Chapter 2

MATERIALS AND METHODS

2.1 Reagents

The reagents and solvents used were of analytical grade and were procured from local vendors. Double distilled water was used throughout the studies. Multiwalled Carbon nanotube (MWCNT), Nafion, Alumina, Cystamine dihydrochloride and PAM Chloride were purchased from Sigma Aldrich Corporation, USA. Except MWCNT, other chemicals were used as
received. L-Cysteine (L-Cys), Lithium perchlorate (LiClO₄) and Tetra-n-butyl ammonium chloride were obtained from Lancaster, UK and were used as such without any further purification. Chloroauric acid was purchased from SRL Chemicals, India. Sodium dihydrogen orthophosphate (NaH₂PO₄) and disodium hydrogen orthophosphate (Na₂HPO₄) were purchased from Merck, Germany and were used as received. All other common reagents used for the studies were obtained from s.d. fine chemicals, India. Pure drugs such as Ambroxol Hydrochloride (AMB), Sulfamethoxazole (SMX), Lamivudine (LAM), Metronidazole Benzoate (MBZ), Nimesulide (NIM) and Trimethoprim (TMP) were obtained as gift samples. Pharmaceutical formulations containing the drugs were purchased from local drug stores.

2.2 Instruments Used

All the electrochemical measurements were made on BAS Epsilon Electrochemical analyzer (Bioanalytical system, USA) interfaced to a PC. A conventional three electrode system, including working electrode, counter electrode and reference electrode was employed. Working electrode used was glassy carbon electrode (GCE) modified with suitable chemical modifications, counter electrode used was platinum wire and Ag/AgCl was used as the reference electrode. All voltammograms were recorded in the polarographic mode. The pH measurements were carried out in a Metrohm pH meter. Electrode cleaning was carried out in an Ultrasonicator (Oscar Ultrasonics, Pvt. Ltd. Mumbai). Scanning Electron Microscopic (SEM) images were recorded using JOEL 6300 LV at Sophisticated Test and Instrumentation Centre (STIC), Kochi. FTIR spectra was recorded on JASCO-4100 FTIR Spectrometer using KBr discs.
2.3 Cleaning of GCE

First the GCE was mechanically polished with alumina on a polishing pad and was cleaned thoroughly with double distilled water. This was then sonicated in methanol, 1:1 HNO₃ solution, acetone and double distilled water sequentially to obtain the cleaned GCE.

2.4 Fabrication of Chemically modified electrodes (CMEs) as sensors for the determination of various pharmaceuticals

2.4.1 Fabrication of MWCNT/Nafion modified GCE

MWCNT was first refluxed in 100 mL 6 M HNO₃ for 10 hours. The resulting suspension was then diluted with 200 mL water. MWCNT was then filtered and washed with double distilled water. The washed nanotube was collected and dried.

MWCNTs were generally insoluble in common solvents; therefore the key step was to disperse insoluble MWCNTs in suitable solvents to perform the electrochemical measurements by using the MWCNT modified electrodes. However nafion, a sulfonated tetra fluoro ethylene copolymer, was found to effectively disperse MWCNTs. Nafion possess several advantages such as excellent ion exchange characteristics, thermal stability, chemical inertness and mechanical strength and has been widely used for the MWCNT modification of electrodes [177]. Hence Nafion was selected as the solvent to disperse MWCNT into water.

5 mg of the treated MWCNT was added to 2 mL of 0.5 % nafion water solution and then sonicated for about 1 h with an ultrasonicator to get a stable and homogenous suspension. Then the GCE was coated with an adequate amount of the resulting MWCNT/Nafion suspension and the solvent was evaporated at room temperature in air to get MWCNT/Nafion modified GCE.
2.4.2 Fabrication of poly(p-toluene sulphonylic acid)/GCE

1mM p-toluene sulphonylic acid (p-TSA) was prepared in 0.1 M NaCl solution. 10 mL of this solution was then pipetted into the electrochemical cell. The cleaned GCE, reference electrode and auxiliary electrode were then immersed in the p-TSA solution. 20 cyclic scans were performed between -2.0 to +2.5 V at a scan rate of 0.1 V s\(^{-1}\) [178]. GCE was then taken out and washed several times with double distilled water and kept in air. A uniform blue film was found to be formed on the GCE surface.

2.4.3 Fabrication of poly(L-Cysteine)/GCE

5mM L-Cysteine (L-Cys) was prepared in 0.1M phosphate buffer solution (PBS). 10 mL of this solution was then pipetted into the electrochemical cell. The cleaned GCE, reference electrode and auxiliary electrode were then immersed in the L-Cys solution. The film was grown on the electrode surface by 30 segments of cyclic voltammetric scans between 0.8 and 2.0V [179]. After immobilization, the film was washed with ethanol to remove the remaining L-Cys monomers. It was observed that after drying in air, a blue thin film was formed at the electrode surface.

2.4.4.1 Fabrication of poly(p-amino benzene sulphonylic acid)/GCE

2mM p-amino benzene sulphonylic acid (p-ABSA) was prepared in 0.1M HNO\(_3\) solution. 10 mL of this solution was then pipetted into the electrochemical cell with glassy carbon as the working electrode, Ag/AgCl as the reference electrode and platinum wire as the counter electrode. The electrochemical deposition of p-ABSA film was carried out by cyclic voltammetry between -0.5 and 2.0V at 0.1V s\(^{-1}\) for 30 cycles [180]. The resulting film was washed with doubly distilled water...
and dried in air. After drying in air, a thin blue film was observed at the electrode surface. Thus poly (p-ABSA) film was electrochemically deposited on the GCE surface.

2.4.4.2 Fabrication of AuNP/poly(p-amino benzene sulphonic acid)/GCE

The poly (p-ABSA) modified electrode was immersed in 0.05M H₂SO₄ solution containing 1mM HAuCl₄. 20 cyclic scans were carried out between 1.3 and 0 V at a scan rate of 0.1Vs⁻¹ [181]. This resulted in the electrochemical deposition of gold nanoparticle (AuNP) on the electrode surface.

2.4.5.1 Fabrication of poly(Cystamine)/GCE

1mM Cystamine hydrochloride (CA) was prepared in 0.1M PBS. 10 mL of this solution was then pipetted into the electrochemical cell. The cleaned GCE, reference electrode and auxiliary electrode were then immersed in the CA solution. The film was grown on the electrode surface by 20 segments of cyclic voltammetric scans between −1.2 and 2.5V [182]. The resulting film was washed with doubly distilled water and dried in air. After drying in air, a blue thin film of poly-CA was formed at the electrode surface.

2.4.5.2 Fabrication of AuNP/poly-CA/GCE

The poly-CA modified electrode was immersed in 0.05M H₂SO₄ solution containing 1mM HAuCl₄ solution. 20 cyclic scans were carried out between 1.3 and 0V at a scan rate of 0.1Vs⁻¹ to deposit AuNPs electrochemically on the electrode surface.

2.5 Preparation of the drug solutions

A 10⁻¹M solution was prepared for each of the drug in suitable solvents. The stock solution was diluted to get the required concentration.
2.5.1 Ambroxol solution

4.146g of AMB was dissolved in methanol and the solution was made upto the volume in a 100mL titrimetric flask.

2.5.2 Sulfamethoxazole solution

2.530g of SMZ was dissolved in methanol in a 100mL volumetric flask and made upto the mark.

2.5.3 PAM Chloride solution

1.726g of PAM Chloride was dissolved in water in a 100mL volumetric flask and made upto the mark.

2.5.4 Lamivudine solution

2.293g of LAM was dissolved in water in a 100mL volumetric flask and made upto the mark.

2.5.5 Metronidazole benzoate solution

2.753g of MBZ was dissolved in methanol in a 100mL volumetric flask and made upto the mark.

2.5.6 Nimesulide solution

3.083g of NIM was dissolved in acetone in a 100mL volumetric flask. The solution was made upto 100mL using acetone.

2.6 Preparation of Buffer Solutions

Phosphate buffer solution (PBS) was used as the supporting electrolyte for carrying out the electrochemical measurements.

2.6.1 Preparation of phosphate buffer solution (PBS)

PBS of different pH was prepared by mixing NaH$_2$PO$_4$ and Na$_2$HPO$_4$ in varying amounts.
Materials and Methods

a) **pH 2**
   1.3799g of NaH$_2$PO$_4$ and 0.0001g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 2.

b) **pH 3**
   1.379g of NaH$_2$PO$_4$ and 0.0003g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 3.

c) **pH 4**
   1.378g of NaH$_2$PO$_4$ and 0.0036g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 4.

d) **pH 5**
   1.3615g of NaH$_2$PO$_4$ and 0.036g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 5.

e) **pH 6**
   1.2143g of NaH$_2$PO$_4$ and 0.3218g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 6.

f) **pH 7**
   0.5836g of NaH$_2$PO$_4$ and 1.5466g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 7.

g) **pH 8**
   0.094g of NaH$_2$PO$_4$ and 2.497g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 8.

h) **pH 9**
   0.01g of NaH$_2$PO$_4$ and 2.6605g of Na$_2$HPO$_4$ was dissolved in 100mL double distilled water to get PBS of pH 9.
2.7 Voltammetric determination of pharmaceuticals

Voltammetric measurements were carried out in a BAS Epsilon Electrochemical analyzer with a 3 electrode system. To the stock solution of drug, appropriate amount of supporting electrolyte (PBS) was added to get the drug solution of suitable concentration and was taken in the electrochemical cell. Voltammogram was recorded and the peak current corresponding to the oxidation/reduction of the drug was measured. The current obtained was plotted as a function of concentration. A linear relationship between the peak current and concentration of the drug could be observed.

2.8 Preparation and analysis of Pharmaceutical Formulations

2.8.1 Ambroxol Formulation – Ambrodil HCl

Ten tablets (‘Ambrodil HCl’, Aristo, India) were weighed and ground to a fine powder. An adequate amount of this powder corresponding to the concentration $1 \times 10^{-3}$M was weighed and transferred to a beaker. The powder was dissolved in methanol and filtered to a titrimetric flask (50 mL) through a Whatman 41 filter paper. The beaker was washed several times and the washings were collected in the volumetric flask and then it was quantitatively diluted. Solutions of different concentrations were prepared by serial dilution of the stock with supporting electrolyte. CV was measured and the unknown concentrations were determined from the calibration graph.

2.8.2 Sulfamethoxazole Formulation – Bactrim and Septra

Ten tablets of each type (‘Bactrim’, Piramal Healthcare, India and ‘Septra’, Burroughs Wellcome, India) were weighed and finely powdered. Then required amount of the powdered drug was weighed to prepare a solution of $5 \times 10^{-3}$ M and was transferred to a beaker. The powder was
dissolved in methanol and filtered to a titrimetric flask (50 mL) through a Whatman 41 filter paper. The beaker was washed several times and the washings were collected in the volumetric flask and then it was quantitatively diluted. Solutions of different concentrations were prepared by serial dilution of the stock with supporting electrolyte. Differential Pulse Voltammogram was recorded and the unknown concentrations were determined from the calibration graph.

2.8.3 Lamivudine Formulation - Lamivir

Ten tablets (‘Lamivir’, Cipla, India) were accurately weighed and finely powdered. An adequate amount of this powder corresponding to the concentration 1×10^{-2} M was weighed and transferred to a beaker. The powder was dissolved in double distilled water and filtered to a titrimetric flask (100 mL) through a Whatman 41 filter paper. The beaker was washed several times with water and the contents were filtered into the flask. The solution was made upto the mark and shaken well. Solutions of different concentrations were prepared by serial dilution of the stock with supporting electrolyte. The sample solution was then taken in the electrochemical cell and the voltammetric studies were carried out.

2.8.4 Metronidazole Formulations – Metrogyl and Flagyl

Ten tablets of each type (‘Flagyl’, Nicholas Piramal India Ltd and ‘Metrogyl’, J.B. Chemicals & Pharmaceuticals, India) were weighed and finely powdered. Then required amount of the powdered drug was weighed to prepare a solution of 1×10^{-2} M and was transferred to a beaker. The powder was dissolved in methanol and filtered to a titrimetric flask (50 mL) through a Whatman 41 filter paper. The beaker was washed several times and the washings were collected in the titrimetric flask and then it was
quantitatively diluted. Solutions of different concentrations were prepared by serial dilution of the stock with supporting electrolyte. Differential Pulse Voltammogram was recorded and the unknown concentrations were determined from the calibration graph.

2.8.5 Nimesulide Formulation – Nise

The mass of ten tablets (‘Nise’, Dr. Reddy’s, India) were taken and then powdered well. The mass of powder required to prepare $1 \times 10^{-2}$ M solution was weighed. It was then transferred to a beaker and dissolved in acetone. The clear solution was transferred to the titrimetric flask through a Whatman 41 filter paper. Then the residue in the beaker was washed several times with acetone and the washings were collected in the titrimetric flask. The solution was then made upto the mark. Solutions of different concentrations were prepared by serial dilution of the stock with supporting electrolyte. The sample solution was then taken in the electrochemical cell and the voltammetric studies were carried out.

2.9 Analysis of Urine Sample

The developed sensors were applied for the determination of the drug in spiked urine samples. Different quantities of drug were added to a fixed volume of urine sample and then quantitatively diluted using the supporting electrolyte. The voltammograms were recorded and the unknown concentrations were determined from the calibration graph.

2.10 Standard Methods

2.10.1 Ambroxol [183]

Ten tablets of ‘Ambrodil HCl’ were accurately weighed and ground to a fine powder. An adequate amount of this powder containing 0.3g of AMB was weighed. It was dissolved in 70 mL of alcohol and 5mL of 0.01 M HCl was
added. It was made up to the volume in a 100mL titrimetric flask. Potentiometric titration was carried out using 0.1M NaOH. The volume added between the two points of inflexion was recorded. 1mL of 0.1 M NaOH is equivalent to 41.46 mg of AMB.

2.10.2 Sulfamethoxazole [184]

Ten tablets of each type ‘Bactrim’ and ‘Septra’, were weighed and finely powdered An adequate amount of this powder containing 0.2g of SMX was weighed. It was dissolved in 50mL of 2M HCl and 3g of KBr was added to it. Then it was ice cooled and titrated against 0.1 M sodium nitrite. The end point was determined potentiometrically.

2.10.3 PAM Chloride [185]

0.5 g of PAM Chloride was accurately weighed and dissolved in 250 mL water. 5mL of this solution was then diluted to 100mL with water. 5mL of this solution was then transferred to a 50mL titrimetric flask. Required amount of urine sample was added and then diluted to 40 mL with water. To this 5 mL of 1M NaOH was added and made up to the mark. The absorbance of the prepared solution was measured. Similarly solutions of various concentrations were prepared and the absorbance was measured.

2.10.4 Lamivudine [186]

Ten tablets of ‘Lamivir’ were accurately weighed and finely powdered. The mass equivalent to the mass of one tablet was accurately weighed and dissolved in distilled water and made up to the volume in a 100mL titrimetric flask and quantitatively diluted. A known volume of the drug was taken and a measured excess of n- bromosuccinimide (NBS) in HCl – acetate buffer was added. After 5 min, a known amount of iron (II)
was added to the mixture which reduces the unreacted NBS. The residual iron (III) is complexed with orthophenanthroline. The absorbance is measured at 510nm. The amount of NBS reacted corresponds stoichiometrically to the amount of LAM.

2.10.5 Metronidazole [184]

Ten tablets of each type ‘Flagyl’ and ‘Metrogyl’, were weighed and finely powdered. An adequate amount of this powder containing 0.08g of MBZ was accurately weighed and dissolved in methanol. 10mL of this solution was diluted to 100mL with methanol and further 10mL of this solution was diluted to 100mL with methanol. The absorbance of the resulting solution was measured.

2.10.6 Nimesulide [183]

The mass of ten tablets of ‘Nise’ were taken and then powdered well. An adequate amount of this powder containing 0.24g of NIM was accurately weighed and dissolved in 30mL of acetone. Then 20mL of double distilled water was added to it and titrated against 0.1M NaOH solution. The end point was determined potentiometrically.