immobilization of avidin indicating the binding of avidin onto CHIT–nFe$_3$O$_4$/ITO surface (Curve iii). Further, the observed enhancement in the redox peak can be attributed to binding of BDNA onto CHIT–nFe$_3$O$_4$/ITO surface in agreement with literature (Curve iv).

The CV investigations conducted as a function of scan rate (v) (10–300 mV/s) (Fig 3.17 (B)) onto CHIT–nFe$_3$O$_4$/ITO electrode. BDNA-Avi-CHIT–nFe$_3$O$_4$/ITO bioelectrode to estimate the concentration of ionic species on the CHIT–nFe$_3$O$_4$/ITO electrode and probe DNA. It can be seen that the anodic potential (Fig 3.17 (B, E)) shifts towards positive side and the cathodic peak potential shifts in the reverse direction suggesting a quasi-reversible process. Besides this, the anodic and cathodic peak currents ($I_{pa}$ and $I_{pc}$) are proportional to the square root of scan rate, $v^{1/2}$ over the range 10-300 mV/s (Fig 3.17 (B, E)), indicating a diffusion controlled process on the given electrodes.

For CHIT–nFe$_3$O$_4$/ITO bioelectrode, the linear regression equations obtained are as follows:

\[ I_{pa} = 2.55371 \times 10^{-5} v^{1/2} + 7.3340 \times 10^{-5}; \quad R=0.9983; \quad SD=6.5527 \times 10^{-5} \quad \ldots \text{Eq. 3.11} \]

\[ I_{pc} = 1.34149 \times 10^{-5} v^{1/2} + 1.0190 \times 10^{-4}; \quad R=0.99299; \quad SD=7.02447 \times 10^{-5} \quad \ldots \text{Eq. 3.12} \]
For BDNA-Avi-CHIT–nFe₃O₄/ITO bioelectrode, the linear regression equations obtained are as follows:

\[ I_{pa} = 9.7258 \times 10^{-6} v^{1/2} + 3.2378 \times 10^{-5}; \quad R=0.9931; \quad SD=5.0253 \times 10^{-6} \quad \text{... Eq. 3.13} \]

\[ I_{pc} = 6.62068 \times 10^{-6} v^{1/2} + 4.6744 \times 10^{-5}; \quad R=0.98136; \quad SD=5.7028 \times 10^{-5} \quad \text{... Eq. 3.14} \]

where R is regression coefficient and SD is standard deviation.

It has been observed that the anodic peak potential \(E_{pa}\) for electrode and bioelectrode varies linearly with logarithm of \(v\) and the trend of the variation in the peak potential with scan rate follows the Eq. 3.15, 3.16:

\[ E_{pa} = 0.07781 \log v + 0.12688; \quad R=0.949; \quad SD=0.00622 \quad \text{... Eq. 3.15} \]

\[ E_{pa} = 0.16633 \log v + 0.08948; \quad R=0.955; \quad SD=0.00991 \quad \text{... Eq. 3.16} \]

Moreover, the concentration of redox probe onto electrode and bioelectrode has been calculated using Laviron’s theory, Eq. 3.17, 3.18:

\[ RT/\alpha nF= 0.07781 \quad \text{...Eq. 3.17} \]

\[ RT/\alpha nF= 0.16633 \quad \text{...Eq. 3.18} \]

where, \(\alpha\) is the transfer coefficient. The value of \(RT/\alpha nF\) has been used to calculate the total surface concentration of the ionic species (I) onto BDNA-Avi-CHIT–nFe₃O₄/ITO bioelectrode using Eq. 3.19:

\[ I_p = n^2F^2\Gamma v A (4RT)^{-1} \quad \text{...Eq. 3.19} \]

where, \(I_p/v\) can be calculated from the slope of \(i_p\) vs. \(v\) plot.
The surface concentration value of redox species of electrolyte [Fe(III)/Fe(IV)] onto the CHIT–nFe₃O₄/ITO electrode has been found to be $0.437 \times 10^{-6}$ mol cm⁻². The total surface concentration value of redox species of electrolyte [Fe(III)/Fe(IV)] has been estimated to be as $7.97 \times 10^{-7}$ mol cm⁻² indicating improved immobilization of BDNA onto Avi-CHIT–nFe₃O₄/ITO electrode.
3.3.2.8. Electrochemical Impedance Spectroscopic (EIS) Studies

Fig 3.18 shows results of the EIS measurements carried out as a function of frequency on an electrochemically deposited CHIT/ITO film (i), CHIT-nFe₃O₄/ITO nanocomposite (ii), an Avi-CHIT-nFe₃O₄/ITO (iii), and a BDNA-Avi-CHIT-nFe₃O₄/ITO bioelectrode (iv) in 0.05 M phosphate buffer saline (PBS) of pH 7.0 containing 5 mM[Fe(CN)₆]³⁻/⁴⁻.

![EIS Spectra](image)

**Fig 3.18:** EIS Spectra of (i) CHIT/ITO, (ii) CHIT-nFe₃O₄/ITO, (iii) Avi-CHIT-nFe₃O₄/ITO (iv) and BDNA-Avi-CHIT-nFe₃O₄/ITO bioelectrodes in 0.05 M PBS containing 5 mM [Fe(CN)₆]³⁻/⁴⁻.

The charge transfer resistance \(R_{CT}\) of CHIT-nFe₃O₄/ITO electrode (Spectra ii) is found to decrease to 2.75 KΩ with respect to the CHIT/ITO electrode \(R_{CT} \approx 5.81\) KΩ, Spectra i) and the lower value of \(R_{CT}\) of CHIT-nFe₃O₄/ITO electrode reveals nanosized structure of Fe₃O₄ particles that provide increased electroactive surface leading to increased electron transfer.

However, when biotinylated ssDNA is immobilized onto CHIT-nFe₃O₄/ITO electrode, \(R_{CT}\) of the electrode increases to 4.18 KΩ that is attributed to the presence
of electro-negative phosphate skeleton that perhaps prevent \([\text{Fe(CN)}_6]^{3-/4-}\) ions from reaching the electrode surface for electron transfer during redox reaction. This implies that BDNA is successfully immobilized onto the electrochemically prepared nanostructured CHIT–nFe_3O_4/ITO electrode (Spectra iv).

### 3.3.2.9. Electrochemical response studies

The BDNA-Avi-CHIT–nFe_3O_4/ITO STD sensor has been used to detect sequence specific DNA based on the DPV current change of MB before and after hybridization. Fig 3.19 shows DPV response of the fabricated STD bioelectrode. The performance of the BDNA-Avi-CHIT–nFe_3O_4/ITO electrode has been studied by incubating with DNA extracted from \(N.\ gonorrhoeae\) culture isolated sample, pus sample spiked with \(N.\ gonorrhoeae\) and \(N.\ gonorrhoeae\) positive male patient sample. The observed significant decrease in the DPV signal shows that the complementary DNA present in the clinical sample hybridizes with the probe DNA (Fig 3.19 (A)). The observed minor variation in DPV signal can be assigned to the sample-to-sample variation arising due to varying bacterial DNA load that cannot be quantified. Fig 3.19 (B) shows results of response studies carried out with respect to concentration of target DNA sequence with BDNA-Avi-CHIT–nFe_3O_4/ITO electrode using MB as a redox indicator. The observed peak current for the reduction of MB after hybridization with cDNA decreases with the target concentration up to \(1\times10^{-6}\) M, and remains constant with further increase of the target concentration indicating that all the immobilized probe molecules on the bioelectrode surface are involved in hybridization at concentration of \(1\times10^{-6}\) M.
Fig 3.19: (A) DPV of (i) BDNA-Avi-CHIT-nFe₃O₄/ITO bioelectrode to detect presence of complementary target sequence in *N. gonorrhoeae* (ii) artificial complementary DNA, clinical samples; (iii) culture isolated, (iv) spiked pus and (v) patient samples; (B) DPV of (i) BDNA-Avi-CHIT-nFe₃O₄/ITO after hybridization with cDNA concentration (ii-xii) 1×10⁻¹⁶ M to 1×10⁻⁶ M DNA at step potential of 3 mV and modulation amplitude of 50 mV in 20 μM MB pretreatment at +0.1 V for 10 s, 0.05 M PBS (pH 7.0, 0.9% NaCl); (C) MB peak height as a function of cDNA concentration, (D) The regeneration of sensor: The sensor in which the addition of a complementary target strand results in a decrease in measured current. Because all of the sensing components are strongly adsorbed to the sensor surface, the sensor is readily regenerated with a 30 s distilled water rinse, as shown.

Prior to this threshold concentration, MB reduction signal decreases with increased target concentration that perhaps prevents interaction of guanine molecules with MB due to duplex formation. The observed constant MB signal reveals that all the hybridization sites on BDNA-Avi-CHIT-nFe₃O₄/ITO electrode are covered. The change in peak current of MB is linearly related to log of cDNA between 1×10⁻⁶ M to
$1 \times 10^{-16}$ M. The detection limit of BDNA-Avi-CHIT–nFe$_2$O$_4$/ITO is found to be $1 \times 10^{-15}$ M (calculated from blank signal). It is found that decrease in the MB peak current (I Target) with respect to cDNA concentration (Fig 3.19 (C)) follows Eq. 3.20 with value of regression co-efficient as 0.99522.

\[ \text{I Target} = 2.67818 \times [\log (\text{cDNA})] + 13.92 \]  
...Eq. 3.20

Sensor surface regeneration is critical in order to ascertain that a signal arises due to specific interactions with the target and is not due to some other non-specific modification of the probe or sensor head. The background current has been measured using DPV. The sensors have been treated with 1 µM target to determine signal suppression. It is found that the sensor is stable enough to allow for ready regeneration: a low ionic strength short wash (30 s at room temperature ($25^\circ$C) autoclaved distilled water ($\sim 18.2$ MΩ) (Fig 3.19 (D)) is sufficient to recover the original sensor signal and it is found that this sensor can be used at least for 6-8 times before significant degradation is observed. For storage, sensors have been fabricated, tested, and regenerated as above. In addition, the electrode can be stored for more than 6 months at 4°C without significant loss in their activity for hybridization and are thus suited for a stable platform for further quantitative measurements.

### 3.3.10. Specificity studies

The ability to discern base mismatch is an important characteristic of a DNA biosensor. The specificity of the biosensor has been investigated by using BDNA-Avi-CHIT–nFe$_2$O$_4$/ITO electrode to hybridize with different target ssDNA (one and two-base mismatches) sequences (Fig 3.20 (A)). The results are summarized in Table 3.4. To evaluate the effect of different mismatches by DPV, we have used five different ssDNA target sequences (Table 3.3). The ssDNA target (C-T) contains base
T instead of base G at the 6\textsuperscript{th} position. The ssDNA target (A-C) contains base C instead of base T at 10\textsuperscript{th} position. The ssDNA target having two-base mismatch is (C-T, C-A), (T-G, A-C), (C-T, A-C) at position (6, 14), (4, 10), (6, 10), respectively. (Fig 3.20 (A)) shows variation of the peak current obtained using DPV measurement after incubating the sensor with equivalent amount of targets each having a single base-pair mismatch, in relation to the immobilized BDNA probe. As expected, the lowest peak current is observed if the target is fully complementary to the immobilized BDNA probe (Fig 3.20 (A)). A significant percentage (%) decrease in current ($I_{pa}$) to 80\% is observed after BDNA-Avi-CHIT–nFe$_3$O$_4$/ITO electrode hybridized with cDNA indicates the successful hybridization. And when the single mismatched base (C-T) is at the 6\textsuperscript{th} position the % decrease in $I_{pa}$ is about 40\%. When the single mismatched base (A-C) is at 10\textsuperscript{th} position, % decrease in $I_{pa}$ is almost 57\%. The contribution of a mismatch to DNA duplex stability depends on the location of the mismatch, its nearest neighbors and its orientation. As both the A-C and C-T mismatches are present internally and stability of C-T and A-C mismatch is almost equivalent, difference in the peak may be attributed to the presence of GTT, ACT bases adjacent to the mismatch, respectively. These factors determine the free energy change of the hybridization reaction. Similarly, the % decrease in $I_{pa}$ is evaluated with two mismatched bases at different points in the DNA sequence. It may be noted that when both the mismatches are at the two ends of the sequence; (C-T, C-A), i.e. at the 6\textsuperscript{th} position and 14\textsuperscript{th} position, the % decrease in $I_{pa}$ is about 6.7\% and the peak current is higher, indicating non-hybridization. With two base mismatches (T-G, A-C), i.e. at the 4\textsuperscript{th} position and 10\textsuperscript{th} position, the % decrease in $I_{pa}$ is about 30\%. With two base mismatches (C-T, A-C) i.e. at the 6\textsuperscript{th} position and 10\textsuperscript{th} position, the % decrease in $I_{pa}$ is about 37\%. It may be noted that the reduction peak current of the STD biosensor in
The presence of cDNA is higher than that of the biosensor in the presence of one-base mismatch and two-base mismatch target DNA. Such difference may originate from the varied binding affinity of MB with cDNA, one-base mismatch and two-base mismatch target DNA. The peak current from a competitive DPV experiment is found to be reproducible and can be quantified for even a single base-pair mismatch detection. Fig 3.20 (B) shows DPV response of the STD sensor to cultures of *E. coli, S. sicca, S. aureus and K. pneumoniae*.

Fig 3.20 (A) DPV of BDNA-Avi-CHIT-nFe₃O₄/ITO bioelectrode to detect one and two-base mismatch-Discriminating DNA Hybridization; (B) DPVs of BDNA-Avi-CHIT-nFe₃O₄/ITO bioelectrodes to detect presence of non-complementary target sequence in other NgNs and other GNBs at step potential of 3 mV and modulation amplitude of 50 mV in 20 μM MB, pretreatment at +0.1 V for 10 s, 0.85 M PBS (pH 7.0, 0.9% NaCl)

The specificity of the bioelectrode has been studied by incubating the probe electrode with DNA extracted from other non-*Neisseria gonorrhoeae Neisseria* species (NgNs) as well as other gram negative bacteria (GNBs). No significant decrease in the signal is obtained in presence of *E. coli, N. sicca, S. aureus and K. pneumoniae* DNA indicating the specificity of probe DNA for *N. gonorrhoeae* detection.
Table 3.4: Percentage (%) decrease of hybridization events between the BDNA-Avi-CHIT-nFe₃O₄/ITO electrode and different base mismatch DNA sequences

<table>
<thead>
<tr>
<th>Target DNA</th>
<th>Iₚₐ of MB (μA)</th>
<th>% Decrease in Iₚₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Target DNA (Only Probe)</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>cDNA</td>
<td>6</td>
<td>80</td>
</tr>
<tr>
<td>OBM1 (C-T) 6th position</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>OBM2 (A-C) 10th position</td>
<td>13</td>
<td>57</td>
</tr>
<tr>
<td>TBM1 (C-T, C-A) 6th &amp; 14th position</td>
<td>28</td>
<td>6.7</td>
</tr>
<tr>
<td>TBM2 (T-G, A-C) 4th &amp; 10th position</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>TBM3 (C-T, A-C) 6th &amp; 10th Position</td>
<td>19</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 3.5 summarizes biosensing characteristics obtained for the electrochemically deposited BDNA-Avi-CHIT-nFe₃O₄/ITO along with those reported in literature for *N. gonorrhoeae* detection.

Table 3.5: Biosensing characteristics obtained for electrochemically deposited BDNA-Avi-CHIT-nFe₃O₄/ITO along with those reported in literature for *N. gonorrhoeae* detection

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Electrode</th>
<th>Method of immobilization</th>
<th>Detection limit</th>
<th>Hybridization time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Oligo (ethylene glycol) SAM onto Au electrode</td>
<td>Thiol-gold</td>
<td>1 pM</td>
<td></td>
<td>Zhang et al., 2008a</td>
</tr>
<tr>
<td>2.</td>
<td>Au nanoparticles</td>
<td>Biotin-avidin coupling</td>
<td>0.16 nM.</td>
<td>7 min</td>
<td>Kalogianni et al., 2006</td>
</tr>
<tr>
<td>3.</td>
<td>Au electrode</td>
<td>Thiol-gold</td>
<td>1 pM</td>
<td>1 h</td>
<td>Wong et al., 2006</td>
</tr>
<tr>
<td>4.</td>
<td>Nanogold electrode</td>
<td>Thiol-gold</td>
<td>10⁻⁰ mol/L</td>
<td></td>
<td>Liu et al., 2005b</td>
</tr>
<tr>
<td>5.</td>
<td>Chitosan-nFe₃O₄/ITO electrode</td>
<td>Avidin-biotin coupling</td>
<td>1×10⁻¹⁵ M</td>
<td>60 s</td>
<td>Present work</td>
</tr>
</tbody>
</table>
3.3.4. Summary

CHIT-nFe$_3$O$_4$/ITO nanocomposite films have been prepared onto ITO plate (0.25 cm$^2$) using electrochemical deposition of quiescent solution of CHIT-nFe$_3$O$_4$. In addition, in a separate experiment, when CHIT solution is drop-cast onto a substrate (which is equivalent to a deposition at potential = 0 V), only featureless CHIT particles are formed, without any specific nanoscale morphology. The results show that application of an electric potential is essential for the formation of CHIT–nFe$_3$O$_4$/ITO nanostructures. It may be noted that the preeminent morphology is known to be dependent on the applied potential. However, this voltage-dependence implies that the formation of CHIT nanostructure requires extra energy. We propose that the extra energy facilitates configurations with induced alignment and uniformity, which was originally restricted to a layered molecular structure bound by the intra and intermolecular hydrogen bonds. Probe DNA specific for *N. gonorrhoeae* has been covalently immobilized onto electrochemically deposited CHIT films using avidin-biotin coupling. These CHIT–nFe$_3$O$_4$/ITO nanocomposite films (BDNA-Avi-CHIT–nFe$_3$O$_4$/ITO) exhibit linear range from 1×10$^{-16}$ M to 1×10$^{-6}$ M, response time as 60 s, stability of 6 months when stored at 4°C and detection limit of 1×10$^{-15}$ M. Besides this, magnitudes of the surface concentration of redox species of electrolyte [Fe(III)/Fe(IV)] onto the CHIT-nFe$_3$O$_4$/ITO electrode and BDNA-Avi-CHIT-nFe$_3$O$_4$/ITO bioelectrode have been found to be 0.437×10$^{-6}$ mol cm$^{-2}$ and 7.97×10$^{-7}$ mol cm$^{-2}$ respectively.

Table 3.6 shows the comparison of electrochemically deposited CHIT film with CHIT-nFe$_3$O$_4$ film as an immobilization matrix for DNA to reveal its implications in DNA hybridization biosensing. It can be seen that CHIT-nFe$_3$O$_4$ can detect lower concentration of target DNA compared to that of CHIT. The better detection limit and stability of CHIT-nFe$_3$O$_4$ can be attributed to the incorporation of nFe$_3$O$_4$ into the CHIT matrix. To enhance the detection limit CNTs can perhaps be
incorporated into the CHIT matrix (Section 3.4) for detection of complementray target DNA via hybridization.

Table 3.6: Comparison table showing the biosensing parameters of electrochemically deposited CHIT with electrochemically deposited CHIT-nFe₃O₄ nanocomposite film for detection of *N. gonorrhoeae*

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Technique used</th>
<th>Detection range (M)</th>
<th>Detection limit (M)</th>
<th>Stability (Months)</th>
<th>Reusability (Times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemically deposited CHIT</td>
<td>DPV</td>
<td>1×10⁻⁹ to 1×10⁻⁶</td>
<td>1.0×10⁻⁹</td>
<td>2</td>
<td>5-6</td>
</tr>
<tr>
<td>Electrochemically deposited CHIT-nFe₃O₄</td>
<td>DPV</td>
<td>1×10⁻¹⁶ to 1×10⁻⁶</td>
<td>1.0×10⁻¹⁵</td>
<td>6</td>
<td>6-8</td>
</tr>
</tbody>
</table>

The results of studies discussed in this section have been published in the "Biosensors and Bioelectronics, 2011, 26, 2967-2974".
3.4. **Chitosan-CNTs platform for DNA sensor for *N. gonorrhoeae* detection**

CHIT is an interesting polysaccharide biopolymer, relatively inexpensive and is a stable material, has excellent film-forming ability, high water permeability and good adhesion, is susceptible to chemical modifications due to the presence of reactive amino and hydroxyl functional groups and provides hydrophilic environment for the immobilization of desired biomolecules (Jiang et al., 2007; Liao et al., 2005). Chitosan is a non-conducting biopolymer, However, it has an excellent biocompatible property and amino functional groups that favors the immobilization of biomolecules on its surface (Zhang et al., 2002) resulting in the stability of the biorecognition element, immobilized on the electrode surface (Darder et al., 2000).

Recent studies indicate that polymers and redox mediators with or without metal nanoparticles exhibit desirable synergistic effects with CNTs and their composites can exhibit hybrid properties of the components (Wang et al., 2008b). In this context, composites of nanomaterials with CHIT have aroused much interest because of attractive structural, mechanical and electronic properties, including improved electrochemical activity. There is a considerable interest in the application of CNTs because of their unique structure, high electrical conductivity, high chemical stability, and high surface-to-volume ratio (Xu et al., 2006b). These properties make CNTs extremely attractive for fabricating electrochemical DNA biosensors (Kawano et al., 2007).

Electrochemical biosensors combine the sensitivity, as indicated by low detection limits, of electrochemical transducers with the high specificity of biological recognition processes. These devices contain a biological recognition element
(enzymes, proteins, antibodies, nucleic acids, cells, tissues or receptors) that selectively reacts with the target analyte and produces an electrical signal that is related to the concentration of the analyte being studied. Electrochemistry is a surface technique and offers certain advantages for detection in biosensors. It does not depend strongly on the reaction volume, and very small sample volumes can be used for measurement (Ronkainen-Matsuno et al., 2002). Electrochemical detection can be used to achieve low detection limits.

Complementary DNA base pairing is the basis for the recognition process of the hybridization biosensor. Short, 20–40 base pairs single-stranded DNA segments with the ability to selectively bind with target analyte are immobilized on a given electrode surface. The DNA fragments are immobilized at a desired surface. An electrical signal is produced when the target DNA binds to the complementary sequence of the capture or probe DNA via hybridization. An electrochemical signal can result due to an electroactive indicator that binds preferentially to the DNA duplexes instead of single-stranded DNA probes such as ruthenium complex, methylene blue.

This section contains results of the studies relating to electrochemical fabrication of CHIT-CNTs onto ITO coated glass substrate for development of electrochemical DNA biosensor for detection of *N. gonorrhoeae*.

### 3.4.1. Experimental

#### 3.4.1.1. Preparation of CHIT–CNTs composite film

Chitosan solution is prepared in acetate buffer. Chitosan-CNTs composite films are fabricated onto an ITO coated glass plates (0.25 cm²) by electro
polymerization of chitosan in presence of acid treated CNTs (1 mg/ml). The polymerization has been performed chronoamperometrically at 4.0 V for 300 s (via intermittent washing using autoclaved Millipore water after every 300 s of polymerization). Then, the electrode is rinsed with autoclaved Millipore water three times and air-dried. As a result, a robust chitosan film doped with CNTs is formed.

3.4.1.2. Immobilization of biotinylated DNA onto CHIT–CNTs/ITO electrode

Fig 3.21: Schematic preparation of BDNA-Avi-CHIT-CNTs/ITO based DNA hybridization sensor

All *N. gonorrhoeae* DNA solutions are prepared in Tris-EDTA buffer. After electrodeposition, biotin labeled DNA (BDNA) is immobilized onto CHIT-CNTs/ITO electrode using avidin under optimized conditions. 5 µl of avidin (1 mg/mL) (activated using 15 mM EDC and 30 mM NHS for 2 h at 25°C) is immobilized onto CHIT-CNTs/ITO surface. The Avi-CHIT-CNTs/ITO electrode is then washed and
subject to 5 min incubation with 5 μl of 20-mer biotinylated oligonucleotide probes (BDNA, 1 ng/mL) in a humid chamber at 25°C (Fig 3.21). The DNA sequences used for the electrochemical DNA hybridization detection are as follows:

Probe: BDNA or biotin- (GenBank accession no: PUID 9716119 snum 2705)

- Probe DNA: Biotin (BDNA): 5’-CCGGTGCTTCATCACCTTAG-3’
- Complementary target DNA (cDNA): 5’-CTAAGGTGATGAAGCACC GG-3’
- One-base mismatch DNA (obmDNA): 5’-CTAAGGTGATGAAGCACC GG-3’
- Non-complementary DNA (nDNA): 5’-GTATGGTGATCAAGCTCC CG-3’

3.4.1.3. Hybridization of BDNA–Avi–CHIT–CNTs/ITO bioelectrode

The BDNA-Avi-CHIT-CNTs/ITO bioelectrode has been optimized for hybridization time and is subject to incubation in desired concentration (1×10^{-6} M to 1×10^{-17} M) of complementary target oligonucleotide solution for 60 s at 25°C. Subsequently, CV and DPV measurements of BDNA-Avi-Chit-CNTs/ITO electrode have been carried out after 20 μM MB pretreatment at + 0.1 V for 10 s, in 0.05 M PBS at pH 7.0.

3.4.2. Results and discussion

3.4.2.1. Fourier Transform Infrared (FT-IR) Spectroscopic studies

Fig 3.22 shows FT-IR spectra of CHIT/ITO electrode (A), CHIT-CNTs/ITO electrode (B) in the region of 4000 to 500 cm^{-1}. The FT-IR spectra of pure chitosan displays bands at 3200-3400 cm^{-1} due to stretching vibration mode of OH and NH₂ group, 1745 cm^{-1} due to the C=O (C=O in carboxylic acid), Peak of C-O stretching along with N-H deformation mode and NH₂ group due to N–H deformation at 1650 cm^{-1} and 1560 cm^{-1}, respectively. The peak seen at 1460 cm^{-1} due to symmetrical deformation of CH₃ and CH₂ group. Besides this, 1151 cm^{-1} is assigned to the special
broad peak of β (1–4) glucosidic band in polysaccharide unit. Fig 3.22 (B) shows the
peaks after immersion of CNTs into chitosan. The following characteristic peaks were
observed i.e., 3125 to 3370 cm\(^{-1}\) (O-H and N-H stretching); 2923 and 2854 cm\(^{-1}\) (C-H
stretching of CH\(_2\) groups); 1631 cm\(^{-1}\) (C=O stretching of carbonyl group); 1556 cm\(^{-1}\)
(C=C stretching of CNTs).

![FT-IR spectra of (A) CHIT/ITO and (B) CHIT-CNTs/ITO electrode](image)

3.4.2.2. Scanning Electron Microscopic (SEM) studies

The morphological studies on CHIT-CNTs/ITO and BDNA-Avi-CHIT-
CNTs/ITO electrodes have been performed using a Scanning Electron Microscope and
the results are shown in Fig 3.23 (A, B, C). A thin coating of gold is applied on the
samples prior to SEM studies for better resolution of nanostructures. The SEM
micrograph of CHIT-CNTs/ITO surface indicates that the nanotubular structure of
CNTs is embedded into the porous structure of chitosan Fig 3.23 (A). The
homogeneously embedded and finely dispersed CNTs not only provide mechanical
stability of the film to maintain the 3-D porous framework, but also improve the film
conductivity and excellent catalytic property. Furthermore, such 3-D porous film can be
utilized as substrate for DNA immobilization with increased DNA loading and retained
DNA activity as compared to the non-porous planar films. In addition, the robust
structure that is highly porous and permeable to fluids (containing analytes) is likely to result in enhanced sensing capabilities of the CHIT-CNTs/ITO based sensor. As can be seen, many bright/light streaks onto surface of BDNA-Avi-CHIT-CNTs/ITO electrode are attributed to the immobilization of DNA molecules Fig 3.23 (C).

Fig 3.23: SEM images of (A) CHIT-CNTs/ITO electrode at lower magnification (15 kx), (B) CHIT-CNTs/ITO electrode at higher magnification (50 kx) and (C) BDNA-Avi-CHIT-CNTs/ITO electrode

3.4.2.3. Contact Angle (CA) measurements

Contact Angle studies have been carried out to characterize the immobilization of DNA onto the electrode surface Fig 3.24 (A, B). The change in contact angle values is related to the immobilization of DNA molecules. The CA value of the CHIT-CNTs/ITO electrode Fig 3.24 (A) is found to be as 70°, and it decreases to 27° after the immobilization of BDNA Fig 3.24 (B). The decrease in the contact angle values can be attributed to the presence of NH₂ and OH groups in DNA, which helps to lower the CA values. The change in contact angle values after immobilization indicates successful binding of the DNA probes.
Fig 3.24: CA images of (A) CHIT-CNTs/ITO and (B) BDNA-Avi-CHIT-CNTs/ITO electrodes

3.4.2.4. Cyclic Voltammetric (CV) studies

Cyclic Voltammetric characterization has been carried out with CHIT/ITO, CHIT-CNTs/ITO, Avi-CHIT-CNTs/ITO and BDNA-Avi-CHIT-CNTs/ITO electrodes in 0.05M PBS containing 5 mM [Fe(CN)₆]³⁻/⁴⁻ at 50 mV/s using Ag/AgCl as the reference electrode (Fig 3.25 (A)). Chitosan absorbs negatively charged [Fe(CN)₆]³⁻/⁴⁻ through electrostatic adsorption, the drastic increase in peak current of the CNTs modified electrode (254 μA) in comparison to that of the CHIT/ITO (103 μA) electrode, indicates increased electron transfer between the redox couple in bulk solution and electrodes revealing the suitability of the matrix for the biosensor fabrication. It can be seen that there is considerable decrease in the intensity of these redox peaks after the immobilization of avidin confirming the binding of avidin onto the CHIT-CNTs/ITO surface. Further, the observed enhancements in the redox peaks can be attributed to the binding of BDNA onto Avi-CHIT-CNTs/ITO surface.

The CV investigations conducted as a function of scan rate (v) (10–300 mV/s) (Fig 3.25 (B)) onto BDNA-Avi-CHIT–CNTs/ITO and cDNA-BDNA-Avi-CHIT–CNTs/ITO bioelectrodes to determine the concentration of ionic species on these bioelectrodes. Both anodic and cathodic peak currents (Iₚa and Iₚc) are proportional to
the square root of scan rate, \( v^{1/2} \) over the range 10-300 mV/s (Fig 3.25 (B, E)), indicating a diffusion controlled process on the electrodes.

For BDNA-Avi-CHIT–CNTs/ITO bioelectrode, the linear regression equations obtained are as follows:

\[
I_{pa} = 1.3097 \times 10^{-6} v^{1/2} + 4.83618 \times 10^{-6}; \quad R=0.98107; \quad SD=5.04231 \times 10^{-8} \quad \text{Eq. 3.21}
\]

\[
I_{pc} = 5.35952 \times 10^{-7} v^{1/2} + 2.0199 \times 10^{-6}; \quad R=0.98235; \quad SD=1.99102 \times 10^{-8} \quad \text{Eq. 3.22}
\]

For cDNA-BDNA-Avi-CHIT–CNTs/ITO bioelectrode the linear regression equations obtained are as follows:

\[
I_{pa} = 4.16825 \times 10^{-7} v^{1/2} + 1.46613 \times 10^{-6}; \quad R=0.96673; \quad SD=2.14184 \times 10^{-8} \quad \text{Eq. 3.23}
\]

\[
I_{pc} = 1.98026 \times 10^{-7} v^{1/2} + 6.71658 \times 10^{-7}; \quad R=0.96031; \quad SD=1.11474 \times 10^{-8} \quad \text{Eq. 3.24}
\]

where \( R \) is regression coefficient and \( SD \) is standard deviation.

It has been observed that anodic peak potential \( E_{pa} \) for the bioelectrode varies linearly with logarithm of \( v \) and the trend of the variation in the peak potential with scan rate follows the Eq. 3.25, 3.26:

\[
E_{pa} = 0.031 \log v + 0.252; \quad R=0.97949; \quad SD=0.00127 \quad \text{Eq. 3.25}
\]

\[
E_{pa} = 0.059 \log v + 0.290; \quad R=0.99303; \quad SD=0.00218 \quad \text{Eq. 3.26}
\]

Moreover, concentration of redox probe present onto bioelectrode has been calculated using Laviron’s theory, Eq. 3.27, 3.28:

\[
RT/anF= 0.031 \quad \text{Eq. 3.27}
\]

\[
RT/anF= 0.059 \quad \text{Eq. 3.28}
\]
Fig 3.25: (A) Cyclic Voltammetric characterization of (i) CHIT/ITO, (ii) CHIT-CNTs/ITO, (iii) Avi-CHIT-CNTs/ITO and (iv) BDNA-Avi-CHIT-CNTs/ITO electrode at scan rate of 50 mV/s in 0.05 M Phosphate buffer (0.9% NaCl, pH 7.0) containing 5 mM[Fe(CN)₆]³⁻/⁻ (B & E) BDNA-Avi-CHIT-CNTs/ITO and cDNA-BDNA-Avi-CHIT-CNTs/ITO bioelectrodes as a function of scan rate (10 to 300 mV/s) (C & F) show plots of peak current vs \( v^{1/2} \) (V/s) of BDNA-Avi-CHIT-CNTs/ITO and cDNA-BDNA-Avi-CHIT-CNTs/ITO bioelectrodes (D & G) exhibit plots of peak potential vs scan rate \( v \) (V/s) of BDNA-Avi-CHIT-CNTs/ITO and cDNA-BDNA-Avi-CHIT-CNTs/ITO bioelectrodes in 0.05 M PBS containing 20 \( \mu \)M MB

where, \( \alpha \) is the transfer coefficient. The value of \( RT/\alpha nF \) has been used to calculate the total surface concentration of the ionic species (Γ) onto BDNA-Avi-CHIT-CNTs/ITO, cDNA-Avi-CHIT-CNTs/ITO bioelectrodes using Eq. 3.29:

\[
I_p = n^2 F^2 v A (4RT)^{-1}
\]  

...Eq. 3.29

where, \( I_p/v \) can be calculated from the slope of \( i_p \) vs. \( v \) plot.
The surface concentration value of redox species of electrolyte onto the BDNA-Avi-CHIT-CNTs/ITO bioelectrode has been found to be $0.08445 \times 10^{-6}$ mol cm$^{-2}$. The total surface concentration value of redox species of electrolyte is found to be $0.93702 \times 10^{-7}$ mol cm$^{-2}$ indicating hybridization of cDNA onto BDNA-Avi-CHIT-CNTs/ITO electrode.

3.2.2.5. Electrochemical Impedance Spectroscopic (EIS) studies

Fig 3.26 shows results of the EIS measurements carried out as a function of frequency on an electrochemically deposited CHIT/ITO (i), CHIT-CNTs/ITO (ii), Avi-CHIT-CNTs/ITO (iii) and BDNA-Avi-CHIT-CNTs/ITO (iv), electrodes in 0.05 M phosphate buffer saline (PBS) of pH 7.0 containing 5 mM[Fe(CN)$_6$]$^{3/4-}$. The $R_{CT}$ of CHIT-CNTs/ITO electrode (Spectra ii) is found to decrease to 2.50 KΩ with respect to that of the CHIT/ITO electrode ($R_{CT}$~9.52 KΩ, Spectra i) and the lower value of $R_{CT}$ of CHIT-CNTs/ITO electrode reveals nanosized structure of CNTs based CHIT matrix.

![Fig 3.26: Electrochemical Impedimmetric Spectra of (i) CHIT/ITO, (ii) CHIT-CNTs/ITO, (iii) Avi-CHIT-CNTs/ITO and (iv) BDNA-Avi-CHIT-CNTs/ITO electrode in 0.05 M Phosphate buffer (0.9% NaCl, pH 7.0) containing 5 mM[Fe(CN)$_6$]$^{3/4-}$](image-url)
However, when biotinylated ssDNA is immobilized onto CHIT–CNTs/ITO electrode, $R_{\text{ct}}$ of the electrode increases to 4.75 KΩ assigned to the presence of electro-negative phosphate skeleton that perhaps prevent $[\text{Fe(CN)}_6]^{3-/-4-}$ ions from reaching the electrode surface for electron transfer during redox reaction. This implies that BDNA is successfully immobilized onto the electrochemically prepared nanostructured CHIT–CNTs/ITO electrode (Spectra iv).

3.4.2.6. Electrochemical response studies

MB can be used as a hybridization redox indicator to distinguish single stranded and double-stranded form of DNA. MB is known to associate with the unpaired nitrogenous bases of single-stranded DNA as compared to the double-stranded DNA. Fig 3.27 (A) describes response of the BDNA-Avi-CHIT-CNTs/ITO electrode after hybridization with complementary target concentration ranging from $1\times10^{-6}$ M to $1\times10^{-17}$ M.

![Graph](image)

**Fig 3.27:** DPV of (A) BDNA–Avi–CHIT-CNTs/ITO after hybridization with cDNA concentration $1\times10^{-17}$ to $1\times10^{-6}$ M, at pulse height of 50 mV and pulse width of 70 ms, after 20 μM MB pretreatment at $+0.1$ V for 10 s in 0.05 M Phosphate buffer of pH 7.0 containing 0.9% NaCl; (B) shows the MB peak height as a function of complementary target DNA concentration.
It can be seen that MB peak height increases with decrease in the complementary DNA concentration indicating enhanced number of double stranded DNA molecules at the surface Fig 3.27 (A). It has been observed that increase in the MB peak with respect to complementary DNA concentration Fig 3.27 (B) follows Eq. 3.30:

\[ I_{\text{comp}} = 900477.6 \left[ 1 / \ln(\text{comp concentration}) \right] - 1.081 \]  

...Eq. 3.30

The observed MB peak of the same height as that for BDNA-Avi-CHIT-CNTs/ITO bioelectrode for complementary DNA concentration \( \leq 1 \times 10^{-17} \) M indicates absence of duplex at the surface. Therefore, it can be concluded that \( 1 \times 10^{-16} \) M is the detection limit. The Fig 3.27 (B) shows plot of the complementary DNA concentration as a function of the MB peak current. The variation of the MB peak current has been found to obey Eq. 3.30 (Fig 3.27(B)).

3.4.2.7. Specificity studies

Fig 3.28 describes results of specificity studies of the electrode using DPV for detection of complementary target, non-complementary and one-base mismatch synthetic oligomers using BDNA-Avi-CHIT-CNTs/ITO bioelectrode by monitoring the redox activity of MB. It can be seen that MB peak seen at \(-250\) mV is almost negligible after hybridization with complementary target suggesting presence of duplex DNA at the electrode surface. The presence of the MB peak of almost same height as that for BDNA-Avi-CHIT-CNTs/ITO bioelectrode has been observed after incubation with nDNA and obmDNA synthetic oligomers indicating absence of duplex at the surface.
Fig 3.28: Differential pulse voltammograms of (i) ITO in MB, (ii) BDNA-Avi-CHIT-CNTs/ITO, (iii) cDNA-BDNA-Avi-CHIT-CNTs/ITO, (iv) nDNA-BDNA-Avi-CHIT-CNTs/ITO and (v) obmDNA-BDNA-Avi-CHIT-CNTs/ITO electrode at pulse height of 50 mV and pulse width of 70 ms, after 20 μM MB pretreatment at + 0.1 V for 10 s in 0.05 M Phosphate buffer of pH 7.0 containing 0.9% NaCl

Table 3.7 summarizes the biosensing characteristics obtained for electrochemically deposited BDNA-Avi-CHIT-CNTs/ITO along with those reported in literature for *N. gonorrhoeae* detection.

Table 3.7: Biosensing characteristics obtained for electrochemically deposited BDNA-Avi-CHIT-CNTs/ITO along with those reported in literature for *N. gonorrhoeae* detection

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Electrode</th>
<th>Method of immobilization</th>
<th>Detection limit</th>
<th>Hybridization time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CHIT/Pt</td>
<td>Tight DNA-CHIT complex</td>
<td>50.0 nmol/L</td>
<td></td>
<td>Xu et al., 2001b</td>
</tr>
<tr>
<td>2.</td>
<td>Ferrocene (Fc)-capped Au NP/streptavidin conjugates onto Au electrode</td>
<td>Thiol-gold</td>
<td>2 pM</td>
<td></td>
<td>Baca et al., 2004</td>
</tr>
<tr>
<td>3.</td>
<td>Au electrode</td>
<td>Thiol-gold</td>
<td>$9 \times 10^{-11}$ M</td>
<td>30 min</td>
<td>Zhang et al., 2008b</td>
</tr>
<tr>
<td>4.</td>
<td>ZrO$_2$/Au</td>
<td>Phosphate-ZrO$_2$</td>
<td>$1.0 \times 10^{-10}$ mol l</td>
<td></td>
<td>Zhu et al., 2004</td>
</tr>
<tr>
<td>5.</td>
<td>Chitosan-CNTs/ITO electrode</td>
<td>Avidin-avidin coupling</td>
<td>$1 \times 10^{-16}$ M</td>
<td>60 s</td>
<td>Present work</td>
</tr>
</tbody>
</table>
3.4.3. **Summary**

The electrochemical technique has been successfully employed to fabricate films of CHIT-CNTs onto ITO coated glass substrates. The morphological studies reveal that the nanotubular structure of CNTs is embedded into the porous structure of chitosan. The homogeneously embedded and finely dispersed CNTs not only provide mechanical stability of the film to maintain the three-dimensional porous framework, but also improve the film conductivity and excellent catalytic property. Probe DNA specific for *N. gonorrhoeae* has been covalently immobilized onto electrochemically deposited CHIT-CNTs films using avidin-biotin coupling. These CHIT-CNTs nanocomposite films (BDNA-Avi-CHIT-CNTs/ITO) exhibit linear range from $1 \times 10^{-17}$ M to $1 \times 10^{-6}$ M, response time as 60 s, stability 4 months when stored at 4°C and detection limit of $1 \times 10^{-16}$ M.

3.5. **Conclusions**

The biosensing properties obtained for CHIT, CHIT-nFe$_3$O$_4$ and CHIT-CNTs are given in Table 3.8.

Table 3.8: Comparison of biosensing parameters of electrochemically deposited CHIT, CHIT-nFe$_3$O$_4$ and CHIT-CNTs for *N. gonorrhoeae* detection

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Biosensing characteristics</th>
<th>Technique used</th>
<th>Detection range (M)</th>
<th>Detection limit (M)</th>
<th>Stability (Months)</th>
<th>Reusability (Times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemically deposited CHIT</td>
<td></td>
<td>DPV</td>
<td>$1 \times 10^{-9}$ to $1 \times 10^{-6}$</td>
<td>$1 \times 10^{-9}$</td>
<td>2</td>
<td>5-6</td>
</tr>
<tr>
<td>Electrochemically deposited CHIT-nFe$_3$O$_4$</td>
<td></td>
<td>DPV</td>
<td>$1 \times 10^{-16}$ to $1 \times 10^{-6}$</td>
<td>$1 \times 10^{-15}$</td>
<td>6</td>
<td>6-8</td>
</tr>
<tr>
<td>Electrochemically deposited CHIT-CNTs</td>
<td></td>
<td>DPV</td>
<td>$1 \times 10^{-17}$ to $1 \times 10^{-6}$</td>
<td>$1 \times 10^{-16}$</td>
<td>4</td>
<td>10-12</td>
</tr>
</tbody>
</table>

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It has been shown that the electrochemically deposited CHIT-CNTs can be used to detect complementary target DNA in a wide concentration range with better detection limit of $1 \times 10^{-16}$ M and higher re-usability.

Inspite of being an interesting matrix for biomolecule immobilization, non-conducting CHIT suffers from disadvantages such as poor electrochemical nature and also the density of CHIT solution changes with time. Moreover, its mechanical properties are not good enough. To overcome these problems, nanostructured conducting polymers, owing to their small size, exhibit unique chemical, physical and mechanical properties e.g., high surface-to volume-ratio, enhanced surface reactivity, better processibility and superior electrochemical behaviour have been utilized for detection of *N. gonorrhoeae*. These exclusive properties of conducting polymers offer excellent prospects for interfacing biological recognition events with electronic signal transaction for designing new generation bioelectronic devices. Among the various conducting polymers, nanostructured polyaniline (nsPANI) is one of the most promising electroactive material, due to its controllable electrical conductivity, unique redox tunability, good environmental stability, low cost and ease of synthesis.

Chapter 4 contains results of the various studies relating to the development of conducting polymer (nsPANI) based DNA sensor for *N. gonorrhoeae* detection.

The results of the studies discussed in present section have been published in "Thin Solid Films, 519, 2010, 1135–1140".