2. Materials and Experimental Methods

2.1. INTRODUCTION

This chapter deals with the experimental procedures used for the preparation of polymeric hydrogel metallic and bimetallic nanocomposites for antimicrobial applications. The nanocomposites were characterized by using UV visible spectrophotometer, Fourier transform infrared spectroscopy (FT-IR), Thermo gravimetric analyses (TGA), Scanning Electron Microscope (SEM), X-ray diffraction (XRD) and Transmission Electron Microscope (TEM) and the details of the instruments were well described.

2.2. Materials

Polymers, monomers, chemicals, metal salts, solvents that were used for the present work along with their abbreviations are presented in Table 2.1. All the chemicals are of analytical grade and were used as received without any further purification. Double distilled water collected in the laboratory was used throughout the research work.

Table 2.1: List of Chemicals and their sources

<table>
<thead>
<tr>
<th>S.No</th>
<th>Polymer</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acrylamide (AM)</td>
<td>s.d. fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>2</td>
<td>2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS)</td>
<td>-do-</td>
</tr>
<tr>
<td>3</td>
<td>N,N1-methylenebisacrylamide (MBA)</td>
<td>-do-</td>
</tr>
<tr>
<td>4</td>
<td>ammonium persulphate (APS)</td>
<td>-do-</td>
</tr>
<tr>
<td>5</td>
<td>N,N1,N1-teramethylethylenediamine (TMEDA)</td>
<td>-do-</td>
</tr>
<tr>
<td>6</td>
<td>Silver nitrate(AgNO₃)</td>
<td>-do-</td>
</tr>
<tr>
<td>7</td>
<td>sodium borohydride (NaBH₄)</td>
<td>-do-</td>
</tr>
<tr>
<td>8</td>
<td>Gelatin</td>
<td>-do-</td>
</tr>
<tr>
<td>9</td>
<td>Acetic acid</td>
<td>-do-</td>
</tr>
<tr>
<td>10</td>
<td>Chitosan (CS) (high MW, &gt;75% deacetylated)</td>
<td>Sigma Chemical, St. Louis, USA</td>
</tr>
<tr>
<td>11</td>
<td>Gold chloride (H.AuCl4. XH₂O)</td>
<td>SRL Pvt Ltd,Mumbai</td>
</tr>
<tr>
<td>12</td>
<td>CopperSulphate(CuSO₄)</td>
<td>SRL Pvt Ltd,Mumbai</td>
</tr>
</tbody>
</table>
2. Materials and Experimental Methods

2.3. Preparation of hydrogels

Acrylamide (AM) hydrogels were prepared by aqueous redox free radical polymerization using AM, a crosslinker N,N-methylene bisacrylamide, MBA and an ammonium per sulphate (APS) initiator the system. In a typical polymerization reaction acrylamide (8.83 mM) was dissolved in 2mL of distilled water and taken in a 250mL beaker. To this 0.2412 of mM AMPS was added as a co-monomer. Then 0.0648 mM of MBA as a hydrophilic crosslinker and also 2.191 mM of APS, 0.172mM of TMEDA as an initiating pair system were added. The temperature of the system was raised to 50°C for 30 min. After the reaction has been completed, the hydrogel was immersed in distilled water at room temperature for 24 h to remove the unreacted materials present in the hydrogel network. Finally, the hydrogel was dried at room temperature for 2 days. Similarly other hydrogels were prepared by Using Natural Polymers chitosan and gelatin.

2.4 Preparation Metal nanocomposite hydrogels

2.4.1 Silver (Ag) nanocomposite hydrogels

In this process dry hydrogels were equilibrated with water for 24 h and the swollen hydrogels were immediately transferred to a beaker containing 50ml of aqueous AgNO₃ solution and then allowed to equilibrate for 1 day. In the step, the silver ions are being exchanged from solution to the gel networks. These hydrogel-loaded silver salts were then finally transferred to a beaker containing 50ml of concentration NaBH₄ aqueous solution and kept in the beaker for 4 h in order to reduce the silver ions into silver nanoparticles. Finally, the hydrogel was dried at ambient temperature for 2 days. The dried hydrogel were kept in a desiccators/ closed container.

2.4.2 Gold (Au) nanocomposite hydrogels
The dry hydrogels were equilibrated with water for 24 h and the swollen hydrogels were immediately transferred to a beaker containing 50 ml of aqueous H.AuCl₄, XH₂O solution and then allowed to equilibrate for 1 day. In the step, the gold ions are being exchanged from solution to the gel networks. These hydrogel-loaded gold salts were then finally transferred to a beaker containing 50 ml of concentration NaBH₄ aqueous solution and kept in the beaker for 4 h in order to reduce the gold ions into silver nanoparticles.

### 2.4.3 Copper (Cu) nanocomposite

Established amounts of dried hydrogels were immersed for 24 hours in large amount of distilled water for 2 days to swollen to full extent. Then, these hydrogels were transferred to a beaker containing 50mL Copper sulphate (CuSO₄) aqueous solutions to permit equilibrating for 1 day. The gel was removed and transferred to NaBH₄ solution for reducing the metals for 4h. The gel was dried at room temperature. It was powdered and used for characterization.

### 2.5 Preparation of Bimetallic Nanocomposites

#### 2.5.1 Au-Ag Nanocomposites

Required amounts of dried hydrogel were immersed in large amount of distilled water for 2 days to swell to full extent. The swollen hydrogel was transferred into a 250mL beaker and immersed in 20mL of 5mM AgNO₃ and 10mL of H.AuCl₄, XH₂O 5mM aqueous solutions to permit equilibrating for 1 day. The hydrogel was removed and transferred to NaBH₄ solution for reducing the metal ions for 4hrs. The hydrogel nanocomposite was dried at ambient temperature. It was powdered and used for characterization.

#### 2.5.2 Cu-Ag Nanocomposites

Established amounts of dried hydrogels were immersed for 24 hours in large amount of distilled water for 2 days to swollen to full extent. Then, these hydrogels were transferred to a
2. Materials and Experimental Methods

beaker containing 50 mL of 5 mM AgNO₃ and Copper sulphate (CuSO₄) aqueous solutions to permit equilibrating for 1 day. The gel was removed and transferred to Na BH₄ solution for reducing the metals for 4Hrs. The gel was dried at room temperature. It was powdered and used for characterization.

2.6. Swelling studies

The equilibrium swelling behaviour of the hydrogels was measured by gravimetric method. The swelling characteristic of the hydrogels provides the information of network integrity before and after loading of metal salt and precipitation of metal nanoparticles inside the networks. Completely dried and accurately weighed hydrogels were immersed in a 100ml beaker containing twice distilled water at room temperature, until the hydrogel reached equilibrium swelling at ambient temperature for 24h. The equilibrium swelling ratio of hydrogels was measured by gravimetric method. The swelling characteristic of the hydrogels provides the information about the hydrogel network integrity after loading metal salts and formation of metal/bimetallic nanocomposites inside the hydrogel networks. To study this phenomenon, the same weights of dried hydrogels were equilibrated in distilled water at ambient temperature for 2 days.

Swelling ratio (Q) = \( \frac{\text{The weight of the swollen hydrogel}}{\text{The weight of the dry hydrogel}} \)

2.6.1. Characterization techniques

2.6. 2 UV-vis spectroscopy

UV-VIS spectroscopy is useful to characterize the absorption, transmission, and reflectivity of a variety of technologically important materials, coatings, thin films and solutions. When white light passes through or is reflected by a colored substance, a characteristic portion of the mixed wavelengths is absorbed. The remaining light will then assume the complementary color to the
2. Materials and Experimental Methods

wavelength(s) absorbed. Colour is the most immediate observable properties for certain metal nanoparticles dispersions. This colour effect is based upon the surface plasmon resonance or particle plasmon resonance. Noble metal nanoparticles such as Au, Ag and Cu have characteristic colours that are related to their particle size, shape, and particle distance and on the matrix material. For the metals, observation of UV-visible spectra will be a useful complement to other methods in characterizing the nanomaterials. For example, information on the formation of bimetallic nanoparticles can be derived from this technique. The uv-visible spectra of the bimetallic nanoparticles are not simple sum of the two monometallic nanoparticles. If two separate metallic phases are present, the absorption spectrum would be characterized by double peaks due to the Surface Plasmon Resonance (SPR) of single metal nanoclusters provided the two metals have characteristic SPR. Comparison of spectra of bimetallic nanoparticles with the spectra of physical mixtures of the respective monometallic particles can confirm the formation of bimetallic nanoparticles. The UV-vis spectra are recorded on an ELICO SL 210 Model UV-vis spectrophotometer (The Elico co, Hyderabad, India) available in the Department of Polymer Science and Technology, Sri Krishnadevaraya University, Anantapur 515003, India.

Fig 2.1 Doublebeam SpectroPhotometer (S.K.University, Anantapur, India)
2.6.3 Application

This Instrument finds place for any spectro photometric or colorimetric measurement where applications require very high-resolution bandwidth and cGLP compliance is mandatory.

Organic compounds in Biological matter.

Glucose, Fructose, Carbohydrates, Proteins etc., in Foods.

Edible dyes, alcohols etc., in Beverages Toxic elements like Cadmium, Lead, Arsenic and Mercury etc., in Effluents.

Micronutrients like N, P, K, Ca, Mg, Zn, Cu, B, Mo, etc., in Agricultural soil, Plants etc.

Constituents in compositions used in Metallurgy, Fertilizer, Pesticide, Chemical, Petro Chemical, Steel, Cement, Glass & Other Industries.

Edible dyes, alcohols etc., in Beverages

Life science applications.

2.6.4 Fourier Transform Infrared (FT-IR) studies

FT-IR is a powerful tool for identifying types of chemical bonds and functional groups present in the compound. FT-IR is an important record which gives sufficient information about the structure of a compound, or it can be employed to establish compounds or to determine the structure of a new compound formed. By interpreting the infrared absorption spectrum, the chemical bonds present in a molecule can be determined. The wavelength of light absorbed is characteristic of the chemical bond.

FT-IR spectral measurements were performed with a Bomem (MB-3000, Canada) spectrophotometer (Fig 2.2). Polymeric MNPs were finely ground with KBr to prepare pellets under a hydraulic pressure of 400kg for MNPs and spectra were scanned between 4000 and
2. Materials and Experimental Methods

600 cm\(^{-1}\).

![FTIR Spectrophotometer](image)

**Fig 2.2:** FTIR Spectrophotometer (Bomem, Model: MB3000, Canada). (S.K.University)

### 2.6.5 Thermal analysis (TGA)

TGA is a simple analytical technique that measures the weight loss (or weight gain) of a material as a function of temperature. This is performed on samples to determine change in degradation temperature. This analysis relies on a high degree of three measurements: weight, temperature, and temperature change. A derivative weight loss curve may be used to analyze the temperature at which weight loss is most apparent. As materials are heated, they may lose weight from a simple process such as drying, or from chemical reactions that liberate gases. Some materials can gain weight by reacting with the atmosphere in the testing environment. Since weight loss and gain are disruptive processes to the sample material or batch, knowledge of the magnitude and temperature range of those reactions are necessary in order to design adequate thermal ramps and holds during those critical reaction periods.
TGA curves of plain hydrogels, MNPs were recorded using Rheometric scientific (STA, TG instruments, SDT Q_{600}, UK) instrument (Fig 2.7). Thermograms were recorded between 500\(^\circ\)C and 500\(^\circ\)C at a heating rate of 10\(^{\circ}\)C/min under nitrogen atmosphere.

**Fig2.3:** DTA analyzer (STA, TA. Instruments, SDT Q_{600}) (S.K.University)

### 2.6.6 X-Ray Diffraction (X-RD) studies

X-ray diffraction (XRD) is a method of choice to investigate the solid structure of metal nanoparticles and morphology of polymer in polymer-metal nanocomposites. The phase changes with increasing diameter of nanoparticles and the crystal structure can be determined with XRD. The polymer and the metal nanoparticles can be identified by their characteristics peaks in XRD patterns. This technique can investigate the formation of bimetallic nanoparticles as opposed to a mixture of monometallic nanoparticles, since the diffraction pattern of the physical mixtures which may consist of overlapping lines of the two individual monometallic
nanoparticles and is distinct from that of the bimetallic nanoparticles. Information on the morphology of the polymer constituent of the composite could be derived also from XRD. The technique can reveal the degree of crystallinity in the polymer component of the nanocomposites as sharper peaks with increased relative nanocomposite. The technique gives information on the change of the nanoparticles content corresponding to the matrix depth from the surface of the nanocomposites.

X-Rd measurements were carried out for dried and finely grounded hydrogel nanocomposite samples on a Rikagu diffractometer (Cu radiation, \( \lambda = 0.1546 \text{ nm} \)) running at 40 kV and 40 mA at Indian Institute of Science, Bangaluru.

### 2.6.7 Scanning Electron Microscope (SEM)

The scanning electron microscope (SEM) is a type of electron microscope capable of producing high resolution images of a sample surface. Due to the manner in which the image is created, SEM images have a characteristic three-dimensional appearance and are useful for judging the surface structure of the sample.

![Fig 2.4 Scanning electron microscopy JOEL MODEL JSM 840A](image-url)
The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. In most applications, data are collected over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques.

Accelerated electrons in an SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. These signals include secondary electrons (that produce SEM images), backscattered electrons (BSE), diffracted backscattered electrons (EBSD that are used to determine crystal structures and orientations of minerals), photons (characteristic X-rays that are used for elemental analysis and continuum X-rays), visible light (cathodoluminescence--CL), and heat. Secondary electrons and backscattered electrons are commonly used for imaging samples: secondary electrons are most valuable for showing morphology and topography on samples and backscattered electrons are most valuable for illustrating contrasts in composition in multiphase samples (i.e. for rapid phase discrimination).

As with other scanning electron microscopy, the JSM-840 examines structure by bombarding the specimen with a scanning beam of electrons and then collecting slow moving secondary electrons that the specimen generates. These are collected, amplified, and displayed on a cathode ray tube (CRT, typically a slower version of the picture tube of a television set) although now, most are driven by PCs and these computer-generated images are displayed on
2. Materials and Experimental Methods

LCDs. The electron beam is scanned using a raster pattern so that an image of the surface of the specimen is formed. Specimen preparation typically includes drying the sample and making it conductive to electricity, if it is not already. Photographs are taken at a very slow rate of scan in order to boost the signal and capture greater resolution.

SEM is typically used to examine the *external* structure of objects that are as varied as biological specimens, rocks, metals, ceramics and almost anything that can be observed in a dissecting light microscope. The JEOL JSM-840 is used for conventional samples of normal sizes using the airlock. These specimens are usually mounted on multiple "stubs" (up to 4 specimen stubs fit) and each are observed individually. An alternative for large specimens is to vent the column and use the specimen drawer, which allows silicon disks and other objects to be viewed, but takes longer to pump as the whole specimen viewing area is brought to room pressure. SEM analysis is considered to be "non-destructive"; that is, x-rays generated by electron interactions do not lead to volume loss of the sample, so it is possible to analyze the same materials repeatedly.

SEM micrographs of (MNP,biMNP)s obtained under high resolution (Mag: 300X, 5kv) Using JOEL MODEL JSM 840A, scanning electron microscope (SEM), equipped with phoenix energy dispersive. SEM micrographs were taken at National Chemical laboratory pune and Hyderabad Central University, Hyderabad.

2.6.8 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy technique whereby a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a
fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera. TEMs are capable of imaging at a significantly higher resolution than light microscopes, owing to the small de Broglie wavelength of electrons. This enables the instrument’s user to examine fine detail—even as small as a single column of atoms, which is tens of thousands times smaller than the smallest resolvable object in a light microscope. TEM forms a major analysis method in a range of scientific fields, in both physical and biological sciences. TEMs find application in cancer research, virology, materials science as well as pollution, nanotechnology, and semiconductor research.

Transmission electron microscopy images for hydrogel-metal nanocomposites (MNPs) were recorded using a Tecnai F 12 transmission electron microscope operating at an acceleration voltage of 15 kV. For TEM measurements, samples were prepared by dropping 10–20 μl of finely grinded MNP solution on a copper grid and dried at room temperature after removing excess solution using filter paper.

Fig 2.5 Transmission electron microscopy Tecnai F 12
2. Materials and Experimental Methods

2.7 Antibacterial activity Test

2.7.1. Disc method

Nutrient agar medium prepared by mixing peptone (5.0 g), beef extract (3.0 g), and sodium chloride (NaCl) (5.0 g) in 1000 ml distilled water and the pH was adjusted to 7.0. Finally, agar (15.0 g) was added to the solution. The agar medium was sterilized in a conical flask at a pressure of 15 lbs for 30 min. This medium was transferred into sterilized petri dishes in a laminar air flan. After solidification of the media, bacillus culture was streaked on the solid surface of the media. To this inoculated petri dish, one drop of gel particle solution (20 mg/10 ml distilled water) was added using 50-μl tip and incubated for 2 days at 37°C in the incubation chamber.

2.7.2 Absorbance count method

The effect of bacterial growth of bacillus in mineral salts medium (MSM) was studied in the presence of nanoparticles (Ag, Au, Au-Ag nanoparticles). This medium was prepared by the following composition:

\[\begin{align*}
\text{NH}_4\text{NO}_3 (1.5 \text{ g}), & \quad \text{KH}_2\text{PO}_4 (2.5 \text{ g}), \quad \text{K}_2\text{HPO}_4 (0.5 \text{ g}), \quad \text{NaCl} (1.0 \text{ g}), \quad \text{MgSO}_4 (1.5 \text{ g}), \quad \text{MnSO}_4 (0.01 \text{ g}), \\
\text{FeSO}_4 (0.05 \text{ g}), & \quad \text{and CaCl}_2 (0.05 \text{ g}) \quad \text{were added to 1000 mL of distilled water and the pH was adjusted to 7.0. Then, yeast extract (0.01\%) was added for bacterial growth. After that the MSM medium was sterilized and 50 mL of solution was transferred into a sterilized 250-mL conical flask. Afterward, 100 μL bacillus bacterium was added into the media. Finally, 100 μL of nanoparticles solution (10 mg/5 mL distilled water) or its equivalent nanoparticles suspension was added, and the optical density of the bacterial medium was measured using a UV-vis spectrophotometer at 600 nm.} \end{align*}\]