CHAPTER 3
MATERIALS AND METHODS

3.1 CHEMICALS AND MATERIALS

3.1.1 Chemicals

The solvents and chemicals were procured from Sisco Research Laboratories (SRL), Merck, and Molychem and are of laboratory and analytical grade. Sodium chloride (NaCl, RANKEM), potassium chloride (KCl, ANALAR), potassium di-hydrogen phosphate (KH₂PO₄, SRL), and sodium hydrogen phosphate (NaHPO₄, ACROS) were procured from Rankem, Analar, SRL, and Acros respectively for preparing PBS (pH 7.2) in double distilled water. Sodium nitrate (NaNO₃), sulfuric acid (H₂SO₄), potassium permanganate (KMnO₄), and hydrogen peroxide (H₂O₂) were procured from Sigma-Aldrich. The PBS was used as a running buffer throughout the whole experiment. GO-coated PCTE nanoporous membrane was used to detect *E. coli* by infrared spectroscopy.

Sulphuric acid ( assay NLT 97.0 %), potassium persulphate GR ( assay 98.0%), phosphorus pentoxide GR ( assay 98%), potassium permanganate ( assay 99%), hydrogen peroxide (30%) and graphite powder were purchased from Rankem, Molychem, Qualigens, Loba Chemie and Aldrich respectively. In addition, Sodium chloride, potassium chloride, sodium hydrogen phosphate, potassium dihydro phosphate, Hydrochloric acid, 1-ethyl-3,3-dimethyl aminopropyl carbodiimide hydrochloride (EDC) (MW = 191.7 g/mol) and N-hydroxysuccinimide (NHS) (115.09 g/mol) were purchased from Sigma-Aldrich.

3.1.2 Materials

A sample of GO was provided by Dr. Rakesh K. Joshi, a fellow at University of Manchester (UK), the concentration of which is 1.6 mg mL⁻¹. This was diluted in doubled distilled water. The gold-coated Polycarbonate Track-Etched (PCTE) membranes were acquired from Whatman (USA). Other, Nuclepore Track-Etch Membrane was also purchased from Whatman (USA) which pore size and diameter were 0.08 µM, and 13 mm, respectively. *E. coli* β-gal antibody and the anti-Pseudomonas antibody were purchased from Bangalore Genein. 2Dm2m CD4 construct (MW = 147.6 kDa, CC = 4 mg.mL⁻¹) (as shown in Figure 3.1a), HIV-1 gp140MS (MW = 140 kDa, CC = 0.3 mg.mL⁻¹) were prepared (as shown in Figure 3.1b and c), purified and characterized following Chen et al 2014 (Chen et al., 2014, 2010). Protein samples were stored in a deep freezer at – 20 °C for long-term storage and to prevent their denaturation. Four positive and two negative human serum samples (Figure 3.2) of HIV were provided by Jindal Institute of Medical Sciences Hospital, Hisar, from the HIV patients and normal patients, respectively. The concentration of serum samples were analyzed using Bradford protein assay method by Multiskan GO microplate spectrophotometer (Thermo Scientific). The protein concentration in human serum sample such as positive sample first, second, third, fourth and negative sample first, second were found to be 46.25 g/L, 46.17 g/L, 75.92 g/L, 60.83 g/L and 74.75 g/L, respectively. The Institutional Biosafety Board of the National Research Centre on Equines (NRCE), India approved the study in the Biosafety Level-3 Laboratory.
Figure 3.1 (a) Shows the 2Dm2m antibody of HIV-1. (b) and (c) Show the Trimer of gp140 (antigen of HIV-1).

Figure 3.2 HIV positive human serum samples (P1, P2, P3, and P4) and negative human serum samples (N1 and N2).

3.2 PREPARATION OF SILVER/SILVER CHLORIDE ELECTRODE

Silver/silver chloride (Ag/AgCl) electrodes were prepared in the laboratory in a variety of shape and size. Firstly, they were cleaned by being swilled abrasive paper and swabbed with ethanol (4 mm side of the square and wide 0.3–0.4 mm as per required). Further, two Ag/AgCl wires were mounted in the beaker filled with the solution of 0.1 M hydrochloric acid, such that the wires immersing in the solution could not come in contact with each other as shown in Figure 3.3. One wire was connected to the positive terminal and another one to the negative terminal with an applied constant voltage of 2V with a maximum current of 10 mA for 30 minutes using interactive source meter 2450. According to this phenomenon, current is admitted to flow the chloride ions towards the positive terminal and hydrogen ions to the negative terminal releasing bubbles of H₂ gas. The chloride was uniformly coated over the surface of the silver wire and appeared as dark grey purple color as shown in Figure 3.4.
3.3 SYNTHESIS OF GO

GO was synthesized according to the modified Hummer’s method using graphite flakes. For this purpose, an approximate quantity (2g) of graphite flake and sodium nitrate (NaNO₃) were mixed with sulfuric acid (H₂SO₄) (100 mL) in an ice bath. Potassium permanganate (KMnO₄) (7.5g) was slowly added to the mixture with constant stirring for 2 hours maintaining the temperature in the range of 8-10°C. 200 mL distilled water and 10 mL of Hydrogen peroxide (H₂O₂) were added to the mixture. The solution was filtered after 12 hrs using a fine filter paper (0.22µm). The reactants washed several times with distilled water. The finally prepared graphite oxide was ultra-sonicated for 24 hrs following centrifugation at 6000 round per minute (rpm) for 10 minutes twice to get few layered GO. The GO sample was properly diluted in deionized water at an aggregated concentration of 0.1 mg mL⁻¹ as shown in Figure 3.5.
3.4 SYNTHESIS OF GO-LAMINATES

Firstly, GO was synthesized by the oxidation of natural graphite powder (GP) according to the modified Hummer’s method (Hu et al., 2010; Marcano et al., 2010). Generally, GP (2.0 g) was accumulated to concentrated sulphuric acid (3.0 ml), potassium persulphate (1.0 g), and phosphorus pentaoxide (1.0 g) at 80 °C for 6 hrs in a hot air oven. In addition, this material was cooled down to room temperature and was subsequently diluted with 200 ml of distilled water. Further, this material was seeped through the filter paper, and purified to remove the residual acid until the pH becomes neutral. This aqueous solution was subjected to desiccation in a dessicator chamber for three days at room temperature. Moreover, the desiccated material known as preoxidized graphite powder (PGP) was subjected to oxidation employing standard Hummer’s method. Typically, the formation of PGP was accompanied by mixing it with concentrated sulphuric acid (23 ml) under constant stirring in ice bath (approx ~ 8 °C) as shown in Figure 3.5b.

Under energetic shaking, potassium permanganate (3 g) was added to maintain the constant temperature of the suspension lower than 20 °C. Hereinafter; the reaction system was relocated to a constantly energetic stirring by the addition of more distilled water (47 ml) at 35 °C for about 2hrs. Distilled water (14 ml) was added again after 15 min and the solution was
stirred for another cycle of 30 min at 35°C. Further, the reaction was terminated by the gentle addition of 2.5 ml of hydrogen peroxide (30%). Finally, the colour of the mixture turned dark brown to yellow. Then, this mixture was filtered using Whatman filter paper and rinsed out with 1:10 solution of hydrochloric acid in order to partially remove the metal ions. The resulting solid was washed and centrifuged at 10,000 rpm for 10 min repeatedly until pH of the distilled water adjusted to neutral. The resulting brown colored GO material was dried at room temperature. The resultant GO (100 mg) powder was diluted in 100 ml distilled water, stirred for 12 hrs and bath sonicated for 8 hrs to exfoliate to single layered GO as shown in Figure 3.6a. The obtained GO dispersion (1 mg.mL\(^{-1}\)) was again centrifuged at 10,000 rpm for 3 min to separate unexfoliated GO from transparent GO solution as shown in Figure 3.6b.

3.5 FABRICATION OF GO COATED NANOSIEVE PLATFORM

![GO sample](image1) ![GO coated PCTE membrane](image2)

**Figure 3.7** (a) Shows the GO- laminates coated PCTE membrane using vacuum filtration assembly. (b) Shows the GO-laminated coated PCTE membrane

GO was prepared by the modified Hummer’s method and brown paste of GO was achieved. The obtained GO (100 mg) was dissolved in 100 mL distilled water and bath sonicated for 8 hours to exfoliate it to GO. Further, GO dispersion was again centrifuged at 10,000 rpm for 3 min to recapture the unexfoliated graphite. The GO dispersion concentration was changed from 1 mg.mL\(^{-1}\) to 0.3 mg.mL\(^{-1}\) (see the Figure 3.6b). This GO dispersion was coated on PCTE membrane using vacuum filtration unit at variable pressure as shown in Figure 3.7a. The flow rate of the first, second, third, fourth, fifth drops of GO was calculated to be 92s, 133s, 160s, 210s, 250s, and so on respectively. Thus, GO-laminated PCTE membrane was obtained in our laboratory (see the Figure 3.7b).

3.6 FABRICATION OF GO COATED NANOSIEVE PLATFORM WITH ANTIBODY (2Dm2m) AND ANTIGEN (gp140\(_{MS}\)).

Moreover, the functionalization method is depicted in Figure 3.8. The functionalized GO-laminates were activated using 100 µl (100 mM) EDC-NHS over 2hrs at room temperature. The unreacted EDC-NHS was removed by rinsing it with deionized water. Subsequently, the 2Dm2m protein (40 µg.mL\(^{-1}\) in PBS) was immobilized on EDC-NHS/GO-
laminated surface for 8 hrs at room temperature. Afterward, the surface was rinsed with PBS solution, finally, the desired 2Dm2m-immobilised GO-laminated nanosieve platform was successfully functionalized and was transferred into the solution chamber as shown in Figure 3.9. 100 µl of gp140MS protein was pipetted dropwise over the nanosieve surface and used for detection of target species. In addition, the nanosieve surface was again rinsed with PBS solution, finally, the desired 2Dm2m-immobilised GO-laminated nanosieve platform was successfully functionalized and was transferred into the solution chamber for positive and negative human serum samples of HIV as shown in Figure 3.10. 100 µl of positive and negative human serum samples were pipetted dropwise over the nanosieve surface and used for detection of target species.

**Figure 3.8** Schematic diagram of (a) EDC-NHS working with GO (b) Immobilization process of the 2Dm2m antibody with linker molecules (EDC-NHS).

**Figure 3.9** Shows the solution chamber with GO-coated PCTE membrane.
3.7 INSTRUMENTATION

3.7.1 UV-Vis Spectrophotometer

Absorption spectra were recorded by the double beam UV-VIS Spectrophotometer UV5704M (Electronics Corporation of India Limited) and with single beam spectrophotometer (Thermo Scientific multi-scan GO microplate reader). The wavelength range was kept at 200–800 nm. 0.1 mg.mL$^{-1}$ and 1 mg.mL$^{-1}$ GO nanocomposites were dispersed in water and ethanol.
with 8 hrs ultra-sonication duration. This solution was then filled with the quartz cuvettes for further measurements. The schematically diagram of UV-Vis spectrophotometer illustrated GO dispersion in the Figure 3.11.

3.7.2 Fourier Transform Infrared Spectroscopy

FTIR is a powerful tool for identifying different types of chemical bonds in a molecule by producing an IR absorption spectrum. An infrared peak shows a fingerprint of a specimen with absorption bands which corresponds to the frequencies of vibrations between the bonds of the atoms of which the material is made. It provides specific information about the vibrations and rotations of the chemical bonds and molecular structures, making it useful for analyzing organic materials and certain inorganic materials. Since each different specimen is an exclusive combination of atoms, no two materials produce the exact same infrared spectrum (Fan et al., 2012; Gorassini et al., 2008; Polovka et al., 2006).

Hence, IR spectroscopy can result in a positive description (qualitative analysis) of different types of samples. Furthermore, the size of the bands in the spectrum is a direct evidence of the amount of sample present. With latest software algorithms, infrared is a superior tool for quantitative and quantitative analysis. Figure 3.12 shows the schematically diagram of FTIR instrument used in our laboratory. The measurement range lies between 400 to 4000 cm\(^{-1}\). The specimen was passed through 64 scans with a resolution of 2.0 cm\(^{-1}\) and the showed data obtained was an average value. Each specimen was analyzed three times and the final spectrum was an average of the three measurements to minimize differences occurred during sample preparation. Furthermore, some FTIR spectra of the membrane were noticed using the install the thin film polystyrene sample labeled “Secondary Polystyrene Standard: 1.5 mil Polystyrene” via the sliding door. Sixteen accumulative scans were noticed.

**Figure 3.12** Shows the schematically diagram of FTIR with GO sample spectrum.

FTIR spectra were recorded using a Thermo Scientific FTIR instrument (Nicolet 6700) in the mid-IR region between 4000 to 400 cm\(^{-1}\) (2.5–25 µm wavelengths) which penetrated the sample with a depth typically in the range of 0.5–5 µm. Attenuated Total Reflection (ATR)-FTIR spectroscopic imaging measurements were taken using a Bruker Equinox spectrometer attached to a Macro chamber (liquid nitrogen cooled) for the gate of Ge (45˚) ATR accessory. Graphite powder and dried GO were mixed with methanol solvent in a.
microcentrifuge tube and then this solution was sonicated using ultrasonication bath at room temperature. Afterward, the mixture was speared onto the surface of Ge crystal at room temperature for 2 min, and the spectrum was recorded by OMNIC software. The schematically diagram of ATR-FTIR spectrometer illustrated GO dispersion in the Figure 3.12.

3.7.3 Field Emission Scanning Electron Microscope

Field emission scanning electron microscope (FESEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons (as shown the schematically diagram of FESEM in Figure 3.13). A FESEM is used to visualize very small topographic details on the surface or entire or fractioned objects. Researchers and co-workers in biology, chemistry, and physics use this technique to analyze the structures that may be as small as one nanometer.

![Schematic Diagram of FESEM Instrument](image)

**Figure 3.13** Shows the schematic diagram of FESEM instrument.

Electrons are released from a field emission source and accelerated in a long electrical field gradient. Within the large vacuum column, these so-called primary electrons are concentrated and deflected by electronic lenses to generate a confined scan beam that bombards the object. As a consequence, secondary electrons are generated from each spot on the object. In this instrument, the angle and velocity of these secondary electrons correlate to the surface design of the object. A detector captures the secondary electrons and generates an electronic signal. The electronic signal is amplified and converted to a video scan-figure that can be seen on a monitor screen or to a digital image that can be saved in our latest desktop and processed further. Normal optimizing parameters for FESEM are: accelerating voltage 10-15kV, beam intensity: 10-12μA, working distance: 8-10mm, Magnification: less than 30,000X for nanomaterials, less than 5,000X for the cementitious system. Figure 3.13 shows the schematic diagram of FESEM system used for morphological studies.

3.7.4 Scanning Electron Microscope
SEM images were obtained using an electron microscope (JEOL) equipped with a thermal field emission emitter and three different detectors. These are EsB detector with filter grid, high-efficiency In-lens SE detector, Everhart-Thornley secondary electron detector. The samples were prepared by casting 20 µL (0.1 mg/ml) aqueous or ethanol suspension of GO composites on the surface of quartz crystal followed by drying in an oven at room temperature to make sure that the solvent was removed. SEM was used to characterize the GO coated over gold surfaces of PCTE membrane. After that, the β-gal antibody was immobilized on the GO-coated surface via EDC-NHS chemistry and finally, the images were recorded.

3.7.5 Thermo Gravimetric/Differential Thermal Analysis

Figure 3.14 Shows the TG/DTA equipment (Make: Perkin Elmer; Model: Diamond (TG/DTA)).

Thermogravimetric/differential thermal analysis (TG/DTA) is an instrument that contains two different techniques TG (Thermogravimetry) and DTA (Differential Thermal Analysis). DTA is a technique, which involves heating a test sample and an inert reference sample under identical conditions and records temperature difference that develops between them. This differential temperature graph is then measured either against time or against the temperature at some fixed points within the instrument. Figure 3.14 presents the TG/DTA apparatus used in our studies. The evolution of heat due to physical or chemical alteration in the target sample will cause the temperature to increase temporarily above that of the reference sample, thus giving rise to an exothermic peak on the DTA plot.

Contrary, a process, which is joined by the absorption of heat, will cause the temperature of the check sample to being left behind that of the reference sample, leading to an endothermic peak. TG is a technique whereby a sample is homogeneously weighted as it is heated to a constant, for preference linear rate. The resulting weight vs. temperature curve gave information on the thermal stability and composition of the real sample, the composition and thermal stability of intermediate compounds and the composition of the residue. The specifications used in TG/DTA analysis are shown in table 3.1.
### Table 3.1 Specifications of TG/DTA

<table>
<thead>
<tr>
<th>Specification</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight Measurement</td>
<td>Horizontal differential balance method</td>
</tr>
<tr>
<td>Sample Weight</td>
<td>Max. 200 mg</td>
</tr>
<tr>
<td>Temperature range</td>
<td>Room temperature ~1500˚C (Normal 1300˚C)</td>
</tr>
<tr>
<td>Heating Range</td>
<td>0.01˚C/min~100.00˚C/min</td>
</tr>
<tr>
<td>TG Measurement Range</td>
<td>± 200mg (0.2µg)</td>
</tr>
<tr>
<td>DTA Measurement Range</td>
<td>± 1000µV (0.06µV)</td>
</tr>
<tr>
<td>DTG Measurement Range</td>
<td>0.5 mg/min ~ 1g/min</td>
</tr>
<tr>
<td>Gas Flow</td>
<td>Max. 200 ml/min</td>
</tr>
</tbody>
</table>

#### 3.7.6 X-ray Diffractometer

![Layered Structure](image)

**Figure 3.15** Shows the powder XRD instrument (Make: Rigaku, Japan Model: Dmax 2200).

X-ray diffraction (XRD) is a recognized methodology. It provides detailed information on nanomaterial chemistry, mineralogical composition and crystallographic structure of materials. It is a versatile and rapid analytical tool for phase identification of a crystalline material and provides information of unit cell dimensions as well. An exclusive spectrum of diffraction angle with respect to the corresponding incident angle is used to identify the material. Implementing the XRD spectra, several compounds in hybrids nanocomposites such as graphene, GO, Co-RGO (Tang et al., 2017), Sr2FeMoO6 (Hu et al., 2017) \((\text{Sr}_{1.55}\text{Gd}_{0.15}\text{Ba}_{0.3})\text{FeMoO}_6\) (Hu et al., 2017), CaCu3Ti4O12 (Xu et al., 2017) and ninhydrin monohydrate (Sasikala et al., 2017) can be identified. The system was operated under an open attenuator run of 40 kV, 40 mA and used a Ni filter at room temperature, with optimized diffraction angle range 5˚-90˚ (2θ). The XRD spectra were acquired rapidly under a rate of 1˚/min. The XRD testing system is shown in Figure 3.15.

#### 3.7.7 Atomic Force Microscopy

The AFM is one of the great resolutions scanning probe microscopy technique that can endow resolution at the nanometer scale. Since the discovery of AFM by Binnig et. al. in 1986 (Feng, 2009), it has been established to be the predominant tool for imaging the surface topography, calculating and manipulating matter at the nanometric scale. The basic AFM working unit contains cantilever with an acute tip, laser, photodiode, detector and data electronics and piezoelectric scanner and is schematically depicted in Figure 3.16. Customarily,
the sample placed on a piezoelectric scanner during the scanning analyses. It can be shifted in the vertical z-direction for keeping a constant force, and in x and y-direction for image scanning. The cantilever tip employs an important role in the AFM system for the scanning of the coated sample surface. The cantilever is commonly silicon or silicon nitride with a tip radius of curvature on the order of nanometers scale. When the cantilever tip is moved into the close proximity of a sample surface, forces between the cantilever tip and the sample brings on a deflection of the cantilever according to Hooke’s Law. A few of the forces that are recorded by AFM contain mechanical contact force, van-der-Waals forces, magnetic forces, Casimir forces, solvation forces, etc. The cantilever deflection is recorded by using a laser spot reflected from the top surface of the cantilever tip into an array of photodiodes into which is connected to the detector. If the cantilever tip is scanned a surface at a constant height, there is feasibility that the tip collides with the surface causing deface to the tip. Thus, in utmost cases, a feedback mechanism is established to control a constant force between the tip and the sample by continuous adaptation of the tip-to-sample distance.

![AFM working unit on GO sample](image)

**Figure 3.16** Schematic diagram of AFM working unit on GO sample.

Generally, the imaging of AFM is working in static and dynamic mode. In static mode, the deflection of the static tip is used as a feedback response. Because the analysis of the static response is prone to noise and drift, low stiffness cantilevers are used to boost the deflection response. Nevertheless, the attractive forces can be quite energetic at closeness to the sample surface, causing the tip to “snap-in” to the surface. Hence, the force between the tip and the surface is kept constant during scanning in contact mode by applying a constant cantilever deflection. In a dynamic process, the cantilever tip is outwardly oscillated at or near to its basic resonance frequency and this oscillation amplitude, phase and resonance frequency are changed by the tip-sample interaction force; these alter in oscillation with respect to the outside reference oscillation endow report about the sample surface characteristics. The Dynamic mode has frequency and amplitude modulations. In case of frequency modulation, the oscillation frequency is modified which endowed information about tip and sample attachments. Generally, stiff cantilever tips are used in this modulation mode and the frequency can be calculated with great sensitivity. In the case of amplitude modulation, changes in the oscillation amplitude endowed the feedback response for imaging. Unlike frequency modulation, alters in the oscillation phase or amplitude are used to probe the tip-sample attachment in amplitude modulation mode.

### 3.7.8 Quartz Crystal Microbalance
Research QCM was purchased from SRS, USA. This apparatus monitors the mass and viscosity of processes occurring within thin films or at surfaces. In this apparatus, quartz crystal, a controller, crystal holder, crystal oscillator electronics, and original Windows software are included. The standard AT-cut (5 MHz), (1 in. 25.4 mm diameter) quartz crystal is generally employed for experiments conducted, RQCM. Here, this instrument was utilized for *E. coli* detection.

### 3.7.9 Electrochemical Measurement

![Electrochemical Measurement setup](image)

*Figure 3.17 Shows the electrochemical biosensor in working condition.*

All electrical measurements were conducted with Keithley 2450 interactive source meter. The measurements used Ag/AgCl electrodes prepared in the laboratory. An L-shaped glass chamber (4 ml volume) was used for (PBS with pH 7.2) electrochemical detection. The alteration current was normalized as $\Delta I/I_0 = (I - I_0)/I_0$, where $I_0$ is the initial ionic current and $I$ is the calculated value in real time, respectively. It is illustrated in Figure 3.17. An electronic pH meter CG 842 (SI Analytics GmbH, www.si-analytics.com) was calibrated with standard buffers of pH 4.00 and pH 7.00 (Sigma Aldrich) at 20-60 (±2)°C. It was used for pH-titrations and for measuring the pH of buffer solutions.