CHAPTER II

EXPERIMENTAL SECTION
II.1. Introduction

This chapter presents details of the chemicals and the experimental procedures used throughout the research programme. We used the techniques such as UV-visible spectrophotometer, Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), thermo gravimetric analysis (TGA), scanning electron microscopy (SEM), and X-ray diffraction studies (X-RD) have been employed. For the characterization methods using to prepare different types of micro beads and microspheres for using drug delivery and silver nano structured hydrogel networks for biological are given in detail.

II.2. Materials

Polymers (Table II.1.), monomers and drugs (Table II.2.), and other chemicals (Table II.3.) used in the research are listed below. The chemicals used were all of analytical grade samples and hence, no attempt was made to purification and double distilled water was used throughout the research.

Table II.1: List of chemicals used in this study and their sources.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Polymers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sodium alginate (Medium M_w.) (NaAlg)</td>
<td>LOBA Chem, Mumbai, India</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan (M_w=800,000) (CS)</td>
<td>Aldrich, USA.</td>
</tr>
<tr>
<td>3</td>
<td>Guar gum (M_w=50,000-8,000,000) (GG)</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>4</td>
<td>Poly(vinyl pyrrolidone) (PVP)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>5</td>
<td>Gelatin (GT)</td>
<td>Fisher Scientific- Mumbai, India</td>
</tr>
<tr>
<td>6</td>
<td>Pectin (PC)</td>
<td>LOBA Chem, Mumbai, India</td>
</tr>
<tr>
<td>7</td>
<td>Poly(vinyl alcohol) (M_w. 1,25,000) (PVA)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
</tbody>
</table>
Table II.2: List of chemicals used in this study and their sources.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Monomers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lignosulfonicacid (LSA)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>2</td>
<td>$N,N'$-Methylene bisacrylamide (MBA)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>3</td>
<td>Acrylamide (AAm)</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td></td>
<td><strong>Drugs</strong></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pyronaridine</td>
<td>Aldrich chemicals, USA</td>
</tr>
<tr>
<td>5</td>
<td>5-Fluorouracil</td>
<td>Aldrich chemicals, USA</td>
</tr>
</tbody>
</table>

Table II.3: List of chemicals used in this study and their sources.

<table>
<thead>
<tr>
<th>Other Chemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Methanol</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>2. Glutaraldehyde (GA)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>3. Hydrochloric acid (HCl)(25% V/V)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>4. Potassium persulphate (KPS)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>5. Potassium dihydrogen ortho phosphate</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>6. Hexane</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>7. Sodium hydroxide</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>8. Tween-80</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>9. Acetone (AR)</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>10. Acetic acid</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>11. Calcium chloride</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>12. Sodium borohydride</td>
<td>Merck, Mumbai-India.</td>
</tr>
<tr>
<td>13. Ammonium persulphate (APS)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>14. Ceric ammonium nitrate (CAN)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
</tbody>
</table>
II. 3. Preparation methods of polymeric micro particulates

The experimental techniques used in this study, preparation of microspheres and micro beads are given below:

II.3.A: Chitosan/guar gum-g-(acrylamide) semi IPN microspheres for controlled release studies of 5-fluorouracil.

II.3.A.1: Synthesis of GG-g-PAm

Guar gum grafted acrylamide was prepared by 2 gms of guar gum was weighed and dissolved in water by stirring overnight. To these solution 0.105 mol acrylamide and $5.47 \times 10^{-4}$ ceric ammonium nitrate were added and stirred well. This reaction mixture is polymerized under
nitrogen atmosphere for 6 h at 70°C. This polymerized polymer was cooled and extracted by precipitating the polymer in acetone and precipitated polymer was dried under vacuum for 24 h.

\[
\% \text{ Grafting} \ (\% G) = \left( \frac{W_1 - W_0}{W_0} \right) \times 100 \quad \text{Eqs.II.1}
\]

\[
\% \text{ Grafting efficiency} \ (\% GE) = \left( \frac{W_1 - W_0}{W_2} \right) \times 100 \quad \text{Eqs.II.2}
\]

Where, \(W_0\), denote the weight of polymer, \(W_1\) graft copolymer and \(W_2\) monomer, respectively.

**II.3.A.2. Preparation of semi IPN microspheres**

Guar gum-g-PAm and Chitosan semi interpenetrating network (semi IPN) microspheres have been prepared a different weight ratio of chitosan and GG-g-PAm was dissolved in the water of certain concentration and left overnight. The two polymer solutions were mixed and stirred well for proper mixing which lead to miscible polymer solution. A known amount of the 5-fluorouracil was dissolved above polymer solution.

The drug loaded blend polymer solution was emulsified into liquid paraffin to form water-in-oil (w/o) emulsion technique \(R_1\) 400 rpm using IXA Motors (India) high-speed stirrer for 30 min in a separate 500 mL beaker containing 100 mL of light liquid paraffin oil, 2 % (w/v) of Tween-80, 1 mL of 0.1 M HCl and the required amount of GA is added. The microspheres formed were filtered, washed repeatedly with hexane and water to remove the oil as well as excess amount of surfactant and the unreacted GA. These microspheres were dried under vacuum at 40°C and stored in desiccators before further analysis.

**II.3.A.3. Estimation of drug loading and encapsulation efficiency**

The drug loaded microspheres (10 mg) were pulverized and incubated in 10 ml 0.02 M phosphate buffer (pH = 7.4) at room temperature for 24 h. The suspension was agitated with agate mortar and filtered through filter paper. The drug solution was assayed.
spectrophotometrically for 5-FU content at the wavelength of 270 nm. The results of % drug loading and encapsulation efficiency were calculated using Eqs. (II.3) and (II.4).

\[
\text{% Drug loading} = \left( \frac{\text{Amount of drug in microspheres}}{\text{Amount of microspheres}} \right) \times 100 - - - - \text{Eqs.II.3}
\]

\[
\text{% Encapsulation efficiency} = \left( \frac{\text{Actual loading}}{\text{Theoretical loading}} \right) \times 100 - - - - \text{Eqs.II.4}
\]

II.3.A.4. Swelling studies

Swelling experiments of semi IPN microspheres were performed in 7.4 pH buffer solution (Figure II). To perform swelling experiments, microspheres were soaked in buffer solution 7.4 pH,(measured with pH meter Thomson Scientific model) several of them were removed from the swelling bottles at different time intervals and blotted carefully with tissue papers (without pressing hard) to remove the surface-adhered buffer solution. The microspheres were then weighed \(W_1\) on an electronic microbalance (Satorous Belam England accurate to ± 0.0001 g).

Figure II.1: pH Mèter, Thermo Scientific.
The microspheres were dried to a constant weight ($W_2$) in an oven maintained at 40 °C for 5 hours. Swelling experiments were repeated thrice for each sample and average values were used in data analysis. The standard deviations (S.D.) in all cases were < 5 %. The weight % water uptake was calculated as:

$$\text{% Water uptake} = \left( \frac{W_1 - W_2}{W_2} \right) \times 100$$

Where $W_1$ is weight of swollen microspheres and $W_2$ is weight of microspheres.

II.3.A.5. In vitro release studies

In vitro release studies were carried out using tablet dissolution tester (LAB INDIA, Mumbai, India) equipped with eight baskets. Dissolution rates were measured at 37± 0.5 °C at constant speed of 100 rpm. Drug releases from the micro beads were carried out in pH 1.2 and 7.4 phosphate buffer solution at 37 °C. At regular intervals of time, sample aliquots were withdrawn and analyzed using UV spectrophotometer (UV-Vis Spectrophotometer, Lab India, UV-3092) at the fixed $\lambda_{\text{max}}$ value of 270 nm.

Figure II.2: Dissolution test apparatus LAB INDIA, DS-8000
After each sample collection, the same amount of fresh medium at the same temperature was added to the release medium to maintain the sink condition. All measurements were carried out in triplicate, and values were plotted with standard deviation errors. The release data fitted into the following Peppas equation [1].

\[
\left( \frac{M_t}{M_\infty} \right) = kt^n \quad \text{Eqs.II.6}
\]

Here, \( M_t/M_\infty \) represents the fractional drug released at time \( t \), \( k \) is a constant characteristic of the drug-polymer system, and \( n \) is an empirical parameter characterizing the release mechanism.
II.3.B: Development of Pectin-poly (vinyl pyrrolidone) blend micro beads for controlled release of 5-FU.

II.3.B.1: Preparation of pectin-poly (vinyl pyrrolidone) micro beads

Pectin (1.8 g) and PVP (0.2 g) were dissolved separately in 25 mL of distilled water until clear solution is formed. The two solutions were mixed to get homogenous solution and taken in hypodermic syringe and added drop wise into aqueous methanol solution (80:20 methanol: water) containing CaCl₂ for 10 sec under constant stirring. The beads were formed and then separated from the solution and washed with water for several times. The resulting beads were dried at 40 °C until to attain constant weight was reached. The 5-FU drug loaded beads were prepared by adding to blend solution before crosslinking. Various formulations were prepared by different amounts of PVP, varying % of drug, and crosslinker variation (Table IV.1).

II.3.B.3. Swelling studies of micro beads

As mentioned in II.3.A.3.

II.3.B.2. Estimation of drug loading and encapsulation efficiency

As mentioned in II.3.A.4.

II.3.B.4. In vitro release studies

As mentioned in II.3.A.5.

II.3.C: Sodium alginate-g-(lignosulfonicacid-co-acrylamide) semi-IPN micro beads for controlled release of pyronaridine

II.3.C.1: Preparation of NaAlg-g-(LSA-co-Am) semi-IPN micro beads loaded with Pyronaridine

The beads were prepared in two steps by ionic gelation method by using calcium chloride as counter ion. Briefly, first step sodium alginate dispersion was prepared in distilled water stirred by overnight and then pyronaridine was added to the NaAlg solution. Second step is to...
prepare the poly (LSA-co-Am) by adding the lignosulfonic acid in 5ml of distilled water, and then acrylamide was added followed by KPS as initiator and stirred at 65 °C for polymerization until the clear solution obtained. This copolymer was added to the NaAlg solution under the constant stirring. The drug-polymer dispersion was added via a 22-gauze needle in to 1:4 ratio of calcium chloride/methanol solution. The CaCl₂ concentrations were used 2%, 3% and 4% w/v for each formulation.

The droplets were gelled in to discrete, spherical beads upon contact with CaCl₂. Each batch of beads was left for 20 min to cure in CaCl₂ solution. Here Na⁺ ions is replaced by the Ca²⁺ ions to form the three dimensional network. The CaCl₂ were decanted and each was washed two times with 200 ml of distilled water, and then dried in hot oven at 40 °C for 24 hours [2, 3].

II.3.C.2. Estimation of drug loading and encapsulation efficiency:
As mentioned in II.3.A.4.

II.3.C.3. Swelling studies
As mentioned in II.3.A.3.

II.3.C.4. In vitro release studies
As mentioned in II.3.A.5.


II.3.D.1. GT-co-LSA microspheres by desolvation

2.0gms of gelatin are dissolved in 20 mL of distilled water under gentle heating. First desolvation step is initiated by the addition of 20 mL acetone. After sedimentation of precipitated gelatin fraction for certain time, the supernatant consisting of some desolvated gelatin as well as gelatin in solution has to be discarded. Now, the sediment is getting dissolved again by the addition of 20 mL water. In situ gelatin microspheres are formed during the second desolvation step by drop wise addition of 30 mL acetone under constant stirring. After 10 min, 400 μL of
glutaraldehyde (25%) are added to reaction mixer to crosslink the gelatin microspheres. Finally after stirring 30 min, 0.1 gm of lignosulfonic acid was added to the above mixer and after 30 min to form GT-co-LSAmicrospheres and redispersion in acetone/water (30/70).

II.3.D.2. Estimation of drug loading and encapsulation efficiency
   As mentioned in II.3.A.4.

   As mentioned in II.3.A.5.

II.4. Characterization Techniques
II. 4.1. Fourier transforms infrared spectroscopic analysis

   Infrared spectroscopy is one of the most powerful analytical tools, which provides the possibility of chemical identification. It provides information regarding the structure of a molecule. FTIR worked based upon the simple principle that a chemical substance shows selective absorption in the infrared region giving rise to absorption bands called an absorption spectrum, over a wide wavelength range.

![FTIR Spectrophotometer (Perkin Elmer Spectrum Two, UK)](image)

Figure II.3: FTIR Spectrophotometer (Perkin Elmer Spectrum Two, UK)
Prepared micro particulates (microspheres and micro beads) were finely ground with spectroscopic grade KBr, and to prepare pellets using a hydraulic pressure of 600 kg/cm². Different bands will be present in the spectrum, which will correspond to the characteristic functional groups and bonds present in a chemical substance. In this study infrared absorption spectra of polymeric matrices were obtained using a FTIR spectrophotometer (PerkinElmer Spectrum Two, UK).

II.4.2. Differential scanning calorimetric (DSC) studies

DSC curves of pure beads, 5-Fluorouracil, and drug loaded beads were recorded using TA instruments sequential thermal analyzer (Model-SDT Q600, UK). The sample was weighed between 10 to 12mg. The samples were heated from 50⁰ to 400⁰C Analysis of the samples was performed at heating rate of 10⁰C/min under N₂ atmosphere at a purging rate of 100 mL/min.

**Fig II.4: Differential scanning calorimetric (DSC)**
II.4.3. X-ray diffraction studies (X-RD)

The X-RD measurements of plain drug, drug-loaded microspheres and plain microspheres were recorded with a Rigaku Geiger flex diffractometer (Tokyo, Japan) equipped with Ni-filled Cu Kα radiation (λ=10518Å°). The dried particulates of uniform thickness were mounted on a sample holder, and the patterns were recorded in the range 0 to 50 degrees.

II.4.4. Scanning electron microscopy (SEM)

To determine the particle size and size distribution, drug loaded micro beads were taken on a glass slide and their sizes were measured using an optical microscope under regular polarized light. SEM images of micro beads were recorded using a JSM 6400 SEM (JEOL Ltd., Akishima, Tokyo, Japan) at X50 and X500 magnifications. Working distance of 8.5-9.5 mm was maintained and the acceleration voltage used was 10 kV, with the secondary electron image (SEI) as a detector.
References

