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## Chapter 2 Methodology and Instrumentations

### 2.1 Methods and Materials

#### 2.1.1 Materials used for Synthesis and other experiments of Hydrogels

Bovine Serum Albumin (BSA) (lyophilized powder,  $\geq 96\%$ ), multiwalled carbon nanotubes ( $\geq 99.9$  wt.%), sodium chloride, hydrochloric acid, sodium hydroxide, disodium hydrogen phosphate, sodium dihydrogen phosphate, dimethyl sulfoxide anhydrous (DMSO), fetal bovine serum (FBS), citrate buffer, ethanol, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), sodium pyrophosphate, phenazine methosulfate, sodium pyruvate, trichloroacetic acid, streptozotocin (STZ), and nicotinamide adenine dinucleotide (NADH) were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Spray-dried aloe vera gel powder 200X (*Aloe Barbadensis*) was obtained from Heilen Biopharm, Gandhinagar, India. Hyaluronic acid (HA) sodium salt (98%, 8-15 kD), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxy succinyl imine (NHS), ammonia, and additional reagents were sourced from SRL. Wheat straw was purchased from Profodd Retail Pvt. Ltd., Varanasi, India. Ultrapure water with a resistivity of  $12 \text{ M}\Omega \cdot \text{cm}$ , produced using a Millipore water purification system, was used for all aqueous solution preparations, and all chemicals used were of analytical grade and employed without further purification.

#### 2.1.2 Synthesis of Hydrogels

To prepare hydrogels, BSA (80 mg/mL) was first dissolved in PBS buffer to create an aqueous solution. For BSA-AV hydrogels, different w/v% concentrations of AV gel powder were

added to the BSA solution, stirred until homogeneous, and then transferred to glass vials. The solutions were heated to 70°C for 30 to 45 minutes, undergoing a vial inversion test to confirm gelation, resulting in hydrogels with varied rheological properties based on AV content.

For the HA hydrogel, sodium salt of HA was dissolved in deionized water, then EDC and NHS were added to activate the carboxylic groups, followed by stirring for 24 hours in the dark. The solution was then dialyzed against deionized water for 48 hours and lyophilized at -80°C overnight. Functional group activation and chemical structure were analysed using <sup>1</sup>H NMR spectroscopy. In preparing HA-BSA hydrogels, BSA was dissolved in PBS buffer with 150mM NaCl, followed by the addition of HA powder at various concentrations. The mixtures were then transferred to glass vials and heated to 70°C to form hydrogels, confirmed by the vial inversion test. The composition for the BSA-HA hydrogel is summarized in Table 2.1.

Table 2. 1 Formulation of composite hydrogels of BSA and HA

Sample Name	HA concentration (%W/V)	BSA concentration (%W/V)
3H-8B	3	8
2H-8B	2	8
1H-8B	1	8
H	3	-
8B	-	8

For cellulose extraction, wheat straw was processed following Ma et al. (2011). The straw was washed, dried at 70°C, ground, and sifted through a 100 mesh. The powder was soaked in 12% ammonia solution (1:12 mass ratio) for two days, followed by nitrate treatment (1 mol/L) at 100°C for an hour, filtered, washed, and dried using a refrigerant dryer to yield wheat straw cellulose (WSC). To prepare the BSA/Cellulose/CNT hydrogel, CNT at varying

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concentrations was added to an aqueous BSA solution (PBS buffer with 150mM NaCl) and probe-sonicated for 30 minutes to achieve functionalization and dispersion. Cellulose was then added to the mixture, which was incubated at 72°C to promote hydrogel formation, confirmed by a vial inversion test.

## **2.2 Physiochemical Characterization Techniques**

### **2.2.1 Rheology**

Rheology is the study of the flow and deformation of materials, playing a crucial role in understanding the properties of hydrogels. Various rheological tests, including amplitude sweep, frequency sweep, and temperature sweep, provide valuable insights into the mechanical behavior and stability of these hydrogels.

The amplitude sweep test evaluates the material's response to increasing strain while keeping the frequency constant[14]. This test helps determine the linear viscoelastic region (LVER) of the hydrogel, where the material behaves elastically and retains its structure. During the test, the hydrogel sample is subjected to oscillatory shear stress at a constant frequency, with the strain amplitude gradually increased until a transition occurs from elastic (solid-like) behavior to viscous (liquid-like) behavior. The critical strain point at which this transition happens indicates the limits of the linear viscoelastic behavior. The results are typically plotted as storage modulus ( $G'$ ) and loss modulus ( $G''$ ) against strain amplitude, with a stable region suggesting that the material can withstand deformation without significant structural changes, while a sharp decrease in  $G'$  indicates the onset of yielding or damage[15].

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In the frequency sweep test, the material's response to varying frequencies is examined while maintaining a constant strain. This test helps to understand the viscoelastic behavior of hydrogels over a range of deformation rates. The hydrogel sample is subjected to oscillatory shear stress with varying frequencies, typically ranging from 0.1 to 10 Hz or more. Measurements of the elastic (storage modulus,  $G'$ ) and viscous (loss modulus,  $G''$ ) responses are taken at each frequency. The results are plotted with  $G'$  and  $G''$  against frequency. A higher  $G'$  compared to  $G''$  indicates solid-like behavior, while a higher  $G''$  suggests a more liquid-like behavior. The crossover point, where  $G'$  equals  $G''$ , provides insights into the material's transition from elastic to viscous behavior.



Figure 2.1 Anton Paar MCR 72 rheometer

The temperature sweep test evaluates how the rheological properties of hydrogels change with temperature, which is crucial for understanding the thermal stability and behavior of the hydrogel under varying environmental conditions. In this test, the hydrogel sample is

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subjected to oscillatory shear at a constant frequency while the temperature is gradually increased or decreased. Measurements of  $G'$  and  $G''$  are taken at each temperature point. The results are plotted with temperature on the x-axis and  $G'$  and  $G''$  on the y-axis, providing insights into the thermal gelation or sol-gel transition temperature of the hydrogel. A significant change in  $G'$  and  $G''$  with temperature can indicate a phase transition, which is essential for applications involving temperature fluctuations. A Rheometer (**Anton Paar MCR 302, Austria**) was used with a 25 mm diameter plate and a 1 mm gap between plates as depicted in figure 2.1.

### 2.2.2 Scanning Electron Microscope (SEM)

Scanning Electron Microscopy (SEM) is an essential imaging technique used to examine surface structure and morphology at a high resolution, offering detailed, three-dimensional images of various materials. SEM operates on the principle of electron-matter interactions, where a focused beam of high-energy electrons is directed onto a sample's surface. Typical experimental setup has been shown in figure 2.2. When these electrons interact with the atoms within the sample, they produce several detectable signals, primarily secondary electrons, backscattered electrons, and X-rays. Secondary electrons (SE), which are low-energy electrons ejected from the sample's outer shell, are highly sensitive to surface features, providing detailed information on surface morphology and textures. Backscattered electrons (BSE), on the other hand, are high-energy electrons reflected directly from the sample's surface, offering insights into compositional contrasts since heavier elements reflect more electrons than lighter elements[15].

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In SEM, an electron gun at the top of the microscope column emits electrons, which are accelerated by a high-voltage field and focused into a narrow beam using electromagnetic lenses. This focused beam scans in a raster pattern over the sample surface. As the electrons interact with the sample, detectors capture the emitted signals and convert them into an image, which is displayed on a computer screen. The interaction of electrons with the sample can also generate X-rays, which can be analyzed to identify the elemental composition of the sample through Energy Dispersive X-ray Spectroscopy (EDS), a complementary technique often coupled with SEM.

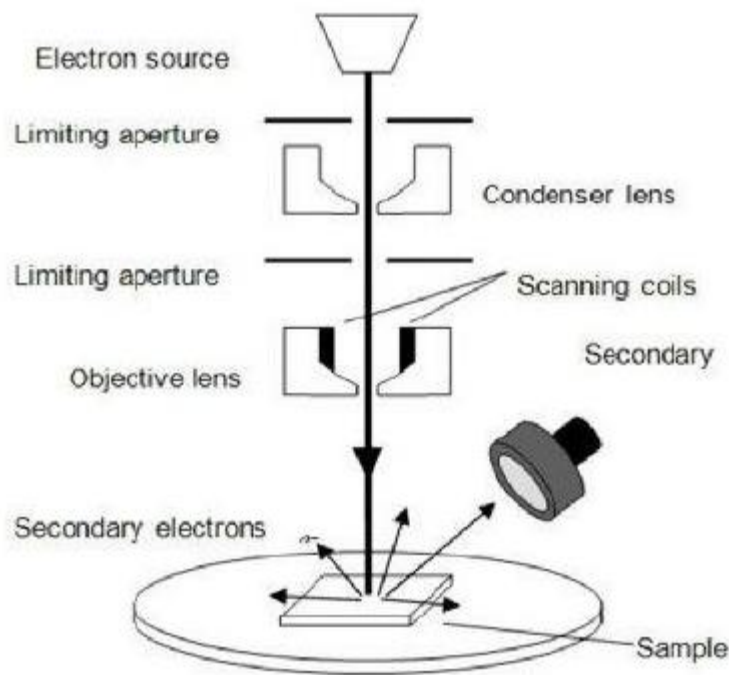


Figure 2.2 Experimental setup for scanning electron microscopy [16]

One of SEM's strengths is its high depth of field, which makes it suitable for imaging rough or uneven surfaces, offering images that appear three-dimensional and detailed across a wide area. SEM images have high spatial resolution, often in the nanometer range, making the

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technique invaluable for applications in materials science, biology, and biomedical engineering. For instance, in biomaterials research, SEM is often used to study surface modifications, particle sizes, and morphological characteristics of hydrogels, polymers, and other materials intended for medical applications.

Imaging was performed with the Scanning Electron Microscope (SEM), **JEOL JSM-7610F model from Japan**, offering a resolution of up to 1  $\mu\text{m}$ .

### 2.2.3 Fourier Transform Infra-Red Spectroscopy

Fourier-Transform Infrared (FTIR) Spectroscopy is an analytical technique widely used for identifying and characterizing materials based on their molecular vibrations. By passing

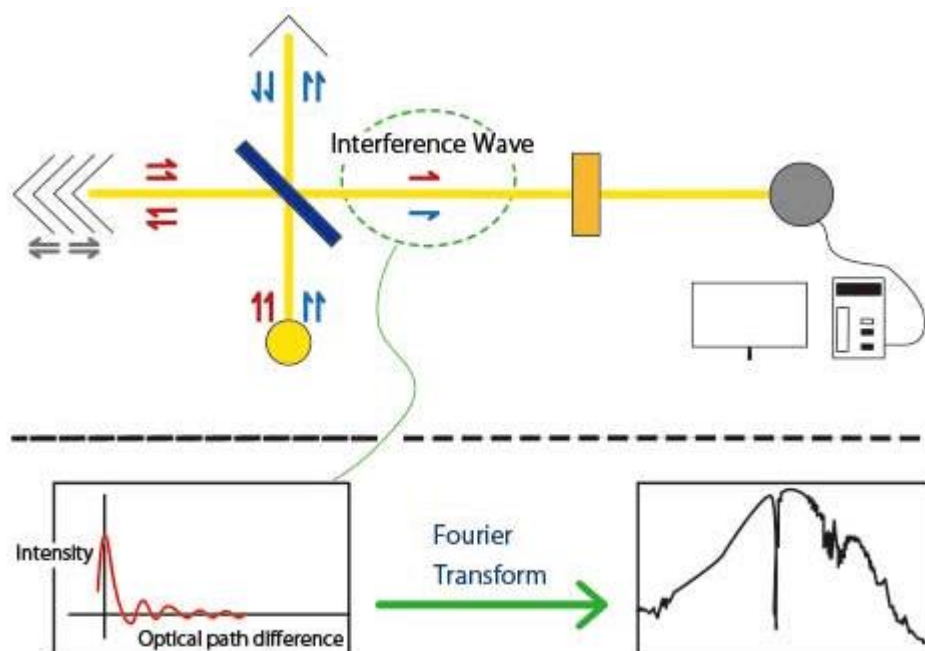


Figure 2.3 Instrumental setup for FTIR spectroscopy [17]

infrared radiation through a sample, FTIR detects specific wavelengths absorbed by the sample's molecules, causing them to vibrate in unique ways. These vibrations correspond to different functional groups within the molecules, producing a unique absorption pattern—or

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"fingerprint"—for each substance[16]. The resulting spectrum, which plots the intensity of transmitted or absorbed IR radiation against frequency (measured in wavenumbers), reveals peaks that correlate with specific molecular bonds, making it possible to identify and analyze the chemical composition of the sample. Typical experimental setup has been shown in figure 2.3.

FTIR operates by applying a Fourier Transform, which converts raw data from the time domain (interferogram) to the frequency domain, producing a detailed spectrum. This mathematical transformation makes FTIR faster and more sensitive than traditional dispersive infrared spectroscopy, allowing for the efficient analysis of complex samples. One of the technique's versatile aspects is that it can accommodate a range of sample types—solids, liquids, and gases—often using Attenuated Total Reflectance (ATR), a method that simplifies sample preparation by enabling surface-level analysis without extensive handling[17].

FTIR spectroscopy has broad applications across scientific disciplines. In materials science, it identifies unknown compounds, monitors chemical changes, and verifies the presence of specific materials by comparing spectra with reference libraries. In pharmaceuticals, it ensures the quality and purity of compounds, while in biomedical research, FTIR is used to characterize functional groups in biomaterials like hydrogels, confirming the successful incorporation of biomolecules or polymers. Additionally, FTIR plays an essential role in environmental science, where it detects pollutants by identifying characteristic absorption bands of contaminants in air, water, and soil. The rapid, non-destructive nature of FTIR spectroscopy, combined with its high specificity, makes it a valuable tool in material characterization and quality control, underscoring its importance in modern scientific research.

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FTIR spectroscopy is particularly valuable in hydrogel research, where it serves as a critical tool for characterizing and confirming the composition and structural properties of hydrogel materials. By identifying the functional groups present, FTIR provides insights into the chemical bonds formed during hydrogel synthesis and crosslinking processes, essential for verifying that desired modifications or components, such as polymers, proteins, or nanoparticles, have been successfully incorporated. For example, in protein-based hydrogels, FTIR can detect amide bonds, a primary indicator of protein structure, as well as secondary structures like  $\alpha$ -helices and  $\beta$ -sheets, which are often linked to bioactive properties in wound healing applications.

The Fourier Transform Infrared Spectroscopy (FTIR) used in this study was the **Nicolet iS5** model from **Thermo Electron Scientific Instruments LLC**, which operates across a wavelength range of 4000–400  $\text{cm}^{-1}$ .

#### 2.2.4 Texture Analyser for Mechanical properties

A texture analyzer is an essential instrument used to evaluate the mechanical properties of hydrogels, providing crucial data on their texture, strength, elasticity, and overall performance, particularly in biomedical applications such as wound dressings and drug delivery systems. This equipment measures various mechanical characteristics, including tensile strength, compression, elongation, and adhesive properties, which are vital for assessing how a hydrogel will behave under physiological conditions, which are vital for assessing how a hydrogel will behave under physiological conditions[18]. Typical experimental setup has been shown in figure 2.4.

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In the context of hydrogels, a texture analyzer typically employs different testing methods, such as compression tests to evaluate the material's ability to withstand external forces without deforming or breaking. This is particularly important for hydrogels used in wound dressings, where the material needs to provide support and protection while remaining flexible and comfortable against the skin. Additionally, tensile tests can be performed to assess the hydrogel's stretchability, which is crucial for applications that require dynamic movements, such as in orthopedic or soft tissue repairs.

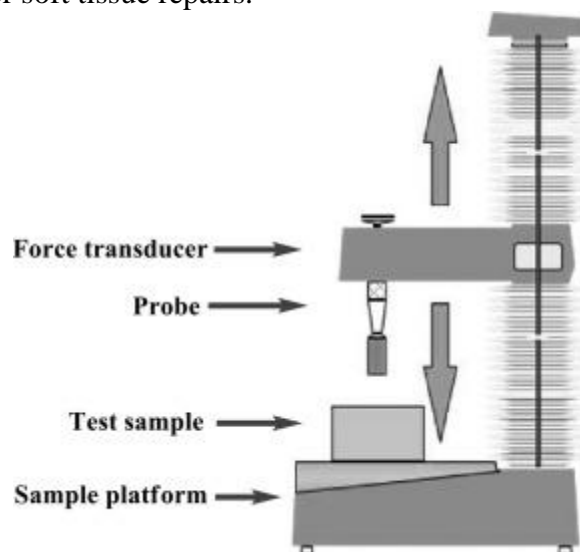


Figure 2.4 Texture analyzer schematic [18]

Moreover, texture analyzers can also be utilized to study the swelling behavior of hydrogels, which directly influences their mechanical performance. By measuring the changes in dimensions or weight as the hydrogel absorbs fluids, researchers can assess its capacity for drug delivery or moisture retention, both vital for effective wound healing. The data generated from texture analysis helps optimize the formulation and design of hydrogels, ensuring that they possess the desired mechanical properties for their intended biomedical applications, ultimately contributing to better patient outcomes.

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Compression test was conducted using a **Shimadzu texture analyser Japan**. The test involved subjecting the hydrogel to a compression test with a maximum load capacity of 500 N, at a constant speed of 1 mm/min and. In addition, adhesion test to determine adhesive property of hydrogels. For that, upon degreasing the inner surface of pigskin, it underwent cleaning and was subsequently cut into rectangular strips measuring  $12 \times 60 \text{ mm}^2$ . A square sample of dimensions  $12 \times 12 \text{ mm}^2$ , with an approximate thickness of 1 mm, was positioned between two pieces of pigskin. The assembly was tightly secured and held firmly for a duration of 5 minutes. Subsequently, the setup was subjected to testing on the fixture of a texture analyser to evaluate the adhesion performance of the sample.

### 2.2.5 Dynamic Light Scattering and Zeta Potential

Dynamic Light Scattering (DLS) and Zeta Potential measurements are crucial techniques in material science and colloidal chemistry, especially for characterizing nanoparticles, colloids, and biomolecules in suspension. These techniques provide insights into particle size, distribution, stability, and surface charge, essential factors in fields like drug delivery, where particle behavior can significantly affect therapeutic effectiveness and bioavailability.

Dynamic Light Scattering (DLS) is based on the principle of light scattering by particles in a liquid undergoing Brownian motion. When a laser beam passes through a colloidal solution, particles scatter light in all directions. Due to their random thermal motion, smaller particles move faster and cause rapid fluctuations in the scattered light intensity, while larger particles move slower, causing slower fluctuations. By analyzing these fluctuations, DLS can determine the particle size distribution, offering valuable information about the hydrodynamic diameter of particles in the solution, which includes any surrounding solvation layers. In a

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typical DLS setup, a monochromatic laser illuminates the sample, and a detector measures the intensity fluctuations of scattered light at a specific angle. This data is processed to calculate the particle size distribution, making DLS particularly useful for analyzing particles within the nanometer to micron range[19]. Typical experimental setup has been shown in figure 2.5.

DLS and Zeta potentials were measured using **Malvern Zetasizer Pro. UK**, for that, all precursor liquid samples of different weight ratios were diluted in deionized water.

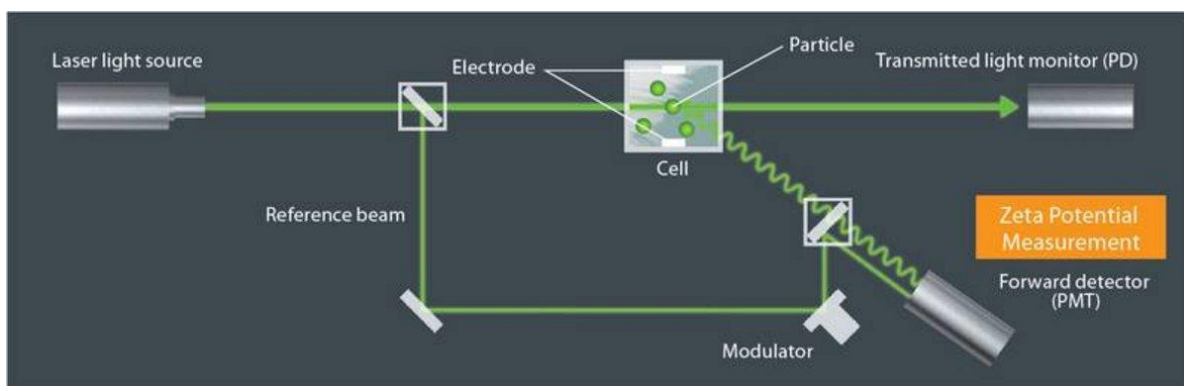


Figure 2.5 Experimental setup for DLS and Zeta Potential [19]

Zeta potential, on the other hand, measures the surface charge of particles within a colloidal system, providing critical information about the stability of the suspension. Particles in aqueous solutions often acquire surface charges, either by ionization of surface groups or through the adsorption of ions from the solution. This surface charge attracts a surrounding layer of oppositely charged ions, forming an electrical double layer around each particle. The zeta potential is defined as the potential difference between the dispersion medium and the slipping plane within this double layer. This potential is an indicator of the electrostatic repulsion between particles; a high absolute value (either positive or negative) usually suggests strong repulsive forces between particles, which helps prevent aggregation and

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enhances suspension stability. Zeta potential is measured through electrophoretic light scattering, where an electric field is applied to the sample, causing charged particles to move toward the oppositely charged electrode. The speed of this movement is then analyzed to determine the zeta potential, providing insight into particle stability in various formulations. Stable suspensions with high zeta potential values are desirable in biomedical applications, as they prevent particles from aggregating, ensuring uniformity and longevity in systems such as drug delivery formulations and therapeutic nanoparticle solutions[20].

Together, DLS and Zeta Potential are complementary techniques that give a comprehensive understanding of particle size, distribution, and stability, which are essential in optimizing the design and performance of biomedical formulati

### 2.2.7 UV Visible and Photoluminescence Spectroscopy

UV-Visible (UV-Vis) spectroscopy and photoluminescence (PL) spectroscopy are invaluable analytical techniques employed to characterize hydrogels, particularly in biomedical applications. UV-Vis spectroscopy involves the absorption of ultraviolet or visible light, leading to electronic transitions within the hydrogel's molecules. This technique is essential for assessing the presence of specific functional groups, monitoring chemical reactions during hydrogel formation, and evaluating the stability of incorporated drugs or bioactive agents. By analyzing the absorption spectra, researchers can determine the concentration of various substances within the hydrogel, facilitating studies of drug release profiles and interactions with light[21]. Moreover, UV-Vis spectroscopy can be applied to evaluate the incorporation of nanoparticles or dyes, which may enhance the properties of the hydrogel or provide

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therapeutic benefits, such as confirming the presence and stability of nanoparticles in antibacterial hydrogels.

In contrast, photoluminescence spectroscopy focuses on the emission of light from a material after it absorbs photons, making it particularly useful for studying the luminescent properties of hydrogels. This technique allows researchers to investigate the distribution and release kinetics of fluorescent probes or dyes incorporated into the hydrogel matrix. By analyzing changes in the PL spectra, researchers can gain insights into the interactions between the hydrogel and the embedded substances, indicating whether drugs are effectively encapsulated and released[22]. Overall, the integration of UV-Visible and photoluminescence spectroscopy provides a comprehensive understanding of the optical properties, stability, and biological interactions of hydrogels, which is crucial for developing effective hydrogel-based systems in drug delivery, wound healing, and tissue engineering applications.

UV absorption of lyophilized hydrogels was performed by **JASCO V770 UV Vis spectrophotometer and Eppendorf bio-spectrometer** between 200 nm to 600 nm range. To investigate fluorescent behavior, Excitation and emission of hydrogel were studied at room temperature using **Fluorolog (Horiba)**.

### 2.2.8 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical technique used primarily for determining the structure of organic compounds and studying molecular interactions. It operates on the principle of nuclear spin, where certain nuclei, such as hydrogen or carbon-13, exhibit magnetic properties. When placed in a strong magnetic field, these nuclei can align in different orientations, resulting in distinct energy levels. By applying radiofrequency

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pulses, these nuclei can transition between energy states, producing signals that are detected to create an NMR spectrum. Key features of NMR spectra include chemical shifts, which indicate the electronic environment around the nuclei; multiplicity, which reveals the number of neighbouring nuclei; and integration, which quantifies the number of nuclei contributing to each signal. NMR spectroscopy is widely used for structural determination, dynamic studies, and quality control in pharmaceuticals[23]. While it provides detailed molecular insights, its limitations include the need for relatively high sample concentrations and the complexity of spectral interpretation. In this study, we employed  $^1\text{H}$  NMR (proton nuclear magnetic resonance) spectroscopy to analyze the structure and dynamics of hydrogels developed.  $^1\text{H}$  NMR is particularly effective in characterizing compounds that contain hydrogen atoms, making it ideal for studying biological materials, such as proteins and polymers.

The chemical structure and activation of functional groups were analysed by  $^1\text{H}$  NMR spectroscopy using **AVH D 500 AVANCE III HD 500 MHz OneBay NMR Spectrometer (Bruker Biospin INTERNATIONAL AG)**.

### 2.2.9 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is a high-resolution imaging technique widely used to study the internal structure, morphology, and crystallography of materials at the atomic or molecular scale. TEM operates on the principle of electron transmission through an ultra-thin sample, enabling it to achieve significantly higher resolution than light microscopy. In a TEM setup, as shown in figure 2.6, an electron gun at the top of the microscope column generates a highly focused electron beam, which is accelerated by high voltage, typically

ranging from 100 to 300 kV. This electron beam passes through a series of electromagnetic lenses to become highly focused before it reaches the sample. Due to the extremely short wavelength of high-energy electrons, TEM can resolve features as small as fractions of a nanometer, making it ideal for analyzing minute details in a sample's structure. When the electron beam encounters the sample, some electrons pass through, while others are scattered due to interactions with the sample's atoms. The transmitted electrons carry information about the sample's density, structure, and composition, which is then magnified and captured on a detector or a screen, generating a highly detailed image of the sample's internal structure[24]. TEM images can reveal individual atomic positions, grain boundaries, crystallinity, and other structural features critical to understanding material properties. Additionally, selected-area

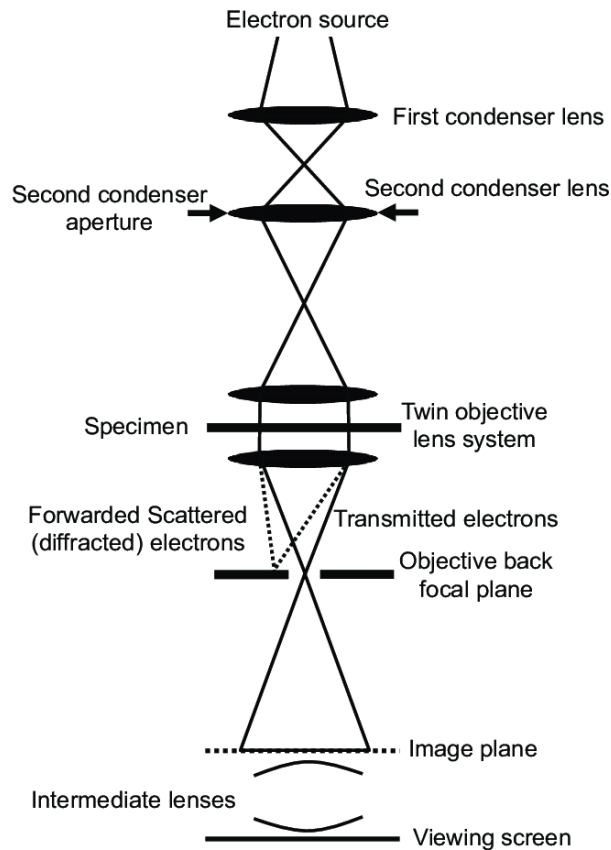


Figure 2.6 Ray diagram of setup of transmission electron microscopy [23]

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electron diffraction (SAED) can be used in TEM to analyze the crystallographic orientation and phase identification of crystalline regions, providing valuable data on the sample's molecular and atomic structure. The Transmission Electron Microscope (TEM) used was the **JEM-2100 model from JEOL, Japan**, with an accelerating voltage range of 80–200 kV, allowing for high-resolution imaging of nanoscale structures.

### 2.2.10 Bioprinter

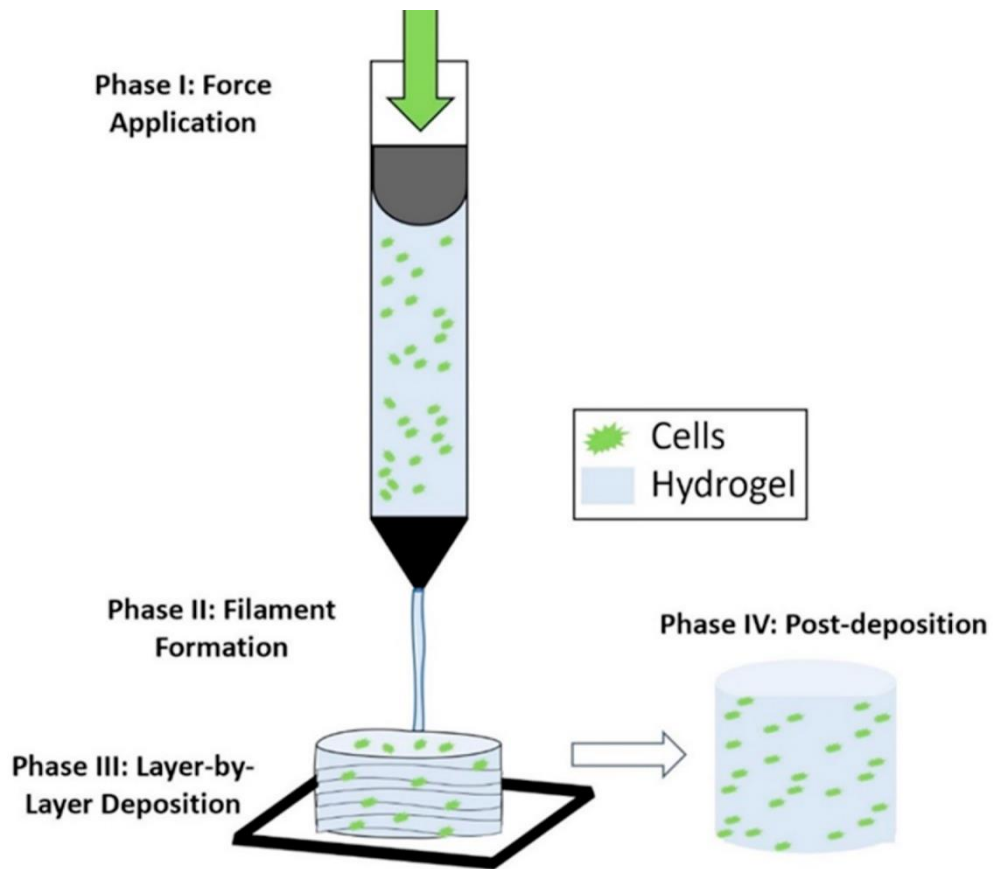


Figure 2.7 Schematic of extrusion-based bioprinter [24]

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Bioprinting is a cutting-edge technology that combines principles from engineering, biotechnology, and materials science to create living tissues through 3D printing. Unlike traditional 3D printers, which print plastics or metals, bioprinters use “bioinks”—materials loaded with living cells and biomolecules, such as hydrogels, growth factors, and scaffolds. These bioinks are carefully designed to mimic the structure and environment of natural tissues, enabling the production of complex, layered architectures that can serve various applications in medicine and research[25]. Typical experimental setup has been shown in figure 2.7. The bioprinting process begins with a digital model of the desired tissue, often created using Computer-Aided Design (CAD) or based on detailed imaging like CT or MRI scans. This model is “sliced” into layers, and the bioprinter deposits bioink layer by layer to build up the structure. The bioink typically solidifies either by exposure to UV light, cross-linking agents, or temperature changes. Specialized software controls the precise placement and thickness of each layer, ensuring that the cells within the bioink are arranged in ways that closely resemble natural tissues. Different bioprinting techniques offer unique benefits: inkjet bioprinting, for instance, deposits droplets of bioink, making it efficient and relatively inexpensive; extrusion bioprinting allows for the creation of thicker structures with high cell density, ideal for more robust tissue constructs; and laser-assisted bioprinting enables highly precise placement of cells, though it’s generally slower and more costly. The choice of technique depends on the specific requirements of the tissue being created, such as its complexity, size, and functional demands.

Bioprinting has transformative potential in fields like regenerative medicine, drug testing, and disease modeling. For instance, it holds promise for developing tissue grafts for wound healing or organ repair, which could one day replace damaged skin, cartilage, or bone.

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Bioprinted tissue models, such as those mimicking liver or kidney tissues, are also proving valuable for drug testing, offering a more accurate and human-relevant method than animal models. For the fabrication of 3D printed dressings, a specially customized extrusion bioprinter manufactured by Alfatek Systems in India was employed. The composite hydrogel was carefully loaded into a syringe with a 22-gauge needle (410 mm) from Alfatek Systems. Subsequently, the syringe was affixed to the extruder of the 3D bioprinter. Using Mendel software, structures (comprising 20 layers) were produced at a printing speed of 5 mm/min. All the experiments were carried out at a temperature 25<sup>0</sup>C and relative humidity of 60 %.

### 2.2.11 Thermogravimetric Analysis

Thermogravimetric Analysis (TGA) is an analytical technique used to assess the thermal stability and composition of materials by measuring changes in mass as a sample is heated, cooled, or held at a constant temperature. During the analysis, a small sample is placed in a controlled atmosphere—typically nitrogen, oxygen, or air—and subjected to a programmed temperature profile in a furnace. As the temperature rises, the sample may undergo thermal decomposition, oxidation, or the release of volatile components, resulting in changes in mass. A highly sensitive balance continuously monitors these mass changes, generating a thermogram that plots mass loss against temperature or time.

TGA is widely employed across various fields, including material science, polymer chemistry, pharmaceuticals, and environmental studies. In polymer analysis, it helps determine thermal stability and quantifies additives or residual solvents. In the pharmaceutical industry, TGA assesses the purity of compounds by detecting moisture and other volatile impurities. The technique also aids in compositional analysis by revealing the breakdown

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profile of complex materials[26]. The interpretation of TGA results is crucial, as the thermogram provides valuable information on the degradation behaviour of materials, with specific mass loss steps indicating different thermal degradation events. By analysing these steps, researchers can identify and quantify the components within a sample, gaining insights into its stability, purity, and suitability for various applications. Overall, TGA is a powerful tool for characterizing the thermal and compositional properties of materials. Thermogravimetric Analysis (TGA) was conducted on the **TGA-50 model by Shimadzu, Asia Pacific**, with a temperature range extending up to 1000°C.

### 2.2.12 Swelling, degradation, and Water vapor transmission rate (WVTR) studies

Swelling, degradation, and water vapor transmission rate (WVTR) studies are crucial for evaluating the performance of hydrogels, particularly in biomedical applications such as drug delivery and wound healing[27].

The swelling study assesses how hydrogels absorb water and swell, which is vital for their functionality in biological environments. The degree of swelling is influenced by factors such as crosslinking density, chemical composition, and the presence of hydrophilic groups. A higher swelling ratio indicates a greater capacity for moisture absorption, which is essential for maintaining a moist environment that promotes wound healing and supports cell migration. The swelling ratio (S) is then calculated as follows:

$$S = \frac{m_t - m_0}{m_0}$$

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Where  $m_t$  represents the mass of swollen hydrogel at time t and  $m_0$  represents the weight of the corresponding dried hydrogel

Degradation studies focus on the stability and longevity of hydrogels in physiological conditions. This is crucial because hydrogels used in biomedical applications should degrade at a rate that matches the healing process or the release of encapsulated drugs. Various methods, including weight loss, mechanical property changes, and structural integrity assessments over time, can evaluate the degradation rate. Understanding the degradation behavior of hydrogels helps predict their lifespan and performance in clinical settings. the weight loss (WL%) using the equation:

$$WL = \frac{m_0 - m_{dry}}{m_0} * 100$$

Where  $m_{dry}$  represents the mass of a thoroughly vacuum-dried sample after the swelling equilibrium was reached. The experiment was conducted three times for each sample, and the average value was calculated from the results of these repetitions.

The WVTR study is essential for determining a hydrogel's ability to facilitate moisture exchange, critical for wound healing. An optimal WVTR ensures that the dressing maintains a moist environment while allowing excess moisture to escape, preventing maceration of the wound. A high WVTR is beneficial for managing exudate levels, while a low WVTR may lead to excess moisture retention, increasing the risk of infection.

$$WVTR = \frac{W_i - W_f}{A \times t}$$

where  $W_i$  is the initial weight of the hydrogel,  $W_f$  is the final weight after a specific time,  $A$  is the surface area of the hydrogel, and  $t$  is the time in hours. A high WVTR is beneficial for

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managing exudate levels, while a low WVTR may lead to excess moisture retention, increasing infection risk.

In summary, swelling, degradation, and WVTR studies provide valuable insights into the performance characteristics of hydrogels, guiding the development of effective biomaterials for various medical applications. These quantitative assessments are critical for ensuring that hydrogels meet the requirements necessary for optimal therapeutic outcomes in wound healing and other biomedical applications.

## **2.2 In vitro studies**

In vitro studies are a crucial step in evaluating the performance and effectiveness of biomaterials, particularly in applications like wound healing. These studies aim to assess various factors, including cell viability, cytotoxicity, and migration (Scratch assay). In these studies, cell lines such as NIH 3T3 and L929, purchased from the National Centre for Cell Science (NCCS) in Pune.

### **2.3.1 MTT Assay**

The MTT assay is a widely used in vitro technique to assess cell viability and cytotoxicity. Named after the yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), this assay is commonly employed to determine the metabolic activity of cells in response to a treatment, material, or environmental change. In this test, live cells convert the MTT compound into insoluble purple formazan crystals through mitochondrial activity, specifically through the action of NAD(P)H-dependent cellular

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enzymes. The quantity of formazan produced is directly proportional to the number of viable, metabolically active cells.

In performing the assay, MTT is first added to cultured cells and incubated, allowing viable cells to metabolize the dye. Following incubation, the cells are lysed, and a solubilizing agent is added to dissolve the purple formazan crystals, resulting in a colored solution. This solution's optical density (OD) is then measured at 570 nm using a spectrophotometer or a microplate reader. The absorbance values allow for quantitative analysis of cell viability, with lower absorbance indicating higher cytotoxicity and reduced cell viability.

In hydrogel studies, the MTT assay is particularly valuable in evaluating the biocompatibility of hydrogels used for biomedical applications. For instance, when testing a hydrogel intended for wound dressing, this assay can help determine whether the hydrogel formulation is non-toxic to human cells and promotes cell proliferation, which is crucial for successful wound healing. This method's straightforward protocol and reliable results make it essential for preliminary biocompatibility assessments in tissue engineering, drug delivery, and regenerative medicine applications.

The color developed was directly proportional to the number of viable cells. The percent (%) viability was calculated using the described formula:

$$\% \text{ cytotoxicity} = \frac{[A]_{\text{control}} - [A]_{\text{test}}}{[A]_{\text{control}}} \times 100$$

% viability = (100 - % cytotoxicity)

Where  $[A]_{\text{test}}$  = Absorbance of the sample to be the test

$[A]_{\text{control}}$  = Absorbance of the control sample

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### 2.3.2 FDA/PI Assay

The FDA/PI (fluorescein diacetate/propidium iodide) assay is a widely utilized method for assessing cell viability and cytotoxicity across various biological studies. This assay leverages the differential ability of live and dead cells to process specific fluorescent dyes, allowing researchers to distinguish between viable and non-viable cells based on membrane integrity and metabolic activity[28]. The FDA/PI assay employs two key dyes: fluorescein diacetate (FDA) and propidium iodide (PI). FDA is a non-toxic, membrane-permeable dye that live cells can convert into a fluorescent compound due to the activity of intracellular esterases. When FDA enters a live cell, esterases cleave the acetate groups, resulting in fluorescein formation, which emits green fluorescence. In contrast, PI is a membrane-impermeable dye that can only penetrate cells with compromised membranes, staining the DNA of dead or dying cells and producing red fluorescence.

The procedure involves preparing and treating cells in a multiwell plate, followed by the addition of a solution containing both dyes. After an incubation period, fluorescence is measured using a fluorescence microscope or flow cytometer, where live cells display green fluorescence and dead cells exhibit red fluorescence. The FDA/PI assay is widely applied in cytotoxicity testing, cell proliferation studies, and drug screening. It offers advantages such as simplicity, speed, and quantitative data on cell viability; however, limitations include the potential inability to differentiate between metabolically inactive live cells and dead cells, as well as the possibility of interference from certain substances. Overall, the FDA/PI assay is a valuable tool in cell biology, pharmacology, and toxicology research, providing critical insights into cell health in response to various treatments.

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### 2.3.3 Hemolysis assay

The hemolysis assay is a laboratory method used to evaluate the potential of substances, such as drugs, biomaterials, or medical devices, to cause damage to red blood cells (RBCs) and induce hemolysis, which is the breakdown of RBCs and the release of hemoglobin into the surrounding fluid. This assay is crucial for assessing the biocompatibility of materials intended for medical applications, particularly those that come into direct contact with blood. The principle of the hemolysis assay relies on the observation that damaging agents can compromise the integrity of red blood cell membranes, leading to hemoglobin release. The degree of hemolysis is quantified by measuring the concentration of free hemoglobin in the supernatant after centrifugation, with higher levels indicating greater cell lysis[29].

For assessing the hemolytic activity of hydrogels, human blood was collected in an EDTA tube. The whole blood was added with an equal volume of PBS at pH 7.4, centrifuged at 1500 rpm for 5 min and plasma was discarded. The collected blood cells after centrifugation were again washed with PBS (7.4) and the supernatant was discarded. The washed red blood cells were added to an equal volume of PBS (7.4). 200  $\mu$ L of these cells were taken in each microcentrifuge tube and lyophilized samples were added at a concentration of 1mg/ml and 5 mg/ml. All the microcentrifuge tubes were kept at room temperature, under continuous stirring on a shaker for 2 hr, 4 hr, and 8 hr. At the end of the 2 hr, 4 hr, and 8 hr of incubation, the tubes were centrifuged at 11,000 rpm for 5 min, the supernatant was shifted to a 96 well plate, and absorbance was read at 540 nm. Triton -X (0.1%) was taken as a positive control and PBS pH 7.4 was taken as a negative control. Percent hemolysis was calculated by the formula:

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$$\% \text{ Hemolysis} = \frac{\{[Abs]_{sample} - [Abs]_{saline}\}}{[Abs]_{control} - [Abs]_{saline}} \times 100$$

The hemolysis assay is commonly used for biocompatibility testing, assessing the safety of biomaterials and medical devices intended for blood contact, as well as in pharmaceutical development to evaluate the hemolytic potential of new drugs. Additionally, it is useful in toxicology studies to investigate the effects of various chemicals on red blood cell integrity. While the assay is relatively straightforward and provides rapid results, it has limitations, such as not fully mimicking the complex interactions occurring in vivo, as it does not account for blood components like plasma proteins and other cells. Therefore, careful optimization of experimental conditions is necessary to ensure accurate results. Overall, the hemolysis assay is an important tool for evaluating the potential hemolytic effects of substances on red blood cells, providing insights into the biocompatibility of materials intended for medical use.

#### 2.3.4 Scratch assay

The scratch assay, also known as the wound healing assay, is a widely used in vitro technique for studying cell migration and proliferation, particularly in the context of wound healing and tissue regeneration. This assay simulates a wound by creating a scratch or gap in a confluent monolayer of cells, allowing researchers to investigate the mechanisms underlying cell movement and the factors that promote or inhibit healing. The principle of the scratch assay is based on the premise that cells in a confluent monolayer will migrate into the created wound area in an effort to re-establish cell density and cover the gap. Researchers can quantitatively measure the rate of wound closure over time, providing insights into various cellular processes, including migration, proliferation, and the effects of different treatments[30].

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The procedure generally involves several key steps, starting with the culture of cells to confluence in a suitable dish or multiwell plate. Once the cell monolayer reaches confluence, a sterile tool, such as a pipette tip, is used to create a straight scratch across the cell layer. After gently washing the dish to remove debris and detached cells, researchers may treat the cells with various substances (e.g., growth factors, drugs) to evaluate their effects on migration and proliferation. The cells are then incubated under standard conditions, allowing them to migrate into the wound area. Images of the wound are taken at designated time points using a microscope, and the extent of wound closure is measured by comparing the width of the scratch, which can be quantified using image analysis software.

### 2.3.5 Antioxidant and antibacterial studies

Antioxidant and antibacterial studies are essential components of research in food science, pharmacology, and biomedical sciences, focusing on evaluating the effectiveness of substances—such as natural extracts, synthesized compounds, or materials—in scavenging free radicals and inhibiting bacterial growth. Antioxidants are molecules that neutralize free radicals, which can cause oxidative stress and damage cells, leading to chronic diseases and aging. Common methods for assessing antioxidant capacity include the DPPH assay, which measures the scavenging ability of a sample by observing the reduction in absorbance of a DPPH solution. In the DPPH assay, a fresh solution of DPPH/ethanol (40 mM) was prepared, and 50 mg of hydrogel was immersed in 3 mL of the solution. The mixture was kept in the dark at room temperature for 30 minutes, and the change in absorbance at 517 nm was measured using a UV-visible spectrophotometer (Biospectrometer, eppendorf). The DPPH radical scavenging activity was calculated using the formula:

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$$\text{DPPH scavenging activity} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100\%$$

where  $A_{\text{blank}}$  is the absorbance of the DPPH solution and  $A_{\text{sample}}$  is the absorbance of the samples mixed with the DPPH solution. Each sample was tested in triplicate.

On the other hand, antibacterial studies focus on evaluating the efficacy of substances against bacterial growth and infection, which is crucial for identifying potential antimicrobial agents for use in medicine, food preservation, and hygiene products. Common methods for assessing antibacterial activity include the disc diffusion method, where filter paper discs impregnated with the test substance are placed on an agar plate inoculated with bacteria to measure the inhibition zone; the Minimum Inhibitory Concentration (MIC) test, which determines the lowest concentration of an antibacterial agent that prevents visible bacterial growth; and time-kill assays, which measure bacterial counts over time after exposure to an antibacterial agent to assess the kinetics of bacterial death. To evaluate the antibacterial activity of the hydrogels against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*), a punch well agar diffusion method was used. The bacteria-cultured agar plates (105 CFU/mL) were punched with 6 mm-diameter wells, and 200 mg of the hydrogels were filled into these pre-punched wells. The plates were then incubated at 37°C for 24 hours, with 100 µL of PBS serving as the control. Each test was performed in triplicate, and the diameter of the zone of inhibition around each well was measured and recorded.

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## 2.3 In vivo Studies

### 2.4.1 Diabetic Rat Model

In vivo diabetic models are critical for studying the effects of various treatments on wound healing in diabetic conditions. In this study, diabetes was induced in rats through a single intraperitoneal injection of streptozotocin (STZ) at a dose of 60 mg/kg body weight, administered in a 0.1 M citrate buffer with a pH of 4.5. Following the induction of diabetes, blood glucose levels were monitored regularly using a glucometer via the tail vein.

On the eighth day post-induction, a full-thickness open-excision wound was created on the back of the animals using a biopsy punch. The wound area was disinfected with 70% ethanol to prevent infection. After wound induction, the diabetic rats were randomly divided into various experimental groups for treatment. The treatment groups included a normal control group, a diabetic wound control group, and three groups receiving different formulations of synthesized hydrogel treatments. These treatments were applied topically on an alternate basis over a duration of 21/15 days. Throughout the experiment, blood glucose levels and body weight were monitored to assess the overall health and metabolic status of the animals.

At the conclusion of the experiment, the animals underwent an overnight fast before being sacrificed. Serum samples were collected and stored at  $-80^{\circ}\text{C}$  for future biochemical analysis. Additionally, wound tissues and other organs were harvested for the estimation of oxidative stress biomarkers and related histopathological studies. All experimental procedures were carried out in accordance with the approved guidelines from the Institutional Animal Ethics Committee, ensuring ethical treatment and care for the animals involved in the study.

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The change in the wound edge diameter was measured (in mm<sup>2</sup>) by tracing the wound boundaries. The wound edge contraction was expressed as decrease in wound size percentage.

The percentage of wound contraction also referred as wound closure area was calculated by using the following equation:

$$\% \text{ wound contraction} = \frac{[(\text{Initial wound area} - \text{final wound area})]}{\text{Initial wound area}} * 100$$

#### 2.4.2 Histopathological analysis

Histopathological analysis is a critical technique used to assess tissue structure and cellular characteristics at a microscopic level, particularly in studies investigating the effects of treatments on wound healing, disease progression, or tissue response[31]. This analysis involves the preparation of tissue samples, which are then examined to gain insights into cellular morphology, tissue architecture, and the presence of pathological changes.

##### **Tissue Preparation**

For histopathological analysis, tissues are typically collected from experimental subjects following a specified treatment or intervention period. The collected tissues are fixed in formalin to preserve their structure and prevent decay. After fixation, the tissues are processed through a series of dehydration steps using increasing concentrations of alcohol, followed by clearing with a solvent such as xylene. The dehydrated tissues are then embedded in paraffin wax, allowing for the creation of thin sections that can be mounted on glass slides for microscopic examination.

##### **Sectioning and Staining**

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The embedded tissues are sectioned using a microtome, producing thin slices usually between 4 to 10 micrometers in thickness. These sections are then placed on glass slides and stained with various histological stains to highlight specific cellular components and structures.

Common stains include:

- **Hematoxylin and Eosin (H&E):** This is the most widely used staining method, where hematoxylin stains nuclei blue, while eosin stains the cytoplasm and extracellular matrix pink. This combination provides a general overview of tissue morphology.
- **Masson's Trichrome:** This stain is used to differentiate between collagen and muscle fibers, providing insights into fibrosis and tissue repair.
- **Immunohistochemistry (IHC):** This technique utilizes antibodies to detect specific proteins within the tissue, allowing for the assessment of cellular markers associated with inflammation, proliferation, or angiogenesis.

### 2.4.3 RNA extraction and real-time qPCR analysis

Quantitative real-time PCR (qRT-PCR) is a powerful technique used to measure gene expression levels by quantifying mRNA abundance in various samples. It is widely utilized in molecular biology to analyze gene expression changes in response to treatments, investigate disease pathways, and understand cellular responses. The process begins with the isolation of high-quality RNA from cells or tissues, as the integrity of RNA is crucial for reliable qRT-PCR results[25]. The extracted RNA is then treated with DNase to eliminate any genomic DNA contamination that could interfere with accurate measurements. Following RNA isolation, reverse transcription is performed to convert the RNA into complementary DNA

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(cDNA) using reverse transcriptase enzymes, ensuring that the RNA sequence can be amplified and quantified.

Skin samples were collected from rats at 7, and 14-days post-injury. Total RNA was extracted from both wounded skin samples using an RNA extraction kit (Gene All, South Korea). TRI Reagent (Sigma) was employed according to the manufacturer's instructions to isolate total RNA from the skin tissue. Subsequently, cDNA was synthesized from 1  $\mu$ g of the extracted RNA utilizing the iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time qPCR analysis was conducted with the iTaq™ Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's protocol. The expression levels of inflammatory genes, namely IL-1 $\beta$  and TNF- $\alpha$ , as well as VEGF were assessed in conjunction with the housekeeping gene 18S rRNA. The relative quantification was performed by determining the  $\Delta\Delta$ CT value, with the fold change calculated as  $2^{-\Delta\Delta$ CT}, indicating the relative upregulation or downregulation of the inflammatory genes.

#### **List of the real time qPCR primers used for the experiment**

**IL-1** Fwd GACAACCTGCACTACAGGCTCC Rev AGGCCACAGGTATTTTGTCG

**VEGF** Fwd ACCTCCACCATGCCAAGT Rev TTGGTCTGCATTCACATCTG

**TGF- $\beta$**  Fwd CTA CTACTGCTTCAGCTCCACAGA Rev ACCTTGGGCTTGCGACC

#### 2.4.4 Biochemical Assay

Biochemical assays for oxidative stress biomarkers are critical in research areas such as toxicology, pathology, and regenerative medicine, as they help quantify oxidative damage and antioxidant defense in cells or tissues. Oxidative stress occurs when the production of

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reactive oxygen species (ROS) exceeds the body's antioxidant defense mechanisms, leading to potential cellular damage. Several assays are commonly used to evaluate oxidative stress biomarkers, including markers of lipid peroxidation, protein oxidation, and antioxidant enzyme activity[32].

Superoxide Dismutase (SOD), Glutathione (GSH), Glutathione S-transferase (GST), Nitric Oxide (NO), and Lactate Dehydrogenase (LDH) are crucial biomarkers for assessing oxidative stress, antioxidant capacity, and cell damage. SOD is an enzyme that protects cells by converting superoxide radicals into hydrogen peroxide and oxygen, helping to reduce oxidative damage. GSH is a major antioxidant that directly neutralizes ROS, while GST aids in detoxification by binding GSH to toxic compounds. NO acts as a signaling molecule but can contribute to oxidative stress at high levels; it is commonly measured by its stable metabolites, nitrate and nitrite. LDH serves as a marker for cellular damage, as it is released from damaged cells. These assays together offer insights into cellular responses to stress and the effectiveness of protective treatments.

#### 2.4.5 Photoacoustic Imaging

Photoacoustic imaging (PAI) is an advanced biomedical imaging technique that combines optical and ultrasound technology to visualize tissues with high resolution and depth based on their optical absorption characteristics. The process begins with a pulsed laser beam directed at the tissue, where the light is absorbed by molecules like hemoglobin or melanin, or by targeted contrast agents, leading to localized, rapid heating. This heating generates a transient pressure rise, producing ultrasonic waves through thermoelastic expansion. These waves are then captured by ultrasound detectors on the surface, which convert them into

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signals to form images representing the distribution of light-absorbing molecules within the tissue[33].

PAI is particularly advantageous for visualizing blood vessels and vascular structures, as hemoglobin's strong light absorption enables clear imaging of blood-rich tissues, making it valuable for detecting tumors, assessing inflammation, and monitoring oxygenation levels as depicted in figure 2.8. With its ability to penetrate several centimetres into soft tissue, PAI is widely used in fields such as oncology, cardiology, and neurology. It provides real-time, high-resolution images with significant depth without the need for ionizing radiation, unlike other imaging modalities[34]. Rats were scanned with an ultrasound/photoacoustic system (**Vevo LAZR\_X Vevo 3100, Toronto, Canada, 40 MHz transducer**) in B-mode for the wound and power Doppler mode for vascularity. Oxygenated and deoxygenated blood was analyzed in photoacoustic mode. Scans were conducted on days 7 and 14 post-treatment. Superimposed ultrasound and photoacoustic images provided information on wound dimensions and oxygen saturation, and Doppler settings were used to assess vascularity.

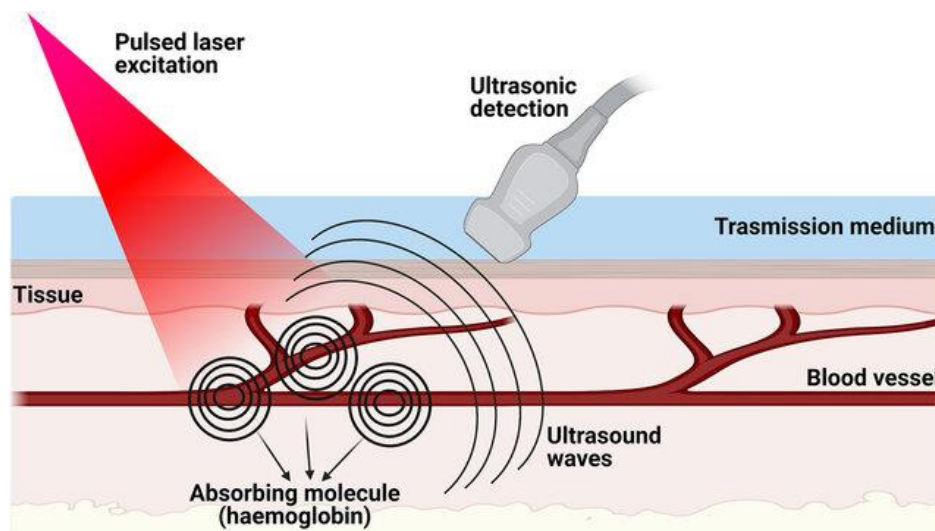


Figure 2.8 Schematic of basic principle of photoacoustic imaging [34]

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## 2.5 Software

Various software tools were utilized for data analysis and image processing in this study. **ImageJ**, a powerful open-source tool, played a crucial role in the characterization of hydrogels by enabling the quantification of structural properties and the evaluation of morphological features from digital images obtained through techniques such as Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). For data visualization, **Origin 9.0** was employed, widely recognized for its robust capabilities in data analysis and graphing. This software allows researchers to easily import, manipulate, and plot data, generating high-quality graphs essential for effectively communicating research findings. Additionally, **Vevo-LAB software** (FUJIFILM VisualSonics, Toronto, Canada) was used for advanced image processing, enhancing the analysis of high-resolution biological images. Finally, **AstraNova** was utilized for Nuclear Magnetic Resonance (NMR) data analysis, facilitating the interpretation and quantification of NMR spectra in the study of hydrogels.

## 2.4 Statistical analysis

The experimental data were presented as mean  $\pm$  standard deviation. Statistical analysis was performed using one-way ANOVA and Student's t-test to assess significance. The significance levels were indicated as follows: \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), and \*\*\* ( $p < 0.001$ ).