EXPERIMENTAL METHODS
CHAPTER 3

EXPERIMENTAL METHODS

This chapter describes the synthesis and characterisation of twenty citric acid based polyesters. Further, it also describes the preparation and characterisation of nanohydroxyapatite and its polymeric composites. This investigation also deals with the characterisation methods such as swelling behaviour and invitro degradation of the composite. Cytotoxicity of the prepared nanocomposite was also evaluated.

3.1 Materials

The materials used for the synthesis and characterisation of all the copolyesters and their composites are presented below.

Citric acid (CA) (Merck AR grade) was used as such. MERCK, AR samples of succinic acid, adipic acid, suberic acid, sebacic acid and maleic acid were used as such.

LANCASTER samples of Ethylene glycol, 1, 4-Butane diol, 1,6-Hexane diol and 1,8-Octane diol were used as such.

Methanol (MERCK, AR) was refluxed over quicklime for six hours and distilled (b.pt. 65°C). ETHANOL (RANCHEM, AR) was purified by similar method (b.pt. 78°C).
AnalaR samples of dimethyl sulphoxide, dimethyl formamide, tetrahydrofuran and 1,4 dioxane were used as such. Spectral grade CDCl$_3$ and DMSO-d$_6$ were used for recording NMR spectra of the copolyesters. Dihydroxybenzoic acid was used for recording MALDI-MASS Spectroscopy.

The materials used for the synthesis of nano hydroxyapatite were Calcium nitrate tetrahydrate (Ca (NO$_3$)$_2$.4H$_2$O) (CNT, ALDRICH), Phosphoric acid (PA, Lancaster AR grade) and ammonia (MERCK, AR grade).

Minimal Essential Media (MEM) reagent was purchased from Hi Media Laboratories. Fetal Bovine Serum (FBS) was purchased from Cistron laboratories Trypsin. Methylthiazolyl diphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals Mumbai. All of the other chemicals and reagents were obtained from Sigma Aldrich Mumbai for Cytotoxicity test.

3.2 Synthesis of polyesters and polymeric nanocomposites

The polymers were synthesized by catalyst free melt poly condensation method.

3.2.1 Reaction flask

The reaction vessel is a 500mL three necked round bottom flask made of pyrex glass. It is equipped with a magnetic stirrer, an air condenser, a nitrogen inlet, thermometer and a quick fit adapter.
3.2.2 Thermostat

The experiments are conducted in an oil bath thermostat. The bath is heated by immersion resistance coil and the temperature of the bath is controlled by a dimmerstat.

3.2.3 Deaeration technique

The nitrogen gas used for the purpose of deaeration may contain traces of oxygen which is removed by passing through Fieser's solution. The gas is then passed through a saturated lead acetate solution to free it from hydrogen sulphide and sulphur dioxide and washed by passing through distilled water. The gas is then dried by passing through a bottle containing anhydrous calcium chloride.

3.2.4 Synthesis of polyesters

The pre polymers were synthesised by catalyst free melt condensation method. As an example, the synthesis of copolyester poly (1,6-Hexanediol-Succinate-Citrate) (P₃), is described as follows.

Equimolar amounts of both the HD and acids [HD:( CA+ SA) =1:1] were taken in a three necked round bottom flask and the monomer mixture was first heated up to 160–165°C followed by mixing at 140–145°C for 1 h under a constant stream of nitrogen. The pre polymer, thus, obtained were dissolved in 1, 4-dioxane (20% w/w solution) and the resulting pre polymer solution was used for film preparation without further purification.¹
Films for structural analyses were prepared by casting into Teflon petri dishes and left in an oven at 80°C for 7 days for solvent evaporation and further polyesterification of the prepolymer.

The diacids and diols used in the preparation of the polyesters along with yields are given in Table 3.1. Citric acid is used as a common monomer in all the polyesters.

Table 3.1

Comonomers used and yield of synthesised polyesters

<table>
<thead>
<tr>
<th>S.No</th>
<th>Polymer</th>
<th>Diol</th>
<th>Diacid</th>
<th>Diacid/Diol</th>
<th>% yield</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>P1</td>
<td>EG</td>
<td>CA</td>
<td>SA</td>
<td>65</td>
</tr>
<tr>
<td>2.</td>
<td>P2</td>
<td>BD</td>
<td>CA</td>
<td>SA</td>
<td>70</td>
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<tr>
<td>3.</td>
<td>P3</td>
<td>HD</td>
<td>CA</td>
<td>SA</td>
<td>65</td>
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<tr>
<td>4.</td>
<td>P4</td>
<td>OD</td>
<td>CA</td>
<td>SA</td>
<td>65</td>
</tr>
<tr>
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<td>P5</td>
<td>EG</td>
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<td>AD</td>
<td>70</td>
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<td>AD</td>
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</tr>
<tr>
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<td>P8</td>
<td>OD</td>
<td>CA</td>
<td>AD</td>
<td>75</td>
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<td>CA</td>
<td>SuA</td>
<td>75</td>
</tr>
<tr>
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<td>BD</td>
<td>CA</td>
<td>SuA</td>
<td>75</td>
</tr>
<tr>
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<td>SuA</td>
<td>70</td>
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<td>OD</td>
<td>CA</td>
<td>SuA</td>
<td>70</td>
</tr>
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<td>EG</td>
<td>CA</td>
<td>SeA</td>
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<td>P14</td>
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<td>CA</td>
<td>SeA</td>
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<td>P15</td>
<td>HD</td>
<td>CA</td>
<td>SeA</td>
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</tr>
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<td>P16</td>
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<tr>
<td>17.</td>
<td>P17</td>
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<td>P20</td>
<td>OD</td>
<td>CA</td>
<td>MA</td>
<td>71</td>
</tr>
</tbody>
</table>
EG: Ethylene Glycol       BD: 1, 4-Butanediol       HD: 1, 6-Hexane diol

OD: 1, 8-Octane diol

CA: Citric acid       AD: Adipic acid       SA: Succinic acid

SuA: Suberic acid     SeA: Sebacic acid     MA: Maleic acid

3.2.5 Preparation of nano-hydroxyapatite powder (n-HAp)

Nano hydroxyapatite powders, calcined at different temperatures, have been synthesised via a sol-gel method.²

0.25M Phosphoric acid (PA) solution was prepared and to this solution ammonia (MERCK AR grade) was added in drops and stirred till a constant pH = 10. 1M Calcium nitrate tetrahydrate (CNT, Aldrich) solution was prepared and was slowly added to the PA-NH₃ solution, maintaining Ca/P ratio of 1.67.³ Further small amounts of ammonia was added to the solution to maintain a constant pH = 10. The solution was vigorously stirred for 1 hr and kept for ageing for 24 hr at room temperature. The gel obtained after ageing was dried at 65°C for 24 hrs in a dry oven. The powders obtained from dried gel were washed repeatedly using double distilled water to remove NH₄⁺ and NO₃⁻. After washing, the powder was calcined in air at 500°C for 30 min in an electric furnace.
3.2.6 Preparation of n-HAp/polymer composite

The Pre-polymer dissolved in ethanol (1:1 w/v) was mixed with the desired amount of n-HAp powder. The Pre Polymer-n-HAp mixture was stirred to get homogeneous solution and cast into Teflon dishes and left in an oven at 110°C for 2 days for post-curing.

3.3 Characterisation of polymers and nanocomposites

The polymers synthesised were characterised by solubility studies, X-ray diffraction and spectral analysis. Thermal and mechanical studies were also made on these polyesters. Swelling characteristics and in vivo degradation of the composite were investigated. Cytotoxicity of the polyester was also studied. The morphology of the polyester film was investigated by SEM.

3.3.1 Solubility

Solubility of all the random copolyesters was determined in various solvents qualitatively. About 100mg of the polymer was taken in small stoppered test tube containing 2ml of the solvent. The mixture was kept for 24 hours with occasional shaking. If insoluble in cold, the mixture was slowly heated up to the boiling point of the solvent, and whether the polymer was dissolved or swollen in the solvent was noted.
3.3.2 FT Infrared spectra

IR spectra of all the copolyesters were recorded by Perkin Elmer IR spectrometer in the range of 4000 to 400 cm\(^{-1}\). The samples were embedded in KBr pellets.

3.3.3 \(^1\)H NMR measurements

\(^1\)H NMR spectra were recorded on AV 3500 MHz Spectrometer by using 7\% wt of DMSO-\(d_6\) Solvent.

3.3.4 \(^{13}\)C NMR measurements

The \(^{13}\)C NMR spectra of the synthesised copolyester were recorded at 300-600 MHz in DMSO-\(d_6\) as solvent.

3.3.5 Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis

MALDI-MS analyses were carried out by using Bruker Daltonics instrument. Dihydroxy benzoic acid was used as matrix and NaI was used as ionizing agent. After optimization, pre-polymers were deposited on MALDI plate by using layer-by-layer method\(^5\)
3.3.6 Thermal analysis

Thermal properties of the copoyesters were analysed on a Perkin-Elmer pyris I Differential Scanning Calorimeter. Each sample is sealed in an aluminum pan and DSC thermogram is recorded at a scanning rate of 20°C/min. Differential scanning calorimetry (DSC) thermograms were recorded in the range of -70 °C to 150 °C under nitrogen atmosphere. Similarly, TGA thermograms were observed under the flow of nitrogen gas (50 ml/min) at a scanning speed of 101°C/min in the range of 50–600°C. The glass transition temperature, T\text{g}, the melting temperature, T\text{m}, and the decomposition temperature, T\text{d}, were measured.

3.3.7 Mechanical properties

The tensile mechanical properties were studied on a Hounsfield tensiometer equipped with a 50 KN load cell at room temperature. The Polymer and Composites thin films samples were prepared according to the ASTM standard (45x5x0.2mm, length x width x thickness) and pulled at a strained rate of 10 mm min\textsuperscript{-1}. The measured values were converted to stress strain and plotted. Young’s modulus was calculated from the initial slope of the curve of the tensile stress versus strain.
3.3.8 Wide angle X-ray diffraction analysis

Wide angle X-ray diffraction studies of the samples were carried out using Siemens D 500 diffractometer with Cu Kα, Ni filtered radiation. The sample is continuously scanned over the 2θ range from 5° to 80° and were utilized for quantitative phase analysis. The crystallite size (D) of the particles was calculated from the XRD line broadening measurement using the scherrer equation:

\[ D = \frac{0.89\lambda}{\beta \cos\theta} \]

Where \( \lambda \) is the wavelength (CuKα), \( \beta \) the full width at half-maximum of the HA line and \( \theta \) the diffraction angle.

3.3.9 Scanning Electron Microscopy

The structure and morphology of the samples were studied using a HITACHI S-3000 scanning electron microscope (SEM). The films were covered with gold coating in order to have good conductivity of the electron beam. Operating conditions were, accelerating voltage 10000V, probe current 45μA and counting time of 60s. The SEM Studies were performed on n-HAp powder, polymer and composite thin films.
3.4.0 Transmission Electron Microscopy

Transmission electron microscope (TEM) (HR-TEM, Model-HITACHI, H-7650) was used to observe the morphology and the particle size of calcined n-HAp powders. For TEM sample preparation, n-HAp powders were ultrasonically dispersed in methanol and drop cast onto a holey carbon support film.

3.4.1 In Vitro assay for Cytotoxicity activity (MTT assay)

Vero Cells (1 × 10⁵/well) were plated in 24-well plates and incubated in 37°C with 5% CO₂ condition. After the cell reaches the confluence, the samples were added and incubated for 24 hours. After incubation, the sample was removed from the well and washed with MEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. The advantages of the MTT procedure are accuracy and reliability and the saving of time. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank.

The % cell viability was calculated using the following formula:

\[
\text{% cell viability} = \left( \frac{\text{Optical density of treated cells}}{\text{optical density of sample}} \right) \times 100
\]
3.4.2 Swelling property and Invitro degradation of the composite

The percentage swelling of the polymers was measured in DMSO. Cylindrical disc of about 7cm were cut using cork borer from purified cross linked polymer film. The discs were pre-weighed to know the initial mass \( W_0 \) and suspended in DMSO. The films were removed from DMSO at different time intervals, blotted dry with filter paper and weighed \( W_s \). The discs were suspended in deionized water for 24 hrs to exchange DMSO and lyophilized for 72 hrs. The dried samples were weighed to find the dry mass \( W_d \). The swelling percentage was calculated using the formula \(^{10}\):

\[
\text{Swelling} \, (\%) = \left[\frac{(W_d - W_s)}{W_s}\right] \times 100
\]

The invitro degradation examined by measuring the mass loss percentage in phosphate buffer solution (PBS) at 37\(^{0}\)C was evaluated by the following procedure: The 10 mm diameter polymer composite discs, punched from the composite thin films and weighed to find initial weight \( W_0 \), were soaked in 10 ml of Phosphate buffer solution (PBS) at 37\(^{0}\)C and kept in a water bath at constant temperature. Samples were taken out on one week intervals, rinsed with de-ionized water and dried to their remaining weight \( W_t \). The PBS was replaced upon sampling each week \(^{11}\).

The mass loss percentage was calculated using the expression:

\[
\text{Mass loss} \, (\%) = \left[\frac{(W_0 - W_t)}{W_t}\right] \times 100
\]
3.4.3 Preparation of POC–HA composite scaffolds

Porous scaffolds were prepared according to the following procedure:\textsuperscript{12}

The porogen NaCl crystals were added to the P$_{15}$–n-HAp-dioxane mixtures to form slurry with a pre-calculated composition of 90\% salt and 10\% (w/v) P$_{15}$–n-HAp(15\%). This P$_{15}$–n-HAp–NaCl-dioxane slurry was then cast into Teflon molds and placed in an oven at 80\(^{\circ}\)C for solvent evaporation. The composite-salt slurry was removed periodically from the oven and mixed thoroughly between 2 and 4 times an hour, a mixing process that generally required 5–7 h depending on the n-HAp content. After mixing, the composites were cured in an oven at 80\(^{\circ}\)C for 7 days. After the curing period, the dried and solidified blocks of mixed P$_{15}$–nHAp and NaCl were removed from the Teflon molds and soaked in distilled water for 4 days in order to leach out the salt. Preparation of porous scaffolds has been successfully processed by using solvent-casting particulate leaching method\textsuperscript{13}. After salt leaching, scaffolds were then taken out of the water and freeze-dried prior to material characterization.
References: