2.1. Introduction

This Chapter describes the experimental methods and the chemicals used in the synthesis and functionalization of nanoparticles, their bioconjugates, electrode and bioelectrodes for the development of immunosensor for AFB1. Also, various characterization techniques used to characterize the nanoparticles; bioconjugates, electrode, and bioelectrode are discussed. The procedures and protocols related to the performance of the immunosensor and optimization of antibody and antibody conjugate concentrations are also described.

2.2 Materials

2.2.1. Chemicals and reagents

The chemicals and reagents used during the different experiments such as, sodium phosphate monobasic (NaH$_2$PO$_4$ Catalog No. S5011), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC, C$_8$H$_{17}$N$_3$. Catalog No. E7750), N-hydroxysuccinimide (NHS, C$_4$H$_5$NO$_3$ Catalog No. 56480), 4 amino thiophenol (4-ATP, C$_6$H$_7$NS, Catalog No. 22967), hexane dithiol (HDT, C$_6$H$_{14}$S$_2$, Catalog No. H12005), cysteamine hydrochloride (C$_2$H$_8$NSCl, Catalog No. 30078), aurochloric acid (HAuCl$_4$ Catalog No. 254169), trisodium citrate (C$_6$H$_5$O$_7$Na$_3$, Catalog No.), ferrous chloride hexahydrate (FeCl$_2$.6H$_2$O Catalog No. 44939), ferric chloride tetrahydrate (FeCl$_3$.4H$_2$O Catalog No. F2877), and sodium hydroxide (NaOH, Catalog No. 221465) were purchased from Sigma-Aldrich. Ethanol (CH$_3$CH$_2$OH, Catalog No. 22931) Sodium phosphate dibasic (Na$_2$HPO$_4$ Catalog No. A11817) was purchased from Alfa Aesar.
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Potassium ferrocyanide (K₄[Fe(CN)₆], Catalog No. 19525), Potassium ferricyanide (K₃[Fe(CN)₆] Catalog No. 26665), Hydrochloric acid (HCl, Catalog No. 29505), Sodium chloride (NaCl, Catalog No. 43935), Methanol (CH₃OH, Catalog No. 43606), Acetone (CH₃COCH₃, Catalog No. 44106) were from Qualigen Glaxo India Ltd. Hydrogen peroxide 30% (H₂O₂, Catalog No. CH3C630449), was from Merck India and sulphuric acid (H₂SO₄, Catalog No. 339741) was purchased from Sigma-Aldrich.

Biomaterials like monoclonal anti-aflatoxin B1 antibody (aAFB1, Catalog No. A8679), polyclonal IgG antibodies from rabbit (r-IgG Catalog No. A0545 ), aflatoxin B1 (AFB1, Catalog No. A6636), ochratoxin A (OTA, Catalog No.O1877) Bovine serum albumin (BSA, Catalog No. A9647), were procured from Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification.

Whatman Nylon membrane was used to obtained extract from corn flakes. De-ionized water (resistivity ~ 18MΩ cm) from Millipore direct Q3 purification system, was used for the preparation of desired aqueous solutions. The gold coated (diameter: 6.7 mm) quartz resonator (AT-cut quartz crystal, 13.7 mm diameter, 6 MHz) was procured from Autolabs, Netherlands.

2.3. Characterization techniques

The synthesized nanoparticles, fabricated electrodes and bioelectrodes were characterized using various techniques such as ultraviolet-visible spectroscopy (UV-vis), dynamic light scattering (DLS), scanning electron microscopy (SEM), atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy, vibrating sample magnetometer (VSM), transmission electron microscopy (TEM), X-ray
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diffractometer (XRD), quartz crystal microbalance (QCM) and
electrochemical quartz crystal microbalance (EQCM). And comparative
studies were carried out using liquid chromatography- mass spectroscopy/
mass spectroscopy (LC-MS/MS).

2.3.1. Ultraviolet-visible (UV-vis.) Spectroscopy

In spectroscopic studies, certain waves of electromagnetic spectrum fall on
material and absorption or transmittance or both phenomena may happen
within the seconds without destroying the materials. The different regions of
electromagnetic radiations have been used for the analysis of the spatial
arrangement of an atom in a molecule and the presence or position of the
certain organic functional group in a given compound. UV-visible ranges
consist of very small part of the electromagnetic region, which is from 180 –
400 nm for ultraviolet range and 400-780nm for visible range. In
ultraviolet/visible spectroscopy, when the photon of UV-vis light falls on the
molecule, a fraction of light energy is absorbed by the molecule depending
on its the electronic structure and the electrons are promoted lower energy
state to the higher energy state. The detector of spectrometer records the
degree of absorption by a sample at a different wavelength and a spectrum is
produced by plotting absorbance (A) versus wavelength (\(\lambda\)). The absorption
band provides two important information:

- The energy difference between electronic levels determines the band
  position
- And the intensity that depends on both the extent of interaction
  between the radiation with the electronic system and the energy
difference between the ground and excited states.
In UV spectroscopy, molecule undergoes electronic transition involving $\sigma$, $\pi$ and $n$ electrons. The $\sigma$ to $\sigma^*$ transition requires high energy wavelength while $\pi$ to $\pi^*$ and $n$ to $\pi^*$ transition requires UV-vis light. Thus UV-vis absorption phenomenon occurs due to $\pi$ to $\pi^*$ and $n$ to $\pi^*$ electron transition. For example, the UV-vis spectrum of tetraphenylcyclopentadienone shows both $\pi$ to $\pi^*$ and $n$ to $\pi^*$ transition at 343 nm and 512 nm, respectively (Figure 2.1). The non-bonding electrons present over the oxygen atom of the carbonyl group of tetraphenylcyclopentadienone, excite to the $\pi^*$ orbital. UV-vis spectra are plotted between the absorbance ($A$) and transmittance ($T$) versus wave number ($\lambda$) in cm$^{-1}$.

![Figure 2.1: UV-vis spectrum of tetraphenylcyclopentadienone](image)

The optical properties of nanoparticles could measure the size, shape, concentration, agglomeration state, and refractive index near the nanoparticle surface. Metal nanoparticles (ex. gold and silver) have unique properties of surface plasmon oscillation of free electron in the visible region.
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(1). The optical properties of nanoparticles change when particles aggregate; the conduction electron delocalized amongst neighboring particles due to which surface Plasmon resonance shift to longer wavelength. UV-Vis spectroscopy is a valuable tool for identifying, characterizing, and studying these materials. In the present study, the nanoparticles {gold nanoparticles (AuNPs), iron oxide nanoparticles (Fe₃O₄NP) (nanoparticles in citrate buffer) and Au-Fe₃O₄ core shell NP} were characterized by UV-visible spectroscopy (Shimadzu Model 1800 A). The formation of secondary antibody- nanoparticles conjugates was confirmed using UV-visible absorption study.

**Sample preparation:**

The experiment was performed in a solution phase after dispersion in water and measurement was repeated in triplet set using water as the reference solution. **Precautions:**

- To eliminate the solvent interference, the background spectrum should be taken prior to the sample investigation.

- Cuvettes should be cleaned properly before taking the UV-vis spectrum.

2.3.2. **Fourier Transform Infrared Spectroscopy (FT-IR)**

FT-IR is a sensitive technique that can be used for the identification of organic compound and some inorganic molecule also (2-5). The IR spectroscopy is a study of the interaction of molecules, and IR light; and the properties of the molecule determine the nature of the interaction. When radiation passes through a sample (solid, liquid, gas), some frequencies of
the radiation are absorbed by the molecules of the substrate leading to the molecular vibrations. IR radiation does not have sufficient energy to excite the electron however it causes atomic vibration about the covalent bonds. Upon interaction of the infrared radiation with a molecule undergoing a change in dipole the infrared absorption occurs, and when the incoming photon has sufficient energy for the transition to the next allowed vibrational energy states. Absorption in the infrared region results in the excitation of bond deformation, either stretching or bending. Various stretching and bending vibrations occurs at the certain quantized frequency. When the infrared light of that frequency is incident on the molecule, the amplitude of that vibration is increased energy absorption. Depending upon the frequencies of molecular vibration and the IR radiation absorbed, an infrared spectrum is obtained.

The frequency of absorbed radiation is unique for each molecule that provides the characteristics of a molecule. For example, butanoic acid contains carbonyl and alcoholic group. FT-IR spectrum of butanoic acid (Figure 2.2) exhibits the band at 1750 cm\(^{-1}\) for C=O stretching and 3230 cm\(^{-1}\) for OH stretching.
Figure 2.2: FT-IR spectra of butanoic acid

In the present thesis, FT-IR technique has been used to investigate the change in various functional groups during the fabrication of electrodes and bio-electrodes (Chapter-III to V). FT-IR analysis reveals the presence of AuNPs, functionalization of AuNPs over the electrode surface and immobilization of antibodies (Chapter-IV). FT-IR spectrum of the samples were carried out by Perkin-Elmer Instrument; Model 2000. Each sample was recorded over 64 scans with wave number ranging from 400-4000 cm\(^{-1}\) and resolution of 1 cm\(^{-1}\). For FT-IR analysis in each case freshly prepared electrodes were used. The performance of IR spectrophotometer was
checked by recording spectra of reference vs. material wherein; the obtained zero line spectra confirm the good performance of spectrophotometer.

**Precautions:**

- Prior to the experiment, IR chamber should be flushed with nitrogen to avoid interference of CO\(_2\) and water.
- The sample should be sufficiently dried to minimize the interference from water molecules.
- The sample spectra should be taken after recording the fresh background.

### 2.3.3. Raman Spectroscopy

The principle of the Raman spectroscopy revolves around the inelastic scattering of monochromatic light, originated from a laser source. The frequency of monochromatic light changes upon interaction with a sample, it may be up (anti-stoke line) or down (stoke line) from the incident light that is called Raman effect. This shift provides information about vibrational, rotational and other low-frequency transitions in molecules. The properties of either solid, liquid or gaseous samples can be studied using Raman spectroscopy. The molecular deformations in the given electric field \(E\) determined by molecular polarizability, \(\alpha\) determine the Raman effect. The electric dipole moment \(P = \alpha E\) deforms the molecules and is induced upon interaction with the samples (6-8).

In the present study, Raman spectroscopy (Varian-FT Raman spectrometer series II) was studied to confirm self-assembled monolayer layer of HDT on Au electrode to fabricate HDT/Au electrode and compared with spectra of
dithiol in solution form (Chapter IV). Also, the Raman spectroscopy was employed to characterize the multilayer fabrication of aAFB1/Cys/AuNPs/HDT/Au immunoelectrode at each step to confirm the fabrication of AuNPs, Cys and aAFB1 antibodies over the electrode. (Chapter IV).

**Sample Preparation**

1. A very dilute solution (4mM) of dithiol in absolute fresh ethanol was employed for Raman spectra and fresh ethanol used as a reference for baseline correction to avoid the noise.

2. For the electrode form, the electrodes are used for Raman analysis.

**Precautions:**

- The instrument should be calibrated with reference material before sample analysis.

- The sample should be Raman active material for accurate measurement.

**2.3.4. X-ray Diffraction (XRD) Technique**

Wilhelm Conrad Rontgen discovered x-ray in 1895. After that in 1912 Max von Laue described the three-dimensional diffraction of the crystalline substance.

In solid materials particularly in crystals, atoms are arranged in a regular pattern, the smallest volume of the atom is called unit that repeat into the whole crystal. This pattern of the atom can be identified with the help of X-
The interatomic distance in crystals and molecules varies from 0.15-0.4 nm that corresponds to the wavelength of X-rays having photon energies between 3 and 8 keV.

The patterning of atoms in each identical crystal should be same. Thus, the x-ray diffraction pattern of a pure substance is considered as a fingerprint of a crystalline molecule. The X-ray diffraction technique can be used to characterize and identify polycrystalline phases of crystalline materials. The peaks areas are related to the amount of each phase present in the sample. When X-ray falls on atom or molecule the electron wave moves in phase, and there will be well defined x-ray beams in various directions, all are collected from collector and pass on to the detector. A beam that is composed of a large number of scattered rays is called as a diffracted beam that equally reinforces one another. The cathode ray tube is the instrument that is required for the generation of X-rays. The X-rays produce monochromatic radiation upon filtration. The X-rays get concentrated and directed towards the sample. This interaction of the incident beam with the sample produces constructive interference (and a diffracted ray) follows the Bragg's Law

\[ 2d \sin \theta = n\lambda \]  \hspace{1cm} \text{Eq. 2.1}

Where, \( \lambda \) is the wavelength of radiation, \( \theta \) is the diffraction angle, and \( d \) is the lattice spacing in a crystalline sample. The possible diffraction directions of the lattice are attained due to the random orientation of the powdered material by scanning the sample through a range of \( 2\theta \) angles.
(I) Crystallite size

P. Scherrer (9) who evaluated the interdependence of the mean crystallite dimension $t$ and the line broadening $B$, which is known as the Scherrer equation (Eq. 2.2), first treated this effect of particle size broadening:

$$d = \frac{k\lambda}{\beta \cos \theta_B}$$

Eq. 2.2

$$t = 0.9\lambda / \beta \cos \theta_B$$

Eq. 2.3

Where $d$ is the grain size, $\beta$ is the full-width in radians at half maximum of the observed peak, from which the instrumental broadening as well as broadening due to sample strain, has to be subtracted. The factor $k$ is called the shape factor and depends on the crystal structure. For cubic structure, its value is about 0.9. In all XRD patterns for all the samples, the total line width according to this equation as a function of crystallite size is calculated for a fixed value.

(II) Lattice Constant

Lattice constant $a$ was calculated from the $d$ values of the most intense peaks using the formula (Eq. 2.4):

$$a = d_{hkl} \sqrt{h^2 + k^2 + l^2}$$

Eq. 2.4

Where, $h, k, l$ is the Miller indices corresponding to different planes.
The XRD analysis of samples (Fe$_3$O$_4$ and Au -Fe$_3$O$_4$) was done in the powdered form. We have used XRD for structural analysis of Fe$_3$O$_4$ and Au -Fe$_3$O$_4$ (Chapter V). XRD measurements have been carried out using Bruker AXS, X-RD with Cu Kα radiation ($\lambda=1.54$ Å). The voltage and the current of X-ray tube were 40 KV and 40 mA, respectively. The scanning was done by step scanning with a step sized of 0.01 steps and the integration time of 8s per step to get noise free XRD graph.

**Sample Preparation**

Fe$_3$O$_4$ and Au -Fe$_3$O$_4$ nanoparticles were synthesized via co-precipitation method described in Chapter V. We have taken fine powdered form for the XRD analysis.

**Precautions:**

- The powder XRD, there should be an adequate quantity of sample so that the perfect diffraction is achieved.
2.3.5. Scanning Electron Microscopy

SEM is an important tool for surface morphology study at a very fine scale by using highly energetic electrons. In a scanning electron microscope, the sample is exposed to an electron beam from an electron gun (Figure 2.4a), which rapidly moves over or scans the surface of the sample. The intensity of the electrons may vary according to the surface shape and chemical composition. These released electrons are secondary electrons that are collected by a detector and are responsible for generating the electronic signals. An image is produced after these electronic signals are scanned by the cathode ray tube (CRT). The CRT captures and records the image. This phenomenon can be compared with the modern day recording of photographs by the digital camera. The structures of microscopic objects can be observed with this type of microscope. Varieties of signals are produced by accelerated electron-sample interactions. These signals include secondary electrons as well as backscattered electrons, diffracted backscattered electrons, photons (characteristic-rays used for elemental analysis), visible light (cathode luminescence-CL), and heat. Secondary electrons and backscattered electrons are commonly used for imaging samples: Here, X-ray photon detect with X-ray spectrophotometer, an attached unit with SEM called Energy dispersive X-ray spectroscopy is also used for the elemental analysis of the samples. The increase in corresponding —energy slot— analyzed by analyzer and shows on a monitor display. The location of the slot is proportional to the energy of the X-ray photon entering the detector. The detector receives the histogram plot of the X-ray energy, each peaks heights of the element are proportional to the amount of a particular element in the sample being analyzed.
Figure 2.4: Shows the (a) A schematic presentation of various component of SEM and (b) SEM instrument
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In the present work, SEM instrument, model **ZEISS, Model EVO-18 (Figure 2.4b)** with magnification in the range **46 X to 100,000 X** and resolution of **2-50 nm**, was utilized to investigate the surface morphology of 4-ATP/Au electrode, antibody immobilized electrode surface (aAFB1/4-ATP/Au), antigen-antibody interacted surface of electrode (AFB1/BSA/aAFB1/4-ATP/Au). (**Chapter-III and Chapter IV**). SEM studies were carried out for AuNP decorated HDT/Au electrodes and immunoelectrode (aAFB1/Cys/AuNP/HDT/Au) (**Chapter-IV**). SEM was employed to confirm sandwiched form of immunoelectrode (r-IgG-Au-Fe$_3$O$_4$/AFB1/BSA/aAFB/4-ATP/Au) after interaction with secondary antibody conjugate (**Chapter-V**) and also to recognize the change in the surface morphology of Au-Fe$_3$O$_4$ and Fe$_3$O$_4$ nanoparticles (**Chapter-V**). The elemental analysis of Fe$_3$O$_4$ and Au - Fe$_3$O$_4$ nanoparticles were carried out with SEM-EDX (**Chapter-V**).

**Sample Preparation**

- SEM analysis of electrodes and bioelectrodes were taken. For nanoparticles: Au-Fe$_3$O$_4$ and Fe$_3$O$_4$ NP were dispersed in deionized water and spread the equal amount over the glass electrodes and dried under N$_2$ stream at room temperature.

**Precautions:**

- A few Å thick conductive gold coating should be done by sputtering before analysis to have better resolution.

- The dimension of the sample should be ~ 0.5 cm × 0.5 cm.
2.3.6. Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) is a unique type of scanning probe microscopy (SPM) with high resolution (Few microns to below 10 Angstroms) and used to obtain topography and morphology of sample surface. In AFM, a very fine tip is scanned across the surface of the sample to measure the morphology and properties to construct a 3D image of the surface. When a tip comes close to the sample surface, vertical and horizontal deflection of the cantilever occurred due to the atomic force of sample acting on the tip. Laser detects this deflection of the cantilever onto a distant photodetector (Figure 2.5a). Force is calculated by measuring the stiffness of the cantilever using Hooke’s law. Tip moves over the sample by a scanner, typically as a piezoelectric element. The high-resolution capacity of the AFM depends upon the combinations of the ultrasensitive optical lever, sharp tip, and the scanner movement by the precise control of probe-sample forces. The microfabricated silicon (Si) or silicon nitride (Si$_3$N$_4$) cantilevers with integrated tips are commonly used for probes.

AFM can be operated in many modes depending on the application (10). In general, possible imaging modes are divided into static (also called Contact) modes and a variety of dynamic (or non-contact) modes. In contact-mode the tip scans sample at the surface with the constant force. The height of the tip is fixed while in the constant height mode. The motion of the scanner in z direction is recorded and the deflection of the cantilever is fixed in the constant-force mode. The —atomic resolution| images are produced in contact-mode AFM. Silicon nitride tips are used for contact mode. In the non-contact mode, the tip placed at a distance from sample, oscillated at the resonance frequency to keep the amplitude of the oscillation constant. The tips mainly used for this mode are silicon probes.
In the present work, AFM analysis was conducted to examine the surface topography of fabricated electrode and bioelectrode based on SAM, nanoparticles and antibody immobilized surface (Chapter III, IV, V). AFM images were taken in air using a silicon cantilever operated in a non-contact mode using Park Systems, Model XE-70. The instrument is kept in vibration less surface to minimize the false signal from disturbance.

**Precautions:**

- Considering the surface topography of the sample, imaging mode should be appropriately selected.
- To avoid the false signal from contamination, the samples are cleaned and dried before taking the image.
- A smooth surface is required.
2.3.7. Transmission electron microscopy (TEM)

In TEM, high energy electron beam is transmitted through a thin sample to image and analyze material at atomic scale resolution. In TEM, electrons are focused with electromagnetic lenses and the image is observed on a fluorescent screen or digital camera and displayed in real time on a monitor. A transmission electron microscope consists of (Figure 2.6a) (1) two or three condenser lenses for focusing the electron beam on the sample, (2) an objective lens for diffraction of beam at back focal plane and for image of the sample at image plane, (3) some intermediate lenses in order to magnify the image or the diffraction pattern on the screen. The TEM has the advantage over the SEM to determine the positions of atoms within materials which has made an indispensable tool for research and development in many fields.
The TEM has been utilized for the shape, size and structural investigation of the Fe$_3$O$_4$ and Au Fe$_3$O$_4$ (Chapter V). For this, a small drop of a well-dispersed solution of the desired material has been put onto a carbon coated copper grid of 3.05 mm in diameter, followed by the solvent evaporation at room temperature. The TEM images were taken from JEOL JEM (Model 1200F) (Figure 2.6b) with a field emission gun (FEG) source at 300 kV. The HRTEM had TEM point resolution as 0.205 nm.

Precautions:

- A selection of the solvent for TEM analysis should be in such a way that it would neither interfere with the sample nor damage the grid.

- The energy of the electron beam should be optimized as per the need of the sample.

- The thickness of TEM specimen must be approximately 1000 Å or less.
2.3.8. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) also called as Quasi-Elastic Light Scattering (QELS), this technique was used for measuring the size of molecule and particles such as proteins, polymers, and colloids. When a laser light passes through the colloidal solution, it is scattered with the different intensity under the effect of the Brownian motion of particles or molecules in suspension. The change in intensity of fluctuations yields the velocity of the Brownian motion and measure the particle size using the Stokes-Einstein relationship. DLS instrument with the fixed scattered angle (at 90 degrees) determine the limited size range while multi-angle instrument can determine the full particle size distribution. Figure 2.7 shows the schematic diagram of DLS measurement.

Figure 2.7: Schematic diagram of DLS measurements
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DLS technique measures the hydrodynamic radius (diameter of a sphere with translational diffusion coefficient) of particles in solution or suspension form. Z- the average value shows the average particle diameter. While polydispersity index (PDI) is dimensionless, in case of highly monodisperse standards very low PDI (smaller than 0.05) obtained. The PDI of greater than 0.7 indicates that sample has very broad distribution. In the present study, the DLS study of the Fe₃O₄, Fe₃O₄ in the buffer and Au-Fe₃O₄ nanoparticles for measuring the particle size (Chapter V) by the NanoPlus particulate system (Model Nano Plus-1) was carried out. The suspension of nanoparticle was prepared in deionized water. The suspension was sonicated for 15 minutes before measuring the zeta potential.

2.3.9. Vibrating sample magnetometer

Vibrating sample magnetometer (VSM) based on Faraday’s law of induction, in which change in a magnetic field produces an electric field. VSM used to characterize the magnetic behavior of magnetic materials. When a sample is placed in magnetometer at the constant magnetic field, then magnetic domain or magnetic spins align according to the magnetic field. Magnetic dipole moment of the sample which creates a magnetic field around the sample is called stray magnetic field. When the sample placed in magnetometer, this stray magnetic field changes with time and can be measured by pick-up coils. After which according to Faraday's Law of Induction an alternating magnetic field produced an electric field between pick-up coils. This produced current will be proportional to the magnetization of the sample. The greater is the magnetization; the greater is the induced current.
The magnetic property of Fe$_3$O$_4$, Fe$_3$O$_4$ in the buffer and Au-Fe$_3$O$_4$ nanoparticles were studied (Chapter V) with VSM Microsense, ADE-Model EV9 at $17^0$K temperature.

**Sample Preparation**

For magnetic characterization of nanoparticles, I cut a rectangular glass slide of 0.5 x 1.5 cm, and the weight of empty slide was measured. A few drops of nanoparticle solution were drop cast on the slide, dried at room temperature to evaporate the solvent and weight was noted. Thus, a thin film of nanomaterial of fixed weight was deposited on the slide for VSM studies.

**Precautions:**

- Sample size should be same for all.
- Weight of each sample should be identically same

### 2.3.10. Quartz crystal microbalance

Quartz crystal microbalance is based on the principle of the resonator frequency. When voltage is applied to a quartz crystal, oscillation starts at specific frequency, the change in mass on the quartz crystal surface is directly related to the change in frequency of oscillating crystal as shown by Sauerbrey equation *(Eq.2.2)*

$$m = -C \cdot f.$$  \hspace{1cm} ...........Eq. 2.2

where $f$ is the observed frequency change, in Hz, $m$ is the change in mass per unit area, g/cm$^2$, and C is the sensitivity factor for the crystal used (i.e. 0.0815 Hz ng$^{-1}$·cm$^{-2}$ for a 6 MHz AT-cut quartz crystal at 20 °C)
A QCM is a shear mode device, consisting of a thin quartz disk with coated electrodes, where the quartz crystal plate must be cut to a specific orientation with respect to the crystal axes, that is, AT or BT cut so that the acoustic wave propagates perpendicularly to the crystal surface. The resonant frequency of the quartz single crystal depends on the angles of the optical axis. The most commonly used angle is AT cut, that is, $35 \pm 15^\circ$ from the Z axis of the crystal. The temperature dependence of the resonant frequency of AT-cut crystal is essentially zero at $25^\circ C$ as they have low temperature coefficient at room temperature, which causes minimum changes in frequency due to variation in temperature. There is decrease in the frequency of QCM due to the deposition of mass on its surface. The mass sensitivity of QCM is dependent on the thickness of the crystal, which determines its resonant frequency. The thinner is the QCM, the higher is its resonant frequency and sensitivity.

In this present study QCM technique used for the characterization of 4-ATP/Au, aAFB1/4-ATP/Au, BSA/aAFB1/4-ATP/Au, immunoelectrodes and exploited the response of BSA/aAFB1/4-ATP/Au immunoelectrode (Chapter III). The QCM measurement was done with Autolab Potentiostat/Galvanostat Model AUT83945 (PGSTAT302N) at room temperature. The AT-cut quartz crystal (Figure 2.8) of 13.7 mm dia coated with Au (6.7mm dia, $1000 \AA$ thickness) of 6MHz was purchased from Autolab with instrument.
Precautions

- Gold coated quartz crystal should be clean with piranha solution.
- The environment should be clean.
- Surface should be vibration free.

2.3.11. Electrochemical Technique

2.3.11.1 Electrochemical quartz crystal microbalance cyclic voltammetry (EQCM-CV).

Cyclic voltammetry is a potentiodynamic electroanalytical technique that is usually applied to study electrochemical properties of electroactive species. A cyclic voltammogram is obtained when a range of voltage is applied to the solution and change in current is measured with respect to the change in voltage. It is an electrolytic method that uses microelectrodes and an unstirred solution so that analyte diffusion limits the measured current at an electrode surface.
In a CV experiment, current response over a range of potentials (a potential window) was measured. The current increases as the voltage reach the oxidation potential of the analyte, after it falls off as the concentration of the analyte decreases at the electrode surface. At this potential, the direction of the potential scan is reversed, and the same potential window is scanned in the opposite direction to complete a cyclic. This reduction peak has a similar shape as that of an oxidation peak in opposite direction. Non-symmetric peaks are attributed to a quasi-reversible reaction (11, 12). If the process is completely irreversible, the anodic peak does not appear in the measurable potential region. Thus, the cyclic voltammetry infers about the redox potential and nature of electrochemical reactions of a material. The important parameters that can be derived from CV are magnitude of peak current \((I_p)\), peak potential \((E_p)\), number of electrons transferred per reactant molecule \((n)\), rate constant, diffusion coefficient \((D)\) and electrochemical reversibility. The peak current \((I_p)\) for a reversible system is described by the \textbf{Randles-Sevcik equation} (Eq. 2.3) given below (13)

\[
I_p = (2.69 \times 10^5 )n^{3/2}AD^{1/2}Cv^{1/2}\text{ Eq. 2.3}
\]

In the present study, the electrochemical technique with quartz crystal microbalance (EQCM) has been used, and the setup is shown in \textbf{Figure 2.9}. This technique provides us both the electrochemical and frequency response of the electrode. For the electrochemical characterization of prepared electrodes and to study the electrochemical response behavior of the fabricated bioelectrodes (Chapters- III, IV&V) Auto Lab Potentiostat/Galvanostat Model AUT83945 (PGSTAT302N) with Ag/AgCl as reference and a Au electrode as counter electrode was used.
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The CV studies of electrodes (4-ATP/Au, HDT/Au, AuNPs/HDT/Au, Cys/AuNPs/HDT/Au) and bioelectrodes (aAFB1/4-ATP/Au, BSA/aAFB1/4-ATP/Au, aAFB1/Cys/AuNPs/HDT/Au, BSA/aAFB1/Cys/AuNPs/ HDT/Au, AFB1/BSA/aAFB1/4-ATP/Au) (Chapter III, IV and V) were carried out on Au-coated quartz crystal in phosphate buffer saline (PBS, 50mM, pH, 7.4, 0.9% NaCl) containing 5 mM [Fe (CN)$_6$]$^{3-/4-}$ within the potential range of $-0.2$ V to $0.8$ V at $100$ mV/s scan rate.

Figure 2.9: Electrochemical quartz crystal microbalance setup with three electrode system

In addition, the cyclic voltammetric studies of 100 µL solution of nanoparticles (Fe$_3$O$_4$, Fe$_3$O$_4$ in buffer, Au-Fe$_3$O$_4$) and nanoparticles - antibodies (rIgG-Au-Fe$_3$O$_4$) conjugate (Chapter V) were conducted with EQCM-CV (PBS, 50mM, pH, 7.4, 0.9% NaCl) containing 5 mM [Fe (CN)$_6$]$^{3-/4-}$ with the potential range of $-0.2$ V to $0.8$ V at $100$ mV/s scan rate.
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Precautions:

- The selection of the scan rate and the potential range for the CV analysis should be done based on the material.

- All measurements are recorded in a clean environment and under unstirred buffer conditions to minimize the errors.

- Freshly prepared buffers in milli-Q water are utilized for bioelectrode testing.

2.3.12. Liquid chromatography- mass spectroscopy/ mass spectroscopy (LC-MS/MS)

LC-MS is a technique of the combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Liquid chromatography separates the sample components depending on their physicochemical interaction with the column, and the mass spectrometer detects each component as charged ions. LC-MS/MS is a powerful analytical technique employed to detect the components present in a mixture even in the ppm label (ng/L). It also gives information about molecular weight, structure and identify the unknown sample.

LC-MS/MS technique was employ for the detection of AFB1 in contaminated groundnut sample and compares these results with the results obtained from developed aAFB1/4-ATP/Au electrode (Chapter VI). Contaminated samples were analyzed by LC-MS/MS Shimadzu 8030, the separation was performed on a Zorbax Eclipse Plus C18 (3x100mm x 3.5 µm) using a linear gradient of 80% methanol in water with 0.5mm ammonium formate for 10 min at a flow rate of 200µL/min. Tandem mass spectrometry was performed in ESI at a source flow at 800L/h nitrogen at 350 °C.
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2.4. Methods

2.4.1. Solution preparation

2.4.1.1. Preparation of Phosphate saline buffer (PBS)

For the PBS, we have used mono (A) and dibasic (B) solution of sodium phosphate (NaHPO$_4$, Na$_2$HPO$_4$, respectively) in different ratio with the NaCl for different pH of PBS. The ratio of A and B solutions for pH from 6 to 7.8 are given in Table 2.1. 5mM solution of potassium ferrocyanide and potassium ferricyanide was exploited as electrolyte solution (in PBS) for electrochemical measurement.

2.4.1.2. Preparation of antibody and antigen solution

All standard solutions of antibodies, BSA, and antigen, were prepared in 50mM phosphate saline buffer (PBS). 1 mg mL$^{-1}$ anti-AFB1 antibody (aAFB1) solution was prepared in 50 mM phosphate buffer (PBS), 50 mM, pH 7.4 and 0.2 mg mL$^{-1}$ anti-immunoglobulin antibody (r-IgG) solution was prepared in 50 mM PBS (50 mM, pH 7.4). The stock solution of AFB1 was prepared in PBS (50 mM, pH 7.4) with 10% (v/v) methanol and dispensed in different working concentrations and stored at -20 °C. A solution of bovine serum albumin (BSA, 1 mg mL$^{-1}$) was prepared in PBS (50 mM, pH 7.0) and used for blocking the nonspecific binding sites.

2.4.1.3. Preparation of Extraction of food sample

Cereal samples (corn-flakes) were crushed to a powder using a hand-held blender. 2g of powdered cereals were added to methanol: water (7:3, v/v) solution on a sonication bath for 45 min. The extract of the sample was centrifuged for seven minutes at 5000 rpm to remove the solids. The supernatants were collected and allowed to evaporate to dryness under
nitrogen at 25°C. Evaporation was necessary to avoid inhibition of the antibody-antigen binding caused by methanol. The residues were re-suspended in 5 mL PBS and filtered through 0.45 µm Whatman Nylon membranes (14).

The coffee sample was prepared by dissolving 1mg in 1 mL deionized water, and the sample was spiked with different concentration of AFB1 (Chapter III).

*Aspergillus flavus* contaminated groundnut sample was extracted with Strata SAX column (Phenomenex). A 10 g contaminated groundnut was ground into a steel grinder (domestic electrical grinder), followed by the sonication with 50 mL methanol : water (ratio 7:3 v/v), 20 mL hexane, and 0.4 g of NaCl in a falcon tube for 30 mins and centrifuged at 10000 rpm for 5 mins. After that supernatant was transferred to a 100mg Strata SAX column (Phenomenex) for extraction of AFB1. Sequentially, the column was conditioned with 1mL methanol and 1mL methanol-water (7:3 v/v) and washed with 3mL methanol-water (7:3 v/v) and 2mL methanol. Finally, samples were evaporated to dryness under a nitrogen stream and redissolved in 200µL acetonitrile-water (3:7 v/v). The extract was examined by UV-vis spectroscopy and observed an absorption peak at 312 nm for AFB1 (Chapter VI).
2.4.2. Synthesis of gold nanoparticles (AuNPs)

The AuNPs suspension was synthesized by reduction of chloroauric solution (HAuCl₄) with sodium citrate. Briefly, 10 mL of 0.01% HAuCl₄ solution was heated to boil with gentle stirring, followed by the dropwise addition of 1% trisodium citrate solution until the color of the solution changed from yellow to red; continued boiling for an additional 10 min. Then, the solution was cooled with continuous stirring until it attained to room temperature (25°C) and stored at 4°C. The resulting solution was characterized by UV-Vis spectroscopy. For AuNPs, the characteristic absorption peak is obtained approximately at 522 nm (Figure 2.10).

![Uv-vis spectra of AuNPs](image.png)

**Figure 2.10:** Uv-vis spectra of AuNPs
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The synthesis of gold-coated iron oxide (Au-Fe₃O₄) core-shell nanoparticle was accomplished by chemical reduction of iron (II) and iron (III) followed by coating of Au over the Fe₃O₄ nanoparticles detail were mentioned in Chapter V.

2.4.3. Fabrication of immuno electrodes

2.4.3.1. Pretreatment of quartz crystals

The quartz crystals were immersed in 1M NaOH for 5 min and 1M HCl for 2 min in sequence. Later, freshly prepared piranha solution {1:3 (30% v/v) H₂O₂–H₂SO₄} was dropped on the gold surface for 2 min, taking special care to avoid contamination of the electrode leads. The quartz crystals were rinsed twice with deionized water followed by ethanol, and dried under a stream of nitrogen after each pretreatment and then the initial resonance frequency (F₀) was recorded. After cleaning, the quartz crystal was ready for surface modification and antibody immobilization.

2.4.3.2. Fabrication of Self-Assembled Monolayer (SAM) on Au electrode

2.4.3.2.1. Optimization of concentration

For the uniform deposition of SAM layer on Au-coated quartz crystal, the concentration of thiol was first optimized. From the previously reported studies, it was required to use a dilute solution of thiol for uniform and unimolecular deposition. Therefore, thiol solutions of 0.5mM - 4mM in ethanol was utilized. The pretreated quartz crystal was exposed to varied concentration of thiol (0.5mM - 4mM) for 24h at room temperature (25°C) and corresponding change in frequency was monitored at a interval of 6h.

It was observed that optimum decrease in frequency was observed at 2mM concentration for 4- amino thiophenol (4-ATP). Beyond this
concentration, frequency change remains almost unaltered. Prior to the frequency measurement, the crystal was washed with ethanol followed by rinsing with water to remove any unbound 4-ATP molecules.

For the hexane dithiol (HDT), it was observed that optimum decrease in frequency was observed at 4mM concentration. Before the frequency measurement, the crystal was washed with ethanol followed by rinsing with water to remove any unbound HDT molecules. For HDT SAM fabrication, the 4mM concentration of HDT for 24 h was found to be optimum concentration to reach equilibrium.

2.4.3.2.2. Optimization of deposition time for SAM

SAM formation is a spontaneous process. For the 4-ATP SAM fabrication pretreated gold-coated quartz crystal electrode was dipped into 2mM ethanolic solution of 4-ATP and frequency change was measured at the interval of 6 h. After 24 h of incubation, optimum frequency change was observed. Before the measurements, the coated (dipped in ethanol solution) crystal was washed with ethanol followed by rinsing with water to remove any unbound ATP molecules. The Same process was repeated for 3-4 times for reproducibility.

For the HDT SAM deposition, the crystal was dipped into 4mM ethanolic solution of HDT and measured the frequency test at a regular time interval of 6 h. For HDT SAM fabrication, we observed that frequency decreases after 16 h of incubation. The same experiment was repeated for 3-4 times for reproducibility.

Fabrication of multilayered electrode of Cys/AuNP/HDT/Au was completed step by step deposition of each layer of HDT, AuNP, and
cysteamine (Cys). Each step was followed by 3-4 time washing with deionised water of PBS (pH7.4). The detailed study was mentioned in Chapter IV experimental section.

2.4.3.3. Fabrication of anti-aflatoxin antibody

Prior to the fabrication of monoclonal anti-aflatoxin (aAFB1) antibody, these were activated with 0.2M EDC and 0.05M NHS for about 2 h.

2.4.3.3.1. Optimization of aAFB1 concentration for immunosensor

The electrodes were investigated with a variation of the concentration of antibody (aAFB1). Different concentrations of aAFB1 monoclonal antibodies (10–70 μgmL\(^{-1}\)) prepared in phosphate buffer, pH 7.4 were tested with frequency test (Figure 2.11). Maximum frequency change was observed with 40 μgmL\(^{-1}\) after that frequency change remained almost constant. The entire investigation was done with 40 μgmL\(^{-1}\) with an aAFB1 solution.
2.4.3.3.2. Immobilization of aAFB1 antibody

10 μL of 40 μg/mL monoclonal aAFB1 antibodies solution was then spread over electrode surface and incubated for 4 h under humid chamber at room temperature (25 °C) for the amide bond formation between aAFB1 and 4-ATP. The non-specific sites of fabricated aAFB1/4-ATP/Au immunoelectrodes were blocked with BSA (1mgmL⁻¹). Thus, BSA/aAFB1/4-ATP/Au electrodes were utilized for AFB1 detection using frequency and electrochemical techniques (Chapter III). The experimental procedure required washing of QCM crystal 3-4 times with PBS buffer (pH 7.0) and preserved at 4°C while not in use.
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Multilayered BSA/aAFB1/Cys/AuNP/HDT/Au immunoelectrode was fabricated following layer by layer deposition process. The detail results were described in Chapter IV experimental section.

2.4.3.4. Response Study of immunoelectrode

2.4.3.4.1. Optimization of pH of PBS

The t optimized pH required for the immunoreactions varied from 6.0 to 8.0 and examined under frequency test and cyclic voltammetry. At pH 7.4, maximum frequency change and anodic peak current were observed (Figure 2.12) revealing the optimum interaction between antigen and antibody. Therefore, the response study of the immuno-bioelectrode was carried out at optimized pH 7.4.

![Figure 2.12: Effect of pH on antigen-antibody binding](image-url)

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2.4.3.4.2. Optimization of incubation time for antibody-antigen interaction (aAFB1- AFB1)

The incubation time for the interaction between aAFB1 and AFB1 was also monitored with the frequency change (Figure 2.13). It was observed that after 35 min of incubation, optimum change in frequency was observed. Thus, 35 min of incubation time at pH 7.4 PBS was used for the entire experiments.

![Figure 2.13](image)

**Figure 2.13:** Frequency change vs. incubation time plot during the formation of antigen-antibody complex on BSA/aAFB1/4-ATP/ Au immunosensor at pH 7.4 PBS buffer

2.4.3.4.3. Control Study

The controlled study of developed QCM electrodes was conducted prior to the immobilization of antibodies with 5 ngmL$^{-1}$ and 2 ngmL$^{-1}$ of AFB1, respectively, in PBS pH 7.4 containing $[\text{Fe (CN)}_6]^{3/-4}$ using.
frequency test and EQCM-CV to find the effect of electrode material with AFB1. It illustrates that the fabricated electrodes did not show any significant recognition towards AFB1. The 4-ATP/Au nano-electrode was allowed to interact with 2 ngmL$^{-1}$ solutions of AFB1 for 35 min to carry out the control experiment (Figure 2.14). After cleaning with PBS buffer, the frequency measurement of 4-ATP/Au electrode, conducted in PBS buffer (pH 7.4, 0.9% NaCl) shows no significant change in frequency (Figure 2.14 (i)) for 4-ATP/Au electrode. This indicates that the 4-ATP/Au electrode does not show an affinity for AFB1. Similarly, negligible change in current was observed with the EQCM-CV testing of the same electrode (Figure 2.14 (ii)).

![Figure 2.14: Frequency change (i) and EQCM-CV (ii) of control testing of (a) 4-ATP/Au electrode (b) 4-ATP/Au electrode with AFB1 (concentration 2ng mL$^{-1}$) in PBS containing [Fe (CN)$_6$]$^{3/-4}$](image)

Figure 2.14: Frequency change (i) and EQCM-CV (ii) of control testing of (a) 4-ATP/Au electrode (b) 4-ATP/Au electrode with AFB1 (concentration 2ng mL$^{-1}$) in PBS containing [Fe (CN)$_6$]$^{3/-4}$. 
Figure 2.15: EQCM-CV (i) and frequency change (ii) of control testing of (a) Cys/AuNPs/HDT/Au electrode (b) Cys/AuNPs/HDT/Au electrode with AFB1 (concentration 5 ng mL\(^{-1}\)) in PBS containing [Fe (CN)\(_6\)]\(^{3-/4}\).

The surface activity of the Cys/AuNPs/HDT/Au electrode towards AFB1 was tested using 5 ng mL\(^{-1}\) concentration of AFB1 at pH 7.4 PBS for 35 min incubation. Figure 2.15 (i) and (ii) reveal that no significant change is observed in the amplitude of current after the interacting with 5 ng mL\(^{-1}\) concentrations of AFB1, revealing that Cys/AuNPs/HDT/Au electrode surface is non-responsive towards AFB1. The increase in peak potential and broadening of the peak (Figure 2.15 (i)) can be assigned to the insulating nature of AFB1, causing hindrance of the electron transfer towards the electrode. Furthermore, the corresponding frequency change with 5 ng mL\(^{-1}\) antigen solution confirms the identical observation (Figure 2.15 (ii)). It is seen that the fabricated Cys/AuNPs/HDT/Au QCM immunoelectrode does not show any significant recognition towards AFB1.
2.4.4. Determination of linear range, LOD and sensitivity

AFB1 detection was carried out by QCM and EQCM techniques. In QCM technique fabricated immunoelectrodes were allowed to interact with different concentration of AFB1 for 35 minutes in QCM cell containing PBS pH 7.4. The crystal was then rinsed with PBS buffer for 3-4 times to remove the unbound AFB1, and the corresponding frequency changes in Hz were monitored after the interaction of aAFB1 with AFB1. The calibration curve was plotted between the frequency change and concentration of AFB1. The EQCM-CV studies were conducted in a three-electrode cell using modified quartz crystal as the working electrode, gold wire (Au) as the counter electrode, and saturated Ag/AgCl as the reference electrode in the phosphate buffer saline (PBS, 50 mM, pH 7.4, 0.9% NaCl) containing 5 mM [Fe(CN)$_6$]$^{3-/4-}$ as a redox species. The resonant frequency and electrochemical cyclic voltammetry (EQCM-CV) studies of immunoelectrodes were carried out simultaneously in PBS at a scan rate of 100 mV/s in the potential range of $-0.2$ to $0.8$ V. With EQCM CV, calibration curve was plotted between the cathodic current (Ip in ampere) versus concentration (ng mL$^{-1}$) of AFB1. Calibration curve shows the linear range of concentration of analyte that can be detected with the electrode. While the sensitivity of the immunoelectrode shows the change in response of the sensor in per unit concentration of the analyte. Linear detection range and sensitivity of immunoelectrode were calculated by plotting linear regression curve between frequency change Chapter III and IV or current magnitude Chapter III, IV and V with respect to the change in concentration of AFB1. The slope of linear regression curve defines the
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sensitivity of immunoelectrode. The low limit of detection (LOD) of immunosensor was measured by following equation 2.4,

\[
\text{LOD} = \frac{3\sigma_b}{m}
\]  \hspace{1cm} \text{Eq.2.4}

Here, \( m \) is the given slope and \( \sigma_b \) is the standard deviation of the linearity curve.

The association constant (\( K_a \)) was determined with the following Eq.2.5

\[
K_a = \frac{B}{A}
\]  \hspace{1cm} \text{Eq.2.5}

Where, \( B \) is a slope, and \( A \) is intercept of a linear curve plotted against the reciprocal of the \( x \)-axis (concentration of AFB1) and \( y \)-axis (current in ampere).

2.4.5. Repeatability and Reproducibility

The repeatability of developed immunoelectrodes was tested with a set of 3-4 electrodes for each reading and plotted the graph with maximum 3-4 % error. The reproducibility was measured with five successive measurements of the same immunoelectrode with the same concentration of AFB1 using EQCM-CV (Chapter III, IV, and V).

2.4.6. Safety consideration

For the safety concern, every time accessories like gloves, safety glasses, and disposable face mask as safety precautions were used during the experiments. The contaminated glasswares were submerged into a sodium hypochlorite solution (5.25%) for 68 - 72 h before washing and re-using.
2.4.7. Effect of interferents

The effect of interferents was studied with different concentration of ochratoxin A (OTA). The fabricated immunoelectrodes were allowed to interact with different concentration of OTA and fixed concentration of AFB1 in 1:1 ratio for 35 m in QCM cell containing PBS pH 7.4 (Chapter III). The crystal was then rinsed with PBS buffer for 3-4 times to remove unbound AFB1 and measured under EQCM-CV. The same experiment was repeated for 3-4 times.

2.4.8. Real Sample Testing

The real food samples were also tested with immunoelectrode. For this, the corn flakes extract was spiked with different concentrations (0.05, 2, 5 ngmL\(^{-1}\)) of AFB1 (Chapter IV and V) and tested with EQCM-CV in phosphate buffer saline (PBS, 50 mM, pH 7.4, 0.9% NaCl) containing 5 mM \([\text{Fe(CN)}_6]^{3-/4-}\) as a redox species. Initially, these electrodes were interacted with spiked extract and incubated for 35 min.

The spiked coffee sample was tested with different concentration (0.6, 1.2 and 2 ngmL\(^{-1}\)) of AFB1. The spiked coffee sample spread over the immunoelectrode surface and allowed to interact. After the incubation and washing with PBS pH 7.4, the frequency change was measured (Chapter III).

The extracts of two samples of contaminated ground nuts and their 50% diluted samples were examined with the immunosensor (BSA/aAFB1/4-ATP/Au) and the results were validated with LC-MS/MS (Chapter VI).
2.4.9. Shelf life studies

The shelf life of immunoelectrode indicates the stability of the biomolecules; it may be affected by the material utilized for the electrode fabrication and method of immobilization of biomolecules. Shelf life of immunoelectrode was measured using EQCM-CV at the regular interval of 5-7 days for about two months, and the electrode response were measured (Chapters III, IV, and V). 30 sets of immunoelectrodes were prepared and stored at 4°C. The response study of three electrodes were checked after 5-7 days via EQCM-CV in PBS (50mM, pH 7.4, 0.9% NaCl) and the current response was measured in presence of 2 ng/mL standard AFB1 solutions and average was noted. After 5-7 days, the response of another three set of electrodes were measured in the same way and so on.

2.4.10. Regeneration of immunoelectrode

Regeneration process followed by the breaking of antibody-antigen (Ab-Ag) interaction via chemical or physical methods. Between Ab-Ag, an electrostatic force of attraction is applied. In the present study, both chemical and physical (magnetic) regeneration methods were followed. In the chemical regeneration method, immunoelectrode was regenerated by 0.1 molL⁻¹ glycine-HCl buffer solution at pH 2.3 for 10 min to break down the immuno-complex interaction(AFB1-aAFB1) followed by washing with PBS for 3-4 times. The frequency response of the regenerated immunoelectrode (BSA/aAFB1/4-ATP/Au) Chapter III and BSA/aAFB/Cys/AuNP/HDT/Au immunoelectrode Chapter IV was investigated with concentration of AFB1 solution and observed the change in activity of immunoelectrode. After regeneration cycles, the used quartz crystals were rinsed with piranha solution thoroughly to remove all materials.
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absorbed on the gold surface of the crystal. Then the crystal was reused for the preparing SAM on a gold surface.

Magnetic regeneration of r-IgG-Au-Fe\textsubscript{3}O\textsubscript{4}/AFB1/BSA/aAFB1/4-ATP/Au immunosensor can be regenerated using an strong external magnet to remove the immuno r-IgG-Au-Fe3O4 conjugate. The detailed study was mentioned in Chapter V.

2.5 Conclusions

The above described techniques such as UV-Visible spectroscopy, Raman Spectroscopy, Fourier Transform Infra-red Spectroscopy, Scanning Electron Microscopy, X-ray Diffraction, Transmission Electron Microscopy, Atomic Force Microscopy, Zeta potential and vibrating sample magnetometer described in this chapter have been utilized for the structural and morphological analysis of AuNP, Fe\textsubscript{3}O\textsubscript{4} and Au-Fe\textsubscript{3}O\textsubscript{4}, SAM fabricated Au electrode and functionalized immunoelectrodes. Quartz crystal microbalance and electrochemical quartz crystal microbalance-cyclic voltammetry techniques have been utilized to reveal the piezoelectric and electrochemical behavior of electrodes and bioelectrodes. QCM and EQCM-CV techniques were used to study the biosensing response of SAM based immunoelectrodes towards the food toxin (AFB1) detection. The protocols used for the estimation of various parameters relating to the performance of the SAM based immunobiosensor has also been discussed in depth in the present chapter.

Chapter III describes the development of a label-free immunosensor based on a self-assembled monolayer of 4-amino thiol phenol on Au-coated QCM electrode (4-ATP/Au), its characterization, response study and testing on real samples.
2.6. References:


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