CHAPTER 3

EXPERIMENTAL PROCEDURE

This chapter describes the experimental procedures adopted for the synthesis and characterisation of sixteen random copolyesters. It also deals with the study of antimicrobial and antitumor activity of those synthesised copolyesters. The important materials used for the preparation of all the copolyesters are given below:

3.1 Materials

Methanol (Merck, AR) was refluxed over quicklime for six hours and distilled (b.pt. 65°C). Ethanol (Ranchem, AR) was purified by same method. Chloroform (Merck, AR) was distilled and the middle fraction (b.pt. 173°C) was collected and used. n-Hexane (Merck, AR and b.pt. 40°C) were distilled and used.

Merck, AR samples of dimethyl sulphoxide, dimethyl formamide, tetrahydrofuran were purified by distillation before use. Spectral grade CDCl$_3$ and DMSO d6 were used for recording NMR (1H and 13C) spectra of the copolyesters.

Adipic acid (Ranbaxy), sebacic acid (SDS), suberic acid (Fluka), azeleic acid (Merck) and thionyl chloride (SDS) were used. Isophthaloyl chloride and
terephthaloyl chloride were purchased from Aldrich and used without further purification.

4-hydroxybenzaldehydes (Merck), 4-hydroxy-3-methoxy benzaldehyde (Merck) were used as received.

Tetra-n-butylammonium bromide (TBABr, Fluka) was purchased and used as such.

The following fungi used for biological study namely, *Aspergillus flavus*, *Botyritis cinerea*, *Curvularia lunata* 46/01, *Aspergillus niger* MTCC 1344, *Trichophyton rubrum* 57/01, *T. mentagrophytes* 66/01 and *Candida albicans* MTCC227 were purchased from HI media Laboratories Ltd, Mumbai, India.

The test organisms were used to test antimicrobial activity using disc diffusion method. *Bacillus subtilis* MTCC 441, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC6538 and *Enterobacter faecalis* were purchased from HI media Laboratories Ltd, Mumbai, India.

The HepG2 hepatocellular carcinoma cell line (HB 8065; American Type Culture Collection), derived from a human hepatoblastoma, were maintained in Dulbecco’s modified Eagle’s (DMEM) with 10% heat-inactivated fetal bovin serum (FBS) at 37°C with 5% CO₂.
3.2 SYNTHESIS OF MONOMERS

3.2.1 1, 3-bis(4-hydroxy-3-methoxyphenyl)prop-2-enone (BHMPP)

Dry hydrochloric acid was passed at a slow rate into a mixture of 4-hydroxy-3-methoxy benzaldehyde and 4-hydroxy-3-methoxy acetophenone kept dissolved in dry methanol. The reaction was allowed to proceed for an hour and then poured into ice cold water, the yellow precipitate of BHMPP formed was filtered, dried and further recrystallised from methanol.

3.2.2 1-(3,5-dihydroxyphenyl)-3-(4-methoxyphenyl)prop-2-enone (DHPMPP)

Dry hydrochloric acid was passed at a slow rate into a mixture of 3, 5-dihydroxy acetophenone and 4-methoxy benzaldehyde kept dissolved in dry methanol. The reaction was allowed to proceed for an hour and then poured into ice cold water, the yellow precipitate of DHPMPP formed was filtered, dried and further recrystallised from methanol.

3.2.3 2,6-bis (4-hydroxy benzylidene) cyclohexanone (BHBCH)

p-hydroxy benzaldehyde and cyclohexanone in the ratio of 2:1 were taken in round bottomed flask and dissolved in methanol. To this concentrated sulphuric acid was added in drops with constant stirring till the solution became dark pink. The diol thus obtained was filtered, washed with water and crystallised from methanol$^{1, 2}$. 


3.2.4 Preparation of acid chlorides

The acid chlorides, adipoyl, suberoyl, azeloyl and sebacoyl chlorides were obtained from the respective diacids by refluxing them with excess of thionyl chloride followed by removing the unreacted thionyl chloride under reduced pressure. Adipoyl, suberoyl, azeloyl and sebacoyl chlorides were redistilled under reduced pressure³.

3.3 SYNTHESIS OF POLYMERS

All the polymers were synthesised by the interfacial polycondensation method using tetra-n-butylammonium bromide (TBABr) as a phase transfer catalyst⁴.

3.3.1 Reaction flask

The 100 mL three necked round bottom flask made of pyrex glass was used as a reaction vessel. It was equipped with a mechanical stirrer, a pressure equalizer and a nitrogen inlet.

3.3.2 Deaeration technique

The purpose of using nitrogen gas for deaeration is to remove traces of oxygen by passing through Fieser’s solution. The gas was then passed through a saturated lead acetate solution to make it free from hydrogen sulphide and sulphur dioxide and washed by passing through distilled water. Then, the gas was dried by passing through a bottle containing calcium chloride.
3.4 POLYMERISATION PROCEDURE

3.4.1 Scheme - I

The equimolar mixture of BHMPP (1mmole) and DHPMPP (1mmole) was dissolved in 25 mL of aqueous sodium hydroxide (0.1 N) solution taken in a three-necked round-bottomed flask (100 mL). The mixture was stirred continuously to get a homogeneous solution which was wine red in colour. After 15 minutes, a solution of 2mL of 2% TBABr was added and stirred. The mixture was stirred continuously at room temperature for 30 minutes in nitrogen atmosphere. About 25 mL solution containing the adipoyl chloride (2 mmole) in distilled dichloromethane (DCM) was added using pressure equaliser with constant stirring. The mixture was maintained at room temperature with continuous stirring for seven hours. The reaction mixture was poured into 100 mL of n-hexane when the solid copolyester PBHR1, was obtained. It was then filtered and dried in vaccum. The crude sample was purified by dissolving in DCM and reprecipitating in n-hexane. All the other three copolyesters (PBHR2, PBHR3 and PBHR4) were also prepared by using sebacoyl chloride, suberoyl chloride and azeloyl chloride in a similar manner.

3.4.2 Scheme - II

The procedure adopted for the synthesis of copolyester PTBHC1 is as follows: About 2 mmole of BHMPP was dissolved in 25 mL of 0.1N NaOH
solution containing a 2% solution of 2 mL of TBABr. To this mixture, adipoylchloride (1 mmole) and terephthaloyl chloride (1 mmole) dissolved in 25mL DCM were added with vigorous stirring at room temperature. After five hours of stirring, the polymer formed between organic and aqueous layer was filtered and dried. The crude sample was purified by dissolving in DCM and reprecipitating in n-hexane. The other copolyesters PTBHC2, PTBHC3 and PTBHC4 were prepared by adopting the same procedure using other acid chlorides namely, suberoyl, azeloyl and sebacoyl chlorides.  

3.4.3 Scheme - III

Equimolar mixture of BHMPP (1 mmole) and BHBCH (1mmole) was dissolved in 25mL of 0.1N NaOH solution containing 2mL of 2% TBABr. This mixture was stirred continuously at room temperature for 30 minutes in nitrogen atmosphere. To this mixture, adipoyl chloride (2 mmole) dissolved in DCM was added with vigorous stirring at normal temperature. After seven hours of stirring, the polymer was formed as solid between organic and aqueous layer and was separated and dried in vaccum. The crude sample of PBHCH1 was purified by dissolving in DCM and reprecipitsting in n-hexane. Three more copolyesters PBHCH2, PBHCH3 and PBHCH4 were also prepared by a similar procedure.
3.4.3 Scheme - IV

In a 100 mL three-necked flask equipped with a mechanical stirrer, BHMPP (2 mmole) mixed with 25 mL of 0.1N aqueous NaOH was taken. This solution was stirred well till it forms a red coloured solution. After 15 minutes, a solution of 2 mL of 2% of TBABr was added and stirred. To this mixture, adipoyl chloride (1 mmole) and isophthaloyl chloride (1 mmole) dissolved in DCM were added with vigorous stirring at room temperature. After five hours of stirring, the copolyester of PIBHC1 produced between the organic and aqueous layer was filtered and dried. The crude sample was purified by dissolving in DCM and reprecipitating in n-hexane. The polymers PIBHC2, PIBHC3 and PIBHC4 from TBABr and BHMPP were also prepared by using suberoyl, azeloyl and sebacoyl chlorides in a similar fashion.8

3.5 CHARACTERISATION OF RANDOM COPOLYESTERS

The synthesised copolyesters in the present work were characterised by solubility studies, viscosity measurements, X-ray diffraction, elemental analysis, molecular weight determination, spectral analysis and thermal studies. Antimicrobial activity of the copolyesters was studied against few fungi and bacteria. A few characteristic samples were also assayed for anticancer activity.
3.5.1 Solubility

Solubility of the synthesised polymer was tested with various organic solvents. About 100 mg of the polymer was taken in a small test tube containing 2 mL of the solvent. The mixture is kept aside for 24 hours with occasional shaking. If the mixture was insoluble in cold, slowly heated upto the boiling point of the solvent, and the solubility was observed.

3.5.2 Viscosity measurements

The inherent viscosity ($\eta_{inh}$) of the polymers was measured in DMSO (0.5 g/dL), using a suspended level Ubbellohde viscometer. The inherent viscosity values of all the copolyesters were determined.

3.5.3 Elemental analysis

Micro elemental analysis of the polymers was carried out at Regional Sophisticated Instrumentation Centre, IIT, Madras.

3.5.4 FT IR Spectra

Infrared spectra were obtained on a Thermomattson satellite model FT-IR spectrometer using KBr pellets. IR spectra of all the copolyesters were recorded in the range of 4000 to 400 cm$^{-1}$.

3.5.5 Nuclear magnetic resonance spectra

High-resolution $^1$H and $^{13}$CNMR spectra were recorded on a Bruker spectrometer at 500 MHz on CDCl$_3$. The$^1$H NMR spectra of the copolyesters
were recorded in CDCl₃ solvent with TMS as the reference using JEOL Model GS X 300. $^{13}$C NMR spectra of the copolyesters were recorded using DMSO-d₆ solvent.

3.5.6 Thermal analysis

The thermal data of the polymers were determining using a DSC Netzsch (TA Instruments, USA) in the temperature range between -50°C to 500°C at a heating rate of 10°C per minute in nitrogen atmosphere.

3.5.7 X-ray diffraction analysis

X-ray diffraction patterns of polymers were recorded at room temperature by monitoring the diffraction angle 2θ from 0.5 to 20° as low angle on a Rich Seifert (Model 3000) X-ray powder diffractometer. The diffractometer was equipped with a copper target ($\lambda=1.5405$ Å) radiation using a Guinier-type camera and a solid state detector. Curved nickel crystal was used as a monochromator. The step width 2θ (scanning speed) used was 0.04 deg/min.

3.5.8 Gel permeation chromatography

The weight average molecular weight ($M_w$) and the number average molecular weight ($M_n$) were determined by gel permeation chromatography. Approximately 0.2 mL of 1% polymer tetrahydrofuran solvent was used at a rate of 2 mL/min. The GPC was calibrated using narrow molecular weight polystyrene standards of known molecular weight. The molecular weights are
not corrected for peak broadening and for the variation of refractive index with molecular weight.

3.6 TESTS FOR ANTIMICROBIAL ACTIVITY

The copolyesters obtained were assayed for antifungal activity using strains of *Aspergillus flavus, Botyritis cinerea, Curvularia lunata, Aspergillus niger, Trichophyton rubrum, T. mentagrophytes* and *Candida albicans* and bacterial strains namely, *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Enterobacter faecalis*.

3.6.1 Antifungal activity

Alan *et al* reported that the antifungal activities of polymers have been assayed towards plant pathogen.

3.6.2 Preparation of fungal spore

The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile doubled distilled water and homogenised. Yeast was grown on Sabouraud dextrose Broth (SDB) at 28°C for 48 hours.

3.6.3 Antifungal assay

**Determination of the minimum inhibitory concentration (MIC)**

Minimum inhibitory concentration is defined as the lowest concentration that inhibits bacterial growth. To determine minimum inhibitory concentration
(MIC), the serial dilution technique\cite{9, 10} was followed using nutrient broth medium.

The antifungal activity was performed according to the standard reference method (NCCLS, 2002). The copolyesters were dissolved in water with 2% dimethyl sulfoxide (DMSO). The initial concentration of the polymer was 1mg/ml. The initial test concentration was serially diluted two-fold\cite{11}. Each well was inoculated with 5 µL of suspension containing 104 spore/mL of fungi. The antifungal agents Fluconazole and Ketoconazole were included in the assays as positive controls.

3.6.4 Antibacterial activity

The antibacterial studies of copolyesters were carried out by disc diffusion\cite{12, 13} method as reported by Cao et al.

3.6.5 Preparation of stock solution

Stock solutions of synthesised polymers were prepared in dimethyl sulfoxide (DMSO). The stock solutions were prepared in single concentration, i.e., 25 mg/mL and they were used in different µL concentrations namely 100 µL, 250 µL, 500 µL and 1000 µL concentrations and immediately dispensed into each agar wells of culture inoculated Mueller Hinton Agar (HMA) plates using sterilised micropipette.
3.6.6 Preparation of inoculums

Bacterial inoculums were prepared by growing cells in Mueller Hinton Broth (HiMedia) for 24 h at 37°C. These cell suspensions were diluted with sterile MHB to provide initial cell counts of about 10⁴/mL CFU/mL. The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile doubled distilled water and homogenised.

3.6.7 Disc diffusion method

Murray et al reported that the antibacterial activity was carried out using disc-diffusion method. Petri plates were prepared with 20 mL of sterile Mueller Hinton Agar (MHA) (Hi-media, Mumbai). The test cultures (100 µL of suspension containing 10⁸ CFU/mL bacteria) were swabbed on top of the solidified media and allowed to dry for 10 min. The tests were conducted at three different concentrations of the polymer solution (5mg, 2.5mg and 1.25mg per disc). The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvent¹⁴. Streptomycin (10µg/disc) was used as positive control. The plates were incubated for 24 hour at 37°C. Zone of inhibition was recorded in millimeters and the experiment was repeated twice.
3.7 Antitumor activity of copolyesters

The chalcone polymers were dissolved in dimethyl sulfoxide and then added to the culture medium.

Anticancer activity and cell selectivity were carried out by Micro culture tetrazolium test (MTT) assay as proposed by Mosmann.

3.7.1 Principle

MTT is cleaved by mitochondrial dehydrogenase in viable cells, yielding a measurable purple product formazan. This formazan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity (Mosmann T, 1983).

3.7.2 Procedure

48 hr monolayer culture of HepG2 cells and VERO cells at a concentration of one lakh cells / well were seeded in 24 well titer plate. The plates were microscopically examined for confluent monolayer, turbidity and toxicity if the cells become confluent. The growth medium minimal essential media (MEM) was removed using micropipette. Care was taken so that the tip of the pipette did not touch the cell sheet. The monolayer of cells was washed twice with MEM without Foetal cough serum (FCS) to remove the dead cells and excess FCS To the washed cell sheet, add 1ml of medium (without FCS)
containing defined concentration of the drug in respective wells. Each dilution of the drug ranges from 1:1 to 1:64 and they were added to the respective wells of the 24 well titer plates. To the cell control wells add 1ml MEM without FCS. The plates were incubated at 37°C in 5% CO₂ environment and observed for cytotoxicity using inverted microscope¹⁵,¹⁶.

After incubation, the medium was removed from the wells carefully for MTT assay. In each well was washed with MEM without FCS for 2 - 3 times, and 200µl of MTT concentration of (5mg/ml) sample was added. And this was incubated for 6-7hrs in 5% CO₂ incubator for Cytotoxicity. After incubation 1ml of DMSO was added in each well and mixed by using pipette and kept for 45sec. If any viable cells present formazan crystals after adding solublising reagent (DMSO) it shows the purple color formation. The suspension was transferred into the cuvette of spectrophotometer and an Optical density values are readed at 595nm by taking DMSO as a blank. Graph is plotted by taking concentration of the drug on X axis and relative cell viability on Y axis.

The percent cell viability with respect to control is calculated using the following formula,

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\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \times 100
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REFERENCES


