Synthesis of ester-diol based polyurethane for hypopharyngeal tissue engineering

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2019

DEDICATION

This finding is dedicated to the people who are self inspired in research and entrepreneurship to change the social scenario.

DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this work is done by undersigned candidate , the study consist literature survey and original research finding, as a part of Mater of engineering in biomedical engineering studies during the academic session 2017-2019. I have also declared that this submission doesn't contain material previously published or written by other researchers of any universities or institute. I have fully cited and referred all the materials that are not original in this submission.

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DATE: IMON CHAKRABORTY

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PREFACE

As the cases of hypopharyngeal cancer are increasing rapidly which strives tissue engineering to make an artificial substitute for the regeneration of hypopharynx tissue where any kind of tissue loss or damage has occurred. Among the other biomaterial, polyurethane has been considered as a suitable material to perform research in this domain. On top of that, polyurethane is well established and the experimental data which we have got to evaluate the true potential of polyurethane to be used as a substitute of hypopharyngeal tissue. This work highlights the positive quality of synthesized polyurethane and its efficacy for the hypopharyngeal tissue engineering.

In this thesis, chapter one deals with the principles which were used in this work. The background mechanisms of all the experiment are explained with proper diagrammatic representation.

Next chapter describes the knowledge behind this work. It also significantly explains the different findings of the researchers related to biodegradable polyurethane and hypopharyngeal tissue research which may be used in somewhere in this study to accomplish the work.

Chapter three clearly explains the objective and proposed plan of this hypopharyngeal tissue engineering research along with describes the reason and potentiality of this finding in social aspect.

Chapter four elaborately illustrates the methodologies to understand the basic foundation of the material synthesis and its various properties. It also elucidates the different characterization protocol to trust the material potency.

In chapter five, the experimental results significantly demonstrate various property of the material with proper representation. Here different kind of reported findings are compared with the results as a discussion of the findings. Discussion in this chapter indicates the critical remark on the material suitability in specific tissue engineering research.

Finally in conclusion, the finding explains that the synthesized polyurethane membrane as a potent candidate for hypopharyngeal tissue engineering.

ABSTRACT

In this study, ester diol based polyurethane was synthesized in two steps: firstly polyethylene glycol 400 (PEG 400) was reacted with lactic acid to prepare ester diol and then it was polymerized with hexamethylene diisocyanate (HDI) in presence of catalyst, dibutyltin dilaurate (DBTDL). The physical, mechanical and biological testing was done to testify the characterization of membrane. The morphology of synthesized membrane was investigated by using FESEM (Field Emission Scanning Electron Microscopy). A highly connected homogeneous network was obtained due to proper cross linking of the synthesized membrane. Functional groups of obtained membrane were characterized by FTIR Spectroscopy (Fourier Transform Infrared Spectroscopy). Mechanical property analysis indicates the membrane has strength 5.15 MPa and strain 124%. The membrane showed high hemocompatibility, no cytotoxicity on peripheral blood mononuclear cell (PBMC) and susceptible to degradation in simulated body fluid (SBF) solution. Antimicrobial activity assessment was investigated against clinical pathogenic bacteria. Primary human hypopharyngeal cell growth on the polyurethane membrane was revealed the cytocompatibility and subcutaneous implantation on the back of wistar rats were given in-vivo biocompatibility of the membrane. So, the overall analysis of the synthesized material leads us to consider it as a potential biomaterial for hypopharyngeal tissue engineering.

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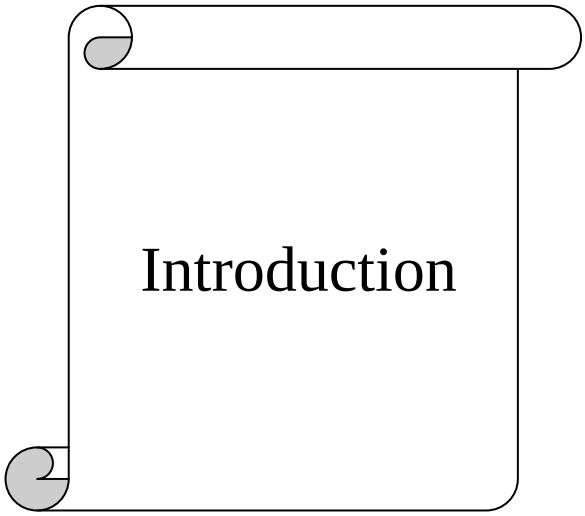
Chapter 1



1. Introduction:

Biomedical engineering is the area to understand medical problems at cellular or molecular level and use the cutting edge technology to develop tissue and organ from individual cells for replacement. Depth knowledge of interdisciplinary domain like anatomy, biochemistry, immunology and the mechanics of cellular structure are essential to infer disease process and to destine interventions [1]. Miniature device has developed to heal or inhibit the disease formation and process progression with concerning precise location. New technologies have introduced for skin tissue replacement, artificial heart valve development and even artificially entire heart formation with high level of biological and physiological accuracy [2]. Synthesization of artificial material for implantation and development of new biomaterials for introduction within the living system results those materials to come in contact with the living cell or protein. So in the quest of new biomaterials, researchers always try to mimic the natural biological and mechanical property of the living tissue [3]. Biomedical engineering is the vast area with multidimensional research and development initiatives. This field is growing exponentially by solving the problems of health care industry and implementation of pre-existing techniques for better living [4].

Orthopedic prosthesis or replacements of different body piece by truly engineered implant or assist device are the vintage innovation of biomedical engineering [5]. Now introduction of tissue engineering covers the replacement of different joints, connective tissues including bone grafting [6]. Biomaterials play an important role in every stage for the development of biomedical engineering. Stem cell technology with tissue engineering has provided an excellent opportunity for multidimensional tissue development with maintaining cells ex-vivo. The potentiality for this combined technology is limitless. This interdisciplinary branch of engineering provides the state of the art applications in various thrust area like [7]-

- Development of new material for implantation and replacement
- Development of new dental material
- Research in tissue engineering for tissue to organ development

- Development of communication aids for handicapped
- Research for material to control the best drug delivery system
- Design of biomedical sensors for physiological component measurement
- Computer modeling and software development for medical research data analysis

Possibilities of this thrust areas are endless with keen research knowledge and developing expert system to make and generate a functional outcome with numerous health care opportunities. Perhaps a huge benefit of biomedical engineering is to understand the ground level health or medical problem including the needs of this system and develop technology, product or system methodology to solve them. So this engineering provides expectancy and built high quality health care at affordable cost towards problem solving with disease battle field and to cook for more efficient biomedical system [3,8].

1.1 Tissue Engineering:

The concept was introduced to create a new field of research highly focused on tissue regeneration from cells with help of biomaterials in form of scaffold or membrane with suitable growth factors. It is a new area that is flourishing rapidly with scope and importance within biomedical engineering. It shows a relationship between cellular and molecular biology in one side and physical, chemical, material and mechanical engineering on the other side. The ability to reconstruct tissue function has numerous clinical implications and supposes to play huge role in cellular and gene therapies. However, tissue can damage in several ways like trauma or cancer. Traditional treatment options include surgical repair or autograph transplantation. Due to this full functional reconstruction of damaged tissue can be quite difficult and the result is not always satisfactory at functional level. In such scenario tissue or organ transplantation reduces the lifelong tissue malfunctioning problems of the patient. Tissue engineering creates the regeneration of patient's own tissue or artificial tissue that shows the precision (shown in figure 1.1) and also reduces the donor tissue related complications. It is also significantly reduce the incompatibility and tissue rejection problem. This field of research includes principal of engineering, process of life sciences towards the synthesis of biological substitute material which

ultimately restore, maintain the tissue functions. Tissue function is really complex which combines the biological and physiological process. To achieve this aspect, three considerations has been taken in account; first, concern about genetic engineering issue; second, cover up cell biology issue and third, concern to check the status of tissue engineering for specific organs [7,9].

Firstly, category includes the common engineering theme towards material property and development including analysis of physical process. Second, stem cell biology, cell motion and microenvironment have considered in this. Third, tissue specific engineering likes bone marrow tissue, liver tissue, and skeleton muscle tissue.

Tissue engineering is the very precise cutting edge technology based on the scientific and physiological principals to design and construct the specific tissue. Tissue engineering can be categorized broadly into two ways [10].

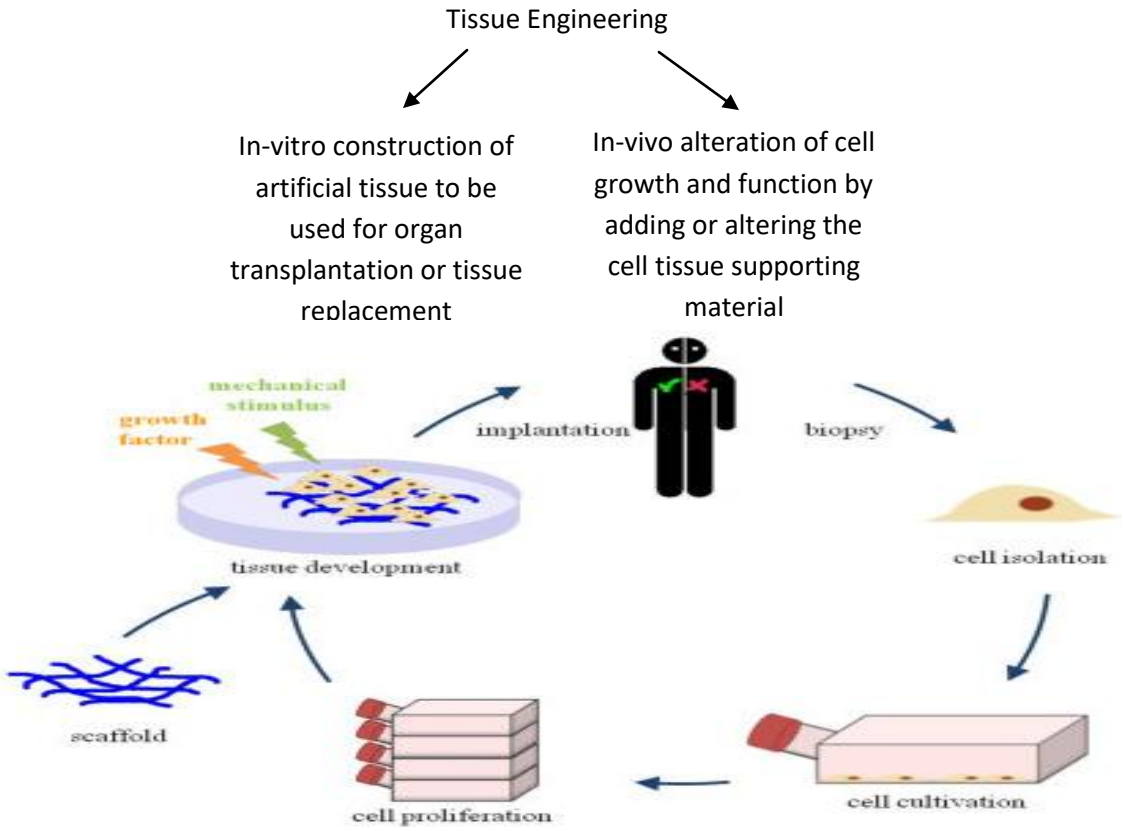


Figure 1.1: Schematic representation of overall process of tissue engineering.

Bio-artificial tissue represents the three dimensional structure with greater magnitude with cell masses than the two dimensional cell culture techniques Most important challenge in tissue engineering is to produce the biomaterial that is structurally and functionally suitable for specific applications. The material should also provide the adequate mechanical strength maintaining the normal flexibility [11].

1.1.1 Cell surface immobilization: Interaction with cell surface greatly drives the biological role in cellular behavior. This attachment depends on the cell membrane receptors which may show the effect on cellular physiology like leukocytes activation on vascular membrane, spatial differentiation of embryonic basement membrane. In the thrust area of tissue engineering, the cells attachment includes many products i.e. dermal dressing, hepatocytes scaffold etc. Significant finding of cell surface interaction are shown in figure 1.2 [12].

1.1.2 Protein surface interaction: Biomaterial interactions with cell surface initiated through protein adsorption. Amount of absorb protein to a surface is significantly measure by level of protein. Cellular level interaction can be jointly compared with amount of protein and its organization on the surfaces [13].

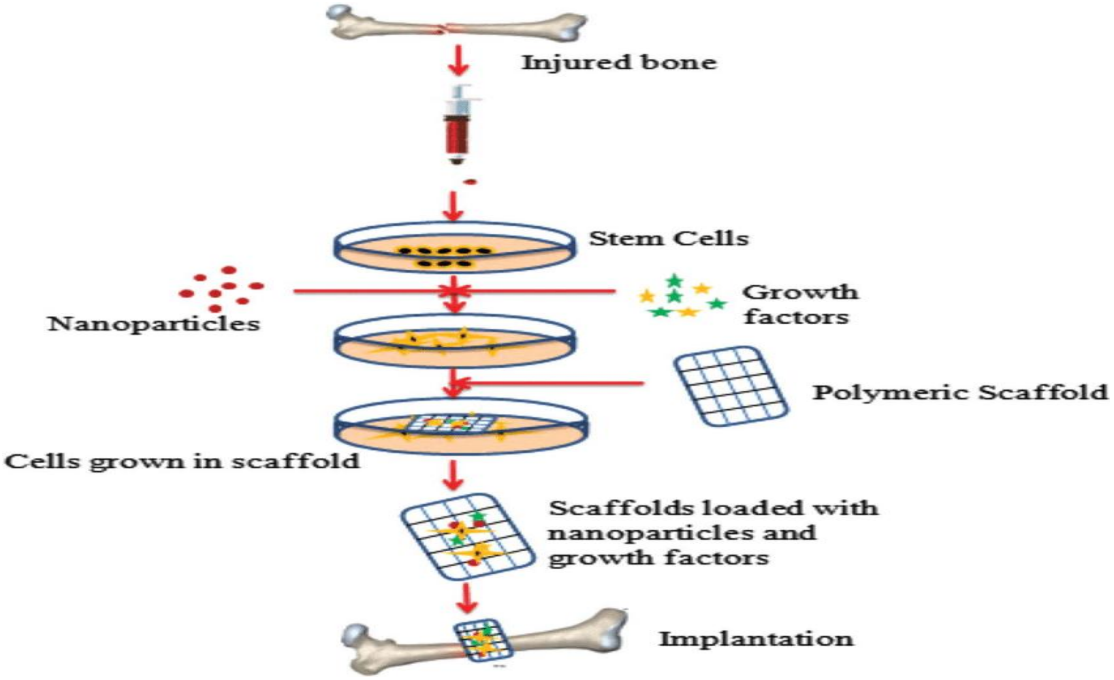


Figure 1.2: Different cellular level interaction in scaffold before implantation.

1.1.3 Tissue engineering of bone marrow: Continuous research effort has been made to regulate mature blood cell production from immature one. Ex-vivo model takes into consideration to reconstruct the hematopoiesis, proliferation and differentiation of mature blood cells [7]. Few important tissues engineering research finding for sophisticated organ and system are-

- Tissue engineering for the liver
- Tissue engineering in the nervous system
- Skeleton muscle tissue engineering
- Cartilage tissue engineering
- Tissue engineering for kidney tissue

1.2 Polymer - Biomaterials:

A biomaterial is a synthetic biological material used to replace the different part of human body and mimic the function of natural living tissue. It is basically inert substance, introduce for implantation solely or along with specific living cell. The effectiveness of biomaterial inside the body is dependent on few factors like point of view of problem area, tissue level, organ level or system level etc [14].

Table 1.1: Different types of material and their applications

Material		Advantage	Example
I.	Polymer (nylon, silicon, polyurethane, polyester)	Resilient , Easy to fabricate	Blood vessel, surgical patches, ear, nose and other soft tissues.
II.	Metal (Ti and its alloy, Co-Cr alloy, stainless steel etc)	Strong, tough and ductile	Joint replacement, bone plates, screw, dental root implants etc.
III.	Ceramics (aluminum oxide, calcium phosphate including hydroxyapatite)	Inert, strong in compression	Dental, femoral head of hip replacement, coating of dental and orthopedic implants. Joint implant, heart valves.
IV.	Composite (carbon-carbon, wire or reinforced bone cement)	Tailor made strong, biocompatible	Joint implant, heart valves

1.2.1 Effectiveness of biomaterials: The performance of biomaterial inside human body depends on several factors like material properties, shape, design, biocompatibility and the factors under the supervision of engineer as well as surgery techniques used by the surgeon including the health condition of the patient [15].

1.2.2 Biocompatibility of material: Acceptance of artificial implant inside our body depends on the compatibility that includes the conditions such as surrounding tissues or body as a whole. Biocompatible biomaterials don't initiate inflammatory response, allergic or immunologic reactions including tissue rejection and also don't initiate or cause cancer [16].

Synthetic polymeric material has been extensively used in various domain of tissue engineering. Main benefit and advantages of polymeric materials are various shapes, manufacturability (latex, sheet, scaffold etc), secondary process ability, cost effective, adequate mechanical and physical property etc. In comparison with other biomaterial, polymeric material shows adequate sterilizing property and biocompatibility. Polymer composed of long chain molecule formed by hydrogen bonds or covalent bonds with backbone chain. The cross linking between the chains maintained by covalent bonding force, vanderwaals force etc [17].

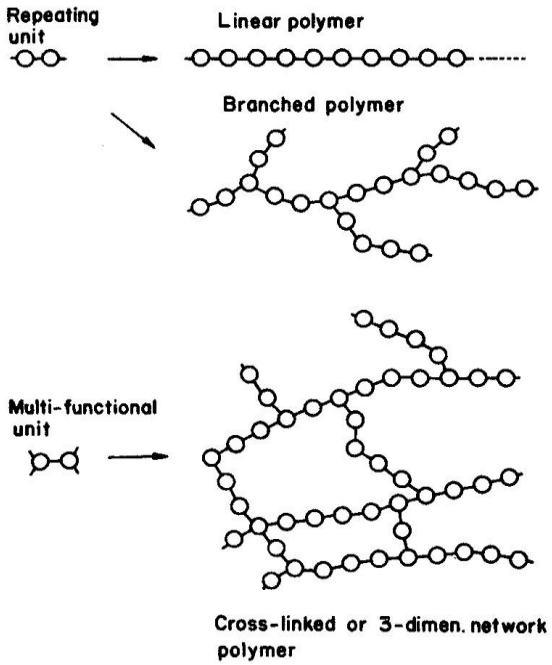


Figure 1.3: Showing the different kind of polymeric chain formation.

1.2.3 Structural modification effects the properties: Physical property modification can structurally and functionally affect the polymer. Chemical composition and chain arrangement also drives the final effectiveness towards functionality of polymeric biomaterial. This tailor made property help to meet the specific application needs like increasing the molecular weight of a polymer can slow down or rigid the mobility of the chains of the polymeric material. Cross-linking of the polymeric chain also reduce the mobility and decrease the crystallization rate. High degree of cross-linking can prevent complete crystallization. Cross-linking density increases cause a material become harder. Al though more than hundred polymers can be easily synthesized but only few of them is effective in biomedical or medical implants.

Table 1.2: List of few polymeric biomaterials having extensive biomedical applications [15,16].

Polymer	Applications
• Polyurethane (PU)	Film, tubing, surgical plates, scaffold, soft tissue material
• Polyamide (Nylon)	Packaging film, catheters etc
• Polystyrene (PS)	Tissue culture flask, roller bottles
• Poly ethylene (PE)	Catheter, pouch, flexible container and orthopedic implants
• Polymethylmeth acrylate (PMMA)	Membrane for blood dialyzer, bone cement and implantable ocular lense.

To improve the biocompatibility and acceptance of polymeric biomaterial, several kind of surface modification phenomena of physical and chemical level has been introduced such as modify hemo-compatibility, influence cell adhesion, growth, proliferation, control protein adsorption, improve lubricity etc. The ultimate aim of polymeric biomaterials is to achieve high degree of natural living tissue level functionality for artificially design biocompatible material to solve the problem of health care discipline [18].

1.3 Polyurethane:

Polyurethane has introduced in several areas of modern life. Polyurethane is used in biomedical fields for its long term in-vivo biostability. This polymeric biomaterial incorporated in tissue engineering research to remodel the fully functional tissue form. Other important benefits of polyurethane like tensile strength, melting point which makes the material more durable. Polyurethane is a material that effectively supports the cell attachment, proliferation and differentiation. Biodegradable polyurethane has been increased in the area of tissue scaffold including drug delivery system. Different aspect of polyurethane depends on wide range of biological, physical and chemical properties that achieved through several selections of intermediates. This keen technological aspect helps in the advancement of polyurethane development for broad range of tissue engineering applications [19].

1.3.1 Physical and chemical properties of polyurethane: It is a polymer in which several urethanes repeating unit exists. These repeating moieties are carbonic acid and derivatives, present on the form of ester linkage. The general structure of urethane group shown in below figure 1.4

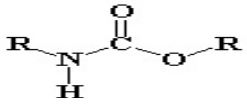


Figure 1.4: General structure of urethane group

The structural validations in R group and amide hydrogen substitution produce different urethanes. Mainly all polyurethane consist of urethane group including different moieties like ester, ether some aromatic etc. The urethane linkage comes from the reaction of any isocyanate moiety (-N=C=O) with an alcohol (-OH). In figure 1.5 the simple linear representation of polyurethane.

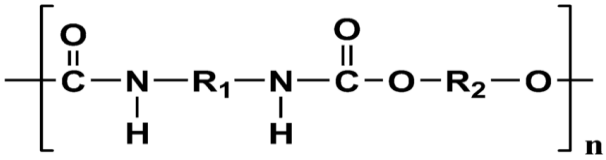


Figure 1.5: Linear Structure of polyurethane

Here n denotes the no of repeating unit, R_2 represents hydrocarbon chain. Diisocyanate is introduced in polyurethane synthesis because it will able to react with the compound containing active hydrogen. Spacing within and branched chain along with number of substitution drives the different range of polyurethane like flexible to rigid or linear to branch could be synthesized [13,20].

1.3.2 Biodegradation Mechanism: Biodegradable polyurethane are synthesized to degrade hydrolytically or enzymatic through in-vivo or in-vitro. Ester linkage has been introduced to hydrolyze in-vitro or in-vivo that yields α-hydroxy acid degraded product including urethane and urea component with terminal groups (for polyester urethane urea shown in figure 1.6). Polyester component of polymer drives the rate of degradation in-vitro. Moreover, amorphous soft segment along with polyurethane have more suitable to degrade rapidly than semi crystalline segment. Hydrophilic soft segment increase the water uptake rate which suggest increasing the rate of degradation [21].

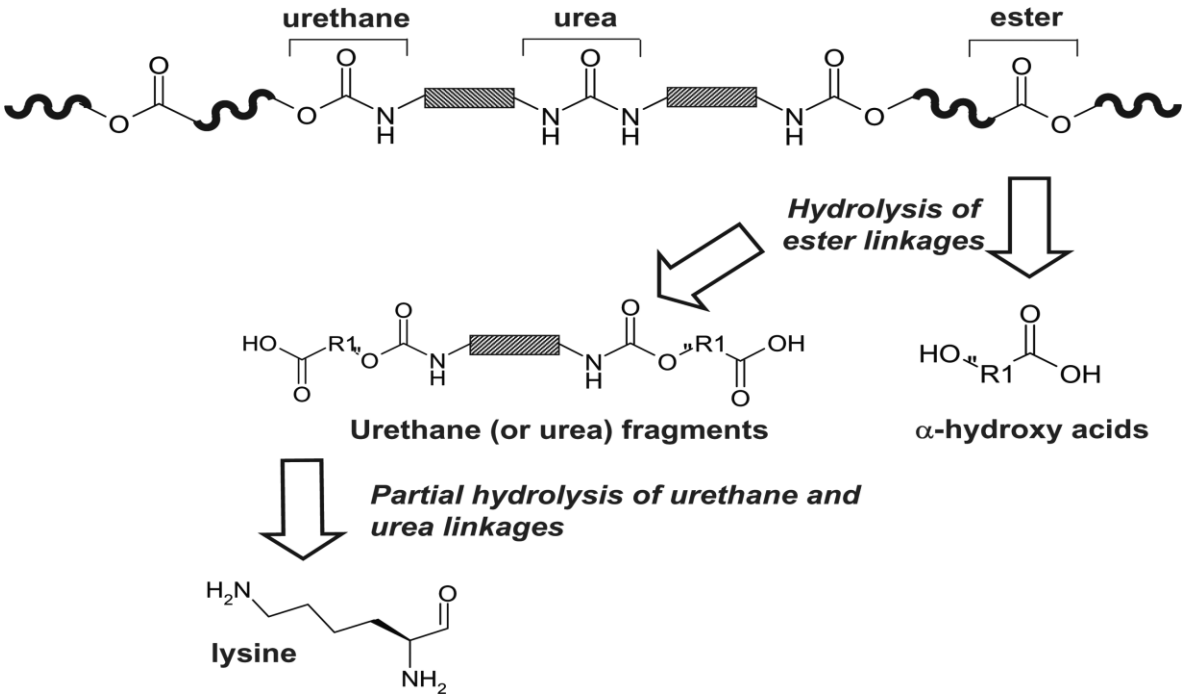


Figure 1.6: Represents the reaction mechanism of a type of polyurethane biodegradation

Several studies suggested that physiological effects of functional group influence the rate of biodegradation of the material, as well as rate of clearance of the degraded product from the implanted tissue.

1.3.3 Scaffold processing: Tissue engineering scaffold has been made from biodegradable polyurethane by using several techniques such as freeze drying, electro spinning of fiber etc [22]. On the basic of application concern, several polyurethane scaffolds has been synthesized, few of them are highlighted below

Polyurethane scaffold for cardiovascular application: Polyurethane has been introduced to engineer the cardiovascular tissue with adequate biodegradable and biocompatible property along with high elongation at break and tensile strength as per natural cardiovascular tissue.

Polyurethane scaffold for musculoskeletal application: Biodegradable polyurethane have been prepared from MDI by controlling of ester linkage in Polyurethane backbone. This elastomer has been studied as a scaffold for bone tissue engineering, with MDI based hard segment to mimic the natural bone. Scaffold synthesized from HDI based polyurethane with a chain extender and soft segment have reported for bone healing.

Polyurethane scaffold for nerve regeneration: Novel biocompatible and biodegradable polyurethane has been investigated for fabrication of biodegradable nerve guidance channel.

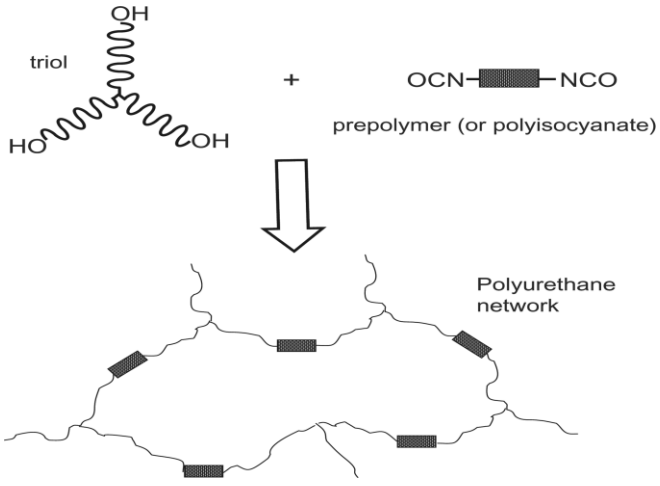


Figure 1.7: Schematic process of isocyanate based polyurethane network preparation

Network of polyurethane was synthesized by at least one isocyanate along with at least one ester linkage. As per material consideration, polyurethane has been commercially used in the medical field since 1960s as a bio-stable implant. In recent years, biodegradable polyurethane is synthesized to control the degradation and promote the new tissue growth with the help of state of art infrastructure of tissue engineering. This material supports the growth of cells and tissue for both in-vitro and in-vivo. These material exhibits tunable biological, physiochemical, mechanical along with excellent control over biodegradation which opens a future opportunity as next generation scaffolds for tissue regeneration [19, 23].

1.4 Pharynx-hypopharynx:

Pharynx is a fibro muscular tube, present behind the mouth and nasal cavity, above the larynx and esophagus. Pharynx is the part of digestive system which consist the part of throat. Anatomically human pharynx is divided into three sections: nasopharynx, oropharynx and larngopharynx [24].

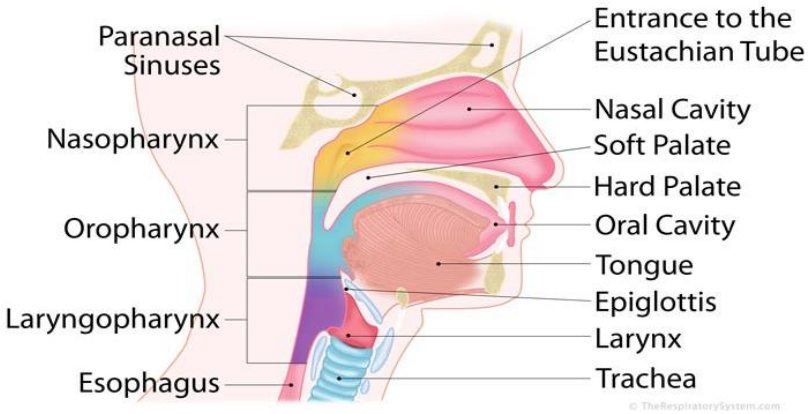


Figure 1.8: Detailed anatomy of human pharynx

Nasopharynx: It is the upper portion of the pharynx. Pharyngeal tonsil or adenoids are known as lymphatic tissue present in the posterior wall of the nasopharynx. Nasopharynx can be obstructed due to congestion by polyps or mucus in case of upper respiratory infection. The auditory tube opens into nasopharynx connected with nasal cavity. The nasopharynx is also involved in breathing and speech [25].

Oropharynx: It is also present posterior of the oral cavity which is extended from ovula. The anterior wall of oropharynx consist of the base of tongue and epiglottic vallecula, the posterior wall consist of tonsil, tonsillar fossa. Food and air both is passing through the pharynx in which a flap of epiglottis closes over glottis during swallowen of food to present aspiration.

Laryngopharynx: It is also referred as hypopharynx which connects to the esophagus. It present to the interior of epiglottis to the common pathway into larynx (respiratory) and esophagus (digestive) canal. Hypopharynx is the combination of three anatomical sites: posterior hypopharyngeal wall, the pyriform sinus and the posterior cricoids region [24,26].

- Posterior hypopharyngeal wall: This is the posterior wall of pharynx. This is made of constrictor for muscles and sits on the tissue covering vertebrae.
- Pyriform sinus: One pyriform sinus is present on each side of larynx. Pyriform sinus is like upside down pyramid in which the tip impinge into the esophagus. This is the main and most common for muscle site for hypopharyngeal cancer.
- Posterior cricoids region: It is present posterior of cricoids cartilage. This is the least common site for hypopharyngeal cancer.

1.4.1 Hypopharyngeal cancer: It is a disease due to cancers (malignant) cell development in the area of hypopharyngeal tissue. In case of cancer the cells originate from the lining of throat surface to form sqamous cell carcinoma [27].

1.4.1.1 Stages of hypopharyngeal cancer:

Stage 0: Only in the lining of hypopharynx but no spread to lymph node.

Stage I: Tumor in the area of hypopharynx around 2 cm in size or smaller. In this stage also no spread to lymph node.

Stage II: Tumor size generally 2cm to 4cm and not spread to larynx (voice box). Generally, it is found in the surrounding area hypopharynx including nearby tissues. In this stage lymph node is also not affected.

Stage III: Size of tumor is more than 4cm in which it is usually subjected to spread over lymph node, maybe some area of neck of that side.

Stage IV: This stage is also sub divided into three categories Stage IV-A, IV-B, IV-C.

IV-A and IV-B: This is the advanced stage of cancer where lymph node must be affected. It is also spread to the surrounding tissue of pharynx such as larynx, thyroid gland etc.

IV-C: It is believe that, cancer is spread over all the surrounding areas of neck including the other part of the body.

1.4.1.2 Symptoms of hypopharyngeal cancer: Following sign and symptoms might be an initiator for the development of hypopharyngeal cancer [27]

- A lumph or tumor in the neck
- Difficulty in swallowing and feel the pain
- Voice change or hoarseness
- Ear pain
- Enlarge lymph node
- Choking
- Unexpected weight loss

1.5 Scanning Electron Microscopy:

Scanning electron microscope uses a focused electron beam over a surface to scan and create an image. Electron beam of the SEM interacts with the sample and produces various signals that are used to get the information about the surface property and composition. Scanning electron microscope has much shorter wavelength then the light microscope to get the better resolution. The principal components of SEM are source of electron, electron detector, sample chamber, column down which electrons travel with electromagnetic lenses and computer to display the images. At the top of column, electron are generated, accelerated down and passes through screening lens and aperture to produce an electron with focused beam which ultimately hit the

sample surface. The sample material is mounted on a stage inside the chamber area. The column and the chamber are evacuated by the pumps till the microscope is designed to operate at low vacuum. This level of vacuum generally depends on the microscope design.

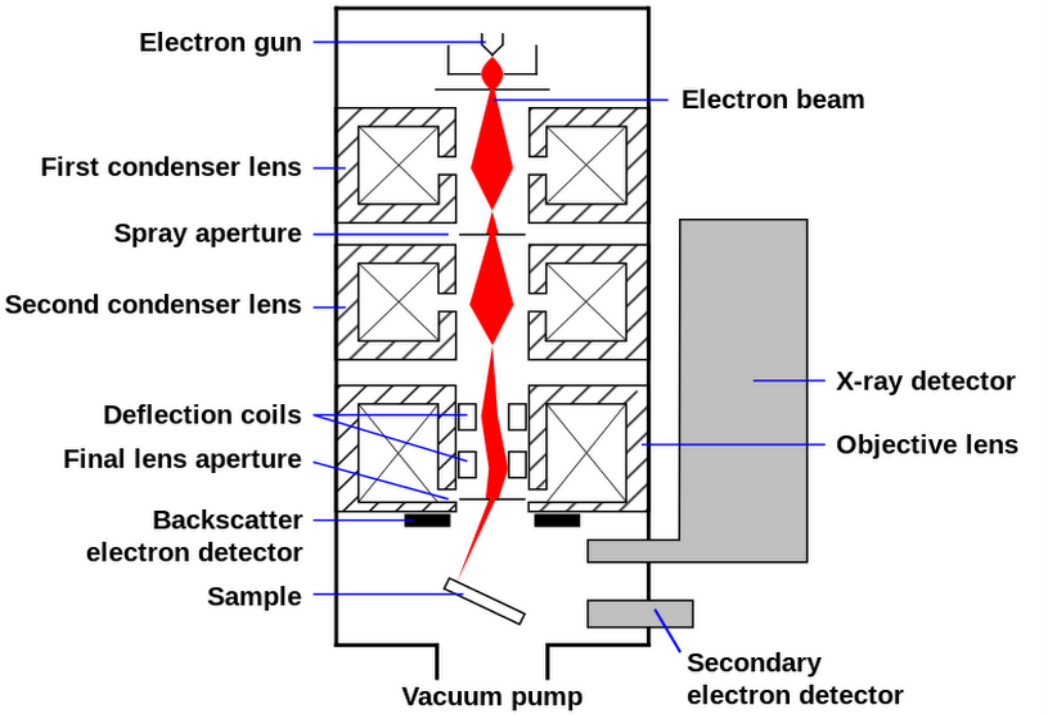


Figure 1.9: Mechanism of Scanning Electron Microscopy

A scanning coil is situated above the objective lenses that control the position of electron beam on the surface of the sample. Then scanning coil allows the electron beam to scan over the sample surface. The beam of scanning enable the information about a define area of the sample to be collected. These electron beams with sample surface are internal and as a result a no of signals are produced. Appropriate detector is used to detect those signals [28].

Electron beam interaction with sample: The scanning electron microscopy produces images with the help of high energy electron beam over the sample surface. Due to this interaction they produce secondary electrons, back scattered electron and characteristics x-rays. This signal was collected and control into an image form with several image processing and displayed on the computer screen. Electron beam penetrates through the sample surface to the depth of few

microns as per the accelerating voltage of electron beam and sample density. Scanning electron microscope can be able to achieve the resolution better than 1nm. Sample is observed by a high vacuum conventional SEM or by low vacuum invariable processor environmental SEM. Secondary electron can generate high resolution images of the specimen surface. Back scattered electron are generally used in analytical SEM along with the characteristics x-ray as the intensity of back scattered electron signal is evidently related to atomic number. The energy of characteristics x-ray is measured by energy dispersive x-ray spectroscopy which results are used to evaluate the abundance of material in the sample. Scanning electron micrograph has a wide depth of field due to very narrow electron beam which yields characteristics three dimensional images for better understanding of the sample surface. Electron amplifier is present in the detector which amplifies the signal to display the high resolution range. Each pixel of computer memory is synchronized with various position of electron beam over the specimen in the microscope. The final image can be represented as a distribution map of signal in truly, emitted from the scanned surface of the sample [28]. Scanning electron microscope has several advantages including the ability to take the larger surface image of the sample and also take the image of bulk material includes the various analytical models to measure the composition and properties of the specimen [29].

1.6 Fourier-Transform Infrared spectroscopy:

Fourier Transform Infrared Spectroscopy is a method of obtaining infrared spectra by collecting the signal for a sample using interferometer and then performs the Fourier transform phenomena (concept) to obtain the specimen. These techniques obtain the infrared spectrum of emission or absorption for a solid, liquid or gas with high resolution data over a broad spectral range. In this analysis, a mathematic process is involved to convert raw data into spectrum image.

FTIR spectrometer are used in the infrared region 2-25µm (5000-400cm-^{1}). Pyroelectric detector is generally used to detect the changes in the temperature as the intensity of IR radiation varies. FTIR has diverge range of applications like in biological material, to investigate the particularly at a gene site along the backbone of trans-membrane protein. Microscopy and imaging studies

using FTIR indicates the different chemical species within the sample and analyzing the tissue sections [30].

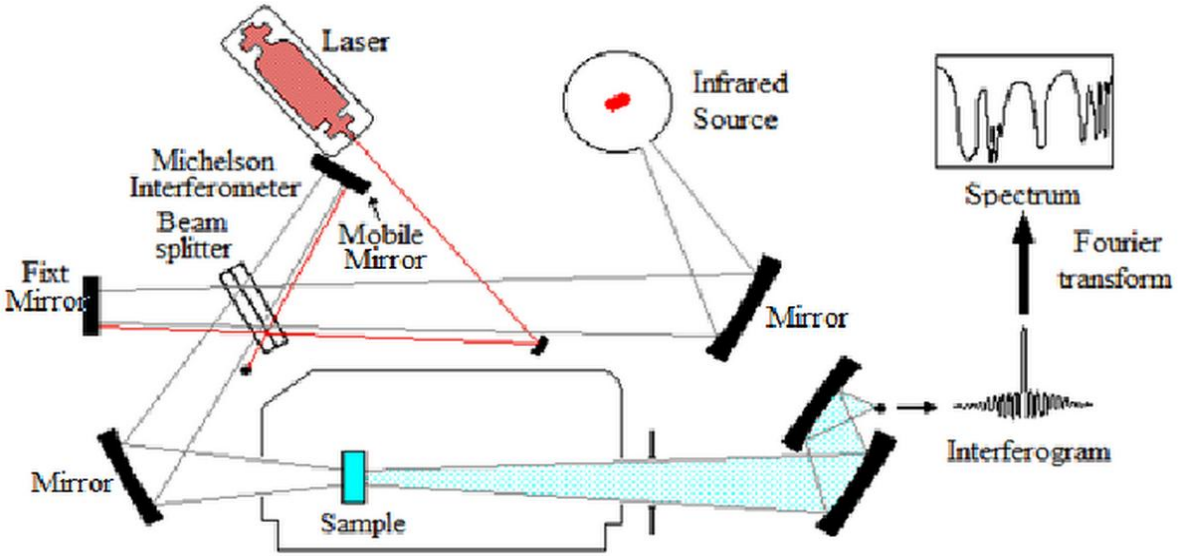


Figure 1.10: Working principal of Fourier Transform Infrared Spectroscopy

FTIR is a highly flexible research instrument which provides the wide range of information:

- Identification of unknown
- Quantative information such as additives
- Evaluate the complex information with other instrument analysis like TGA.
- Kinetic information of the growth and decay.
- Detect the range of functional group.

1.7 Universal Testing Machine:

A universal testing machine (UTM) is used to test compressive and tensile strength of the sample material. It is versatile machine which perform different standard tensile and compressive test on components and material such as metal, concrete, rubber, textile etc. This diversity as per elastomer's need makes UTM as a universal tensile testing machine in any manufacturing industry. Universal testing machine evaluates the material property like tensile strength, elasticity, yield strength and strain etc. Several variations are observed in general component of

UTM like load frame which consist of two strong supports but in case of small machine, one single support has provided. Load cell is a force transducer requires for measuring the load and a movable cross head is control the movement of up and down with constant speed. The testing result has recorded via dial or digital display and chart recorder. Now days, most advanced UTM have a computer interface for data analysis of tested material. If important conditioning can be possible using UTM like temperature, humidity and pressure etc [31].

This testing depends on test method published by standard organization (like ASTM). They have specified the procedure of sample preparation, fixing, gauge length, analysis etc. As the machine is started, the load is begun to apply on the specimen and data is recorded by the control system and associated software's including the extension or compression of the specimen [32].

1.8 Antimicrobial activity:

Antimicrobial activity is a process of inhibiting or killing the disease causing pathogenic microbes. Antimicrobial testing can be used for the material susceptibility and suitable therapeutic outcomes. Antimicrobial activity is generally tested in common infection causing pathogenic bacteria strain such as E. coli and S. aureus [33].

1.8.1 E. coli: It is basically a gram negative bacterium, framed with the peptidoglycan layer and having an outer membrane that provides a barrier for certain antibiotic such as penicillin.

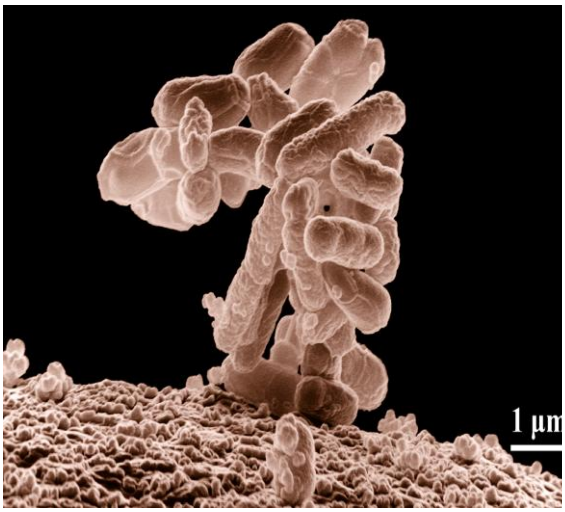


Figure 1.11: Represents the shape and size of E.coli

It is facultative anaerobic, rod shaped, coli form bacteria is generally found in lower intestine of warm blooded organisms. E. coli takes the safranin staining (counter stain of gram staining) and show the pink colour. E. coli can grow in different kind of laboratory media like lysogeny broth or the media containing glucose, magnesium sulfate, dibasic, potassium phosphate, water etc [34].

1.8.2 S. aureus: It is a group of positive, round shaped bacteria which is one of the major human pathogenic microbes causing a wide range of clinical manifestations. This bacterium is generally found in upper respiratory tract and on the skin.

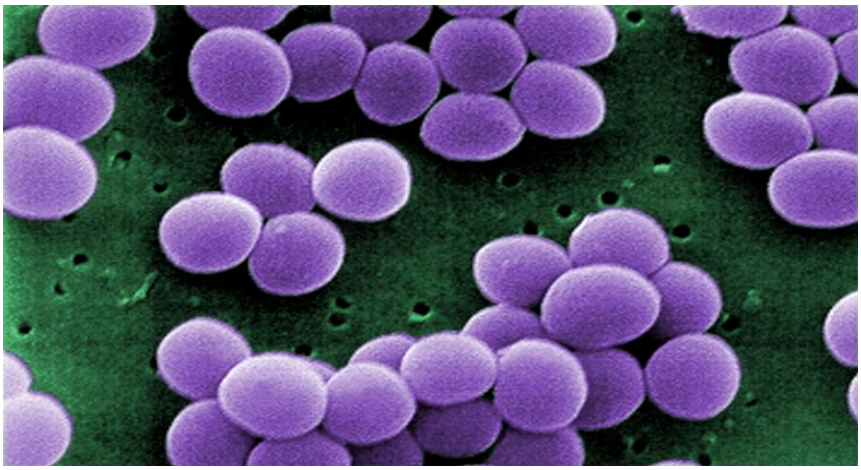


Figure 1.12: Shows the shape of S.aureus

This bacterium is also resist to penicillin and can easily proliferate in different kind of laboratory media. It is basically non motile bacterium so it doesn't form spores. This gram positive bacterium takes up the crystal violet staining (purple strain of gram staining) [35]. This bacterium forms a grape like structure show in the figure.

Among the different method of antimicrobial activity assay, agar disc diffusion and agar well diffusion methods are mostly used.

Agar disc- diffusion method: In this method, agar media plates are inoculated with the test microbes. Then filter paper disc of standard size with test compound is placed on the plate (surface of media).

The petri dishes are inoculated and maintain the proper growth condition for the particular bacteria. Finally the zone of inhibition (shown in figure 1.13) is measured to check the material potency against that particular pathogenic bacterium [36].

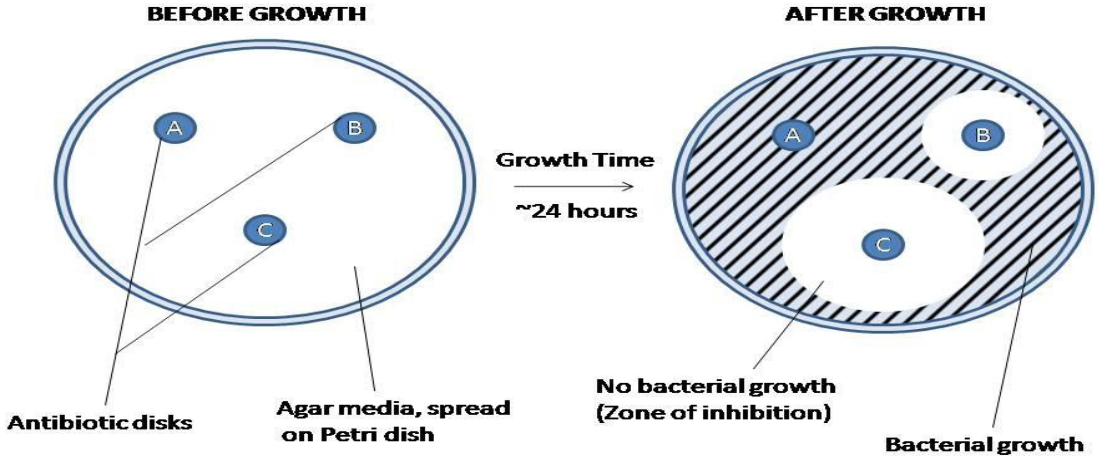


Figure 1.13: Showing the zone of inhibition after bacterial growth

Agar well diffusion method: This technique has few similarities with disc diffusion. In technique a hole is made as per the protocol (standard size) and antimicrobial agent or material is added into that well. All the growth conditions are maintained and finally the zone of inhibition are measured which shows the significance of the material activity against that pathogen [36].

1.9 Cell Culture:

Cell culture is a process in which cell are proliferate and grows in controlled conditions. Firstly, cells of interest are isolated from living tissue and then maintain in controlled conditions.

1.9.1 Peripheral Blood Mononuclear Cell (PBMC): These cells have a round nucleus and the cells include monocytes and lymphocytes (B cells, T cells and NK cells). These blood cells are the important component in the immune system to fight against pathogenic infections. Blood with ficoll (hydrophilic polysaccharide) have undergoes gradient centrifugation from where a separated white layer isolated, containing PBMC. In this gradient centrifugation top layer contain plasma, followed by a white PBMC layer and bottom contains the layer of erythrocytes [37, 38].

1.9.2 Fibroblast cell: Fibroblast is a type of cell that synthesizes the collagen and extracellular matrix. This fibroblast (shown in figure 1.14) is the structural framework for animal tissue and also plays an important role in tissue healing. This is the most common connective tissue cells in animals. Primary fibroblast cell culture is experimented to optimize the synthesis of collagen and extracellular matrix that drives the tissue healing. Primary fibroblasts isolated form skin, pharynx, heart, uterus and hypopharynx etc. Cells are cultured in proper growth media to ensure the optimal growth throughout optimum possible number of passages for cell types [39].

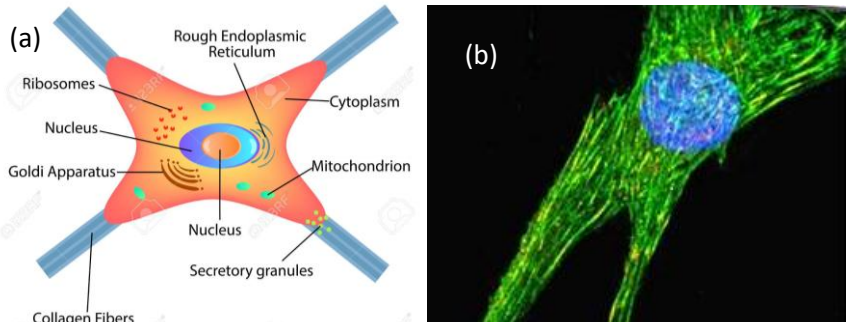


Figure 1.14: (a) represents the basic structure of fibroblast cell; (b) showing the fibroblast cell after staining

1.9.3 Cytotoxicity evaluation using MTT assay: It refers the toxicity to cells. Various types of phenomena occur if the toxic element is treated with cells. Due to toxic effect, cell can lose their membrane integrity and die rapidly, sometimes they can stop growing and proliferating or undergo apoptosis by activity controlled program of cell death [40]. MTT assay is generally used to check the cytotoxicity of different material on cell culture. First the assay performs to check the homogenous cell viability. These viable cells undergo formazan production due to active metabolism using MTT. Dead cells are not capable to form formazan (purple colour). This phenomenon differentiates the healthy cells from the dead cells, represented in figure 1.15.

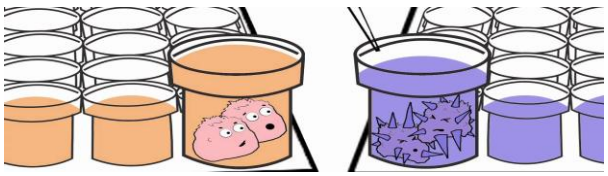


Figure 1.15: Shows the colour changes due to cell death using MTT assay

Exact mechanism of this formagen formation is still unclear to the researcher. This MTT react with mitochondrial dehydrogenase of live cell to form formazan which insoluble precipitate deposited in the close proximity of the cell surface. Finally, this formazan is solubilized to record the absorbance value using different solvent such as DMSO, dimethyl formamide, isopropanol etc [41].

1.10 In vivo biocompatibility study using animal model:

To evaluate toxic effect of any synthesized material it should undergoes in vivo study to understand the compatibility using animal model like rat or mice. The animals are used to test the biocompatibility of the material following the regulations of animal ethical committee. First stage of any material compatibility can be checked by wound creation mechanism. Several kind of wound model is classified like excision wound model, incision wound model, burn wound model, superficial wound model etc [42, 43]. In the healing compatibility study the material provides a potential matrix to promote and enhanced the proliferation of fibroblast. This help in the granulation tissue formation which is the beginning phase of new tissue formation. This preliminary indicates the biocompatibility of material. Upon synthesizing the new matrix element, principal element of extracellular matrix including the collagen indicates the proper care of any wound which ultimately explains the full proof biocompatibility of the material [44].

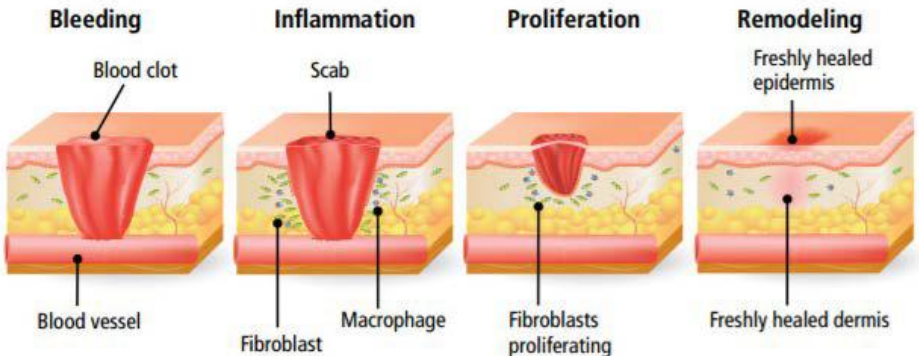


Figure 1.16: Represents the tissue remodeling process

In figure 1.16, the picture explains the normal tissue recovery process for any kind of wound: In case of material compatibility study, the material is generally placed on the wound area and if it

promotes the healing without causing any inflammatory, immunogenic or carcinogenic effect then considered as biocompatible material [37].

1.11 Histopathology:

It is basically the microscopic examination and evaluation of tissue for studying the disease manifestations. Micro anatomy also refers, histology is the anatomical study of cells and tissues (plants or animal) using microscopy. To evaluate tissue section generally used the light microscope but for specialized study electron microscope is also taken into consideration. The most common and popular strain of histopathology study is hematoxylin and eosin (H and E) stain [45].

1.11.1 Hematoxylin: It is extracted from the bone of 'hematoxylom campechiaum'. To strain the nucleus, hematoxylin oxidizes to hematin which is a weak anionic dye of purple colour. Anionic hematin has no affinity for nucleus so metallic salt is mixed with hematoxylin to get positively charged metal action. Ultimately cationic dyes bind to nuclear chromatin (DNA or RNA) and strain them to purple.

1.11.2 Eosin staining: It is counter staining, achieved with an aqueous or alcoholic solution of eosin. It is a red or pink colour strain which is acidic and negative in nature. It is built with positive charged amino acid (lysine, arginine).So its build with the protein and strain them pink, also includes cytoplasmic filaments such as muscle cells, intracellular membrane etc [46].

1.11.3 H and E strain, the studied cell can be observed as-

- Nuclear in purple
- Cytoplasm in red
- Muscle in dark red
- Collagen pale pink
- Mitochondria pale pink
- Erythrocytes in cherry red
- Basophils in purplish red.

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Chapter 2

2. Tissue is the cellular level organization for the development of organ and whole body. Every year, millions of patients suffer the failure or loss of an organ or tissue as a result of any disease or accidents. A paradigm shift has taken place in medicine from using artificial implants and tissue grafts to an engineering approach that uses degradable porous biomaterial or scaffolds integrated with biological cells or molecules to regenerate or repair tissues [1]. Tissue engineering can be utilized to restore or enhance tissues and organs. The potential impact of this engineered tissue could reduce the need for organ replacement, and could greatly accelerate the development of natural organ [2].

2.1 Tissue reconstruction, revolution of medical science:

In the soft tissue engineering scaffold with high strength and elasticity are necessary mostly with controllable biodegradation property. Polymeric materials with high biocompatibility have been extensively used for fabricating living tissue construct. In case of hypopharynx tissue engineering, skeleton muscle plays an important role as a part of organ [3]. A tissue flap is basically persists blood supply and doesn't rely on receipt bed to perfuse donor tissue. Tissue flap are very versatile which can be used to cover large defects to recreate the structure. In case of higher value of tissue transfer demands constant perfusion is needed and be compromised if the supply of arterial or venous drainage is interpreted during the tissue transfer process. Autologous recombination uses tissue like skin, fat and sometimes muscle from another place of the body like to form the breast shape etc. The tissue also referred as a flap generally comes from the back, belly or inner thigh to create the reconstructed tissue. Normally, the tissue can be completely separated from the natural blood vessel to pick up and moved to new place in the appropriate position. This is also referred as a free flap. Some time tissue can transfer with all the blood vessel and placed under the skin, this kind of flap is pedicled flap [4].

Flap reconstruction using tissue from the back-latissimus dorsi muscle is located in the back, below the shoulder and posterior of armpit. It's the muscle that helps for twisting movement like swimming. Latissimus dorsi flap produces an oval flap of muscle; skin including blood vessels from upper back is used for reconstruction. The flap contains the significant amount of muscle present in the latissimus dorsi flap can be considered as a muscle transfer type of flap. Latissimus

dorsi flap helps in the breast reconstruction as it look very natural in terms of colour and texture of breast skin. In the hip flap surgery, skin, fat including blood vessel are cut from upper hip or buttock and placed to the chest to rebuild the breast. Though this kind of flap surgery is technically more difficult than other flap and it is usually take more time. Transverse upper gracilis flap located in the upper thigh which uses gracilis muscle. This muscle starts at pubic bone and ends along with the upper leg. This flap is taken from the upper thigh to reconstruct several areas tissue reconstruction [5].

Soft tissue reconstruction applies to restore both the functional and aesthetic appearance of hand. Skin grafts and substitutes both are useful for reconstruction options to treat certain defects. Flap from the dorsal metacarpal artery system finds applicatiom for hand tissue reconstruction. Using of this material is increased rapidly in impact due to surgical success dependent on strict legal labeling [6]. Zinconia based biomaterial have taken the attention as a potent material for hard tissue engineering. This hard tissue reconstruction basically includes bone and dental tissue including osteogenic scaffolds. Zincornia becomes one of the most popular ceramic material in healthcare practices due to its extraordinary mechanical property, biocompatibility etc [7]. Regeneration medicine utilizes cell based approaches to regenerates diseased or damaged tissue. In the development of biomaterial the strategies for soft tissue reconstruction potentially stimulate the adipose tissue regeneration. Various kind of adhesive material is used as an adhesive biomaterial for tissue reconstruction. Autologus fat grating with biomaterial is a new approach that involves the harvesting of tissue by liposuction from one side of the body and placed to fill a soft tissue defect. Adipogenic differential stem cells encapsulated in alginate and implanted subcutaneously. In combined with collagen it shows the better efficacy to mimic the native extracellular matrix environment [8].

Laboratories are experimenting with different types of biodegradable and biocompatible scaffold for achieving the cartilage healing. Various techniques include the scaffold in their bare form or association with cells, growth protein that stimulates the proliferation and tissue reconstruction. Now tremendous new strategies are developed to promote the regeneration of musculoskeletal, skin, vascular and tissue which drastically enhance the cell viability and communication with

host. It is also clearly found that cells associated with innate and adaptive immunity played a crucial role in tissue damage recovery. Role of various T cell populations in regeneration and angiogenesis are similarly being explored by using biomaterials [9].

2.2 Biodegradable and Biocompatible material:

The functional understanding of the biodegradation phenomena as well as in corporation of cellular and tissue responses determines the biocompatibility of biodegradable material, which is important component in design of biodegradable microscope containing bioactive agents for therapeutic applications [10].

Biodegradation: It is generally considered that mechanism of material biodegradation is based mainly on hydrolytic degradation mechanism. The main factors that show the effect on hydrolytic biodegradation such as water permeability, solubility, chemical composition, porosity, glass transition temperature, molecular weight, physico-chemical factors etc. It has been found that chemical composition alteration by increasing glycoside mole ratio in the polymeric material increases the rate of biodegradation. Additives including their acidic or basic nature as well as loading level in case of therapeutic agent may have remarkable effect on the biodegradation rate. It has been widely accepted that the head group of cation has role in toxicity. Longer side chain also have more impact on living cells specially those incorporate an ester group significantly increase their biodegradation [11]. Research also revealed that additions to chemical structure of polyurethane may also decrease the biodegradation [12]. It has been explored that amount of degradation varies by physical makeup of polyester polyurethane, containing on glass tube. Microbial degradation of polyurethane is hypothesized to be mainly due to hydrolysis of ester bond [13].

Porosity of the material may play a major role in enhancing the biodegradation rate, specifically when the dimension of pore is significantly larger enough for cellular migration. Investigation shows diverse polyurethane has different chemical structure. The rate of biodegradation varies on the method such as hydrolytic degradation or enzymatic degradation by lipase. Finding of the research has conducted that rate of biodegradation increases in accordance of diisocyanate like

MDI<H_{12}MDI<HDI. It has been also found that polyurethane composed by aliphatic diisocyanate shows a greater rate of biodegradation than composed by aromatic diisocyanate [14].

Biocompatibility: Evaluation of the biocompatibility of implantable material requires a deep understanding of the inflammatory and healing response of that material. Wound healing, inflammation recovery due to material response are normally considered as the component of tissue or cellular host response to any kind of injury. Size, shape, chemical, physical property of the synthesized biomaterial including the dimension may be responsible for variation in the intensity and duration of healing process that drives the biocompatibility of the material inside the host [15].

Several event of implantation of a microsphere drug delivery system clearly explains the active inflammatory response, chronic inflammatory response, foreign body reaction to implanted biomaterial. It has been found that myoblast culture and interleukin 1 in air pouch model with in vitro monocytes culture were used to investigate the biocompatibility of different polyurethane membrane. Another study reveals the interaction of biomaterials with various cell types that has been experimented from different viewpoints to evaluate the biocompatibility of polyurethane membrane [16].

In the literature study, it has been found that dose dependent surface endothelium greatly affect the biocompatibility of polyurethane. Biocompatibility of medical grade biomaterial, polyurethane coated polyaniline is reported also. Another study reveals the polyurethane glycol containing polyurethane hydrogel coating improves the biocompatibility of neural electrons. Chitin based polyurethane elastomer were synthesized by step growth polymerization shows tunable biocompatibility to use as a potent biomedical implants [17]. Recent study showed a polyurethane biomaterial composed of urethane hard segment and amorphous soft segment to copolymers of lactide and Ɛ-caprolactone to produce excellent in-vitro and in-vivo biocompatibility. Scaffold of polyurethane urea with appropriate elastic modulus shows good biocompatibility, blood compatibility and durability for long term use in cardiac device. Monocytes activation has showed the role on nano composites with proper surface microphase

separation and suggesting the significant role of nanostructure surface modification on biocompatibility of polyurethane [18]. It has been found that plane and ion beam techniques improve the polyurethane surface biocompatibility. Sulfonated polyethylene glycol grafted polyrotaxanes were prepared in order to surface modification of polyurethane for improved the biocompatibility [19].

2.3 Polyurethane-future material:

In the soft tissue engineering, scaffold with high strength and elasticity with control biodegradation including sufficient biocompatible are necessary. Polyester urethane and polyether urethane from polycaprolactone have synthesized to fulfill such criteria. Hyper branched polyurethane are developed using TDI, PCL, butanediol with or without monoglyceride of sunflower oil to tailor biodegradation, physio-mechanical and thermal property. This hyper branched polyurethane was studied in-vitro, in-vivo interaction of cell and material to draw their potency as a scaffold in tissue engineering [20].

The studies have reported that polyurethane with incorporation of polyether or polycarbonate polyols undergoes enzymatic and oxidative degradation which drives by the inflammatory cells interaction with the material. Polyester urethane fabricated scaffold network with segmented elastomers undergo significantly control degradation to non cytotoxic breakdown of the product which supports the in growth of new tissue in clinical model for hard and soft tissue regeneration. Cholesterol esterase has been reported that it can degrade ether and urethane linkage with low degradation rate [21].

Two component polyurethane network were synthesized using polyester triol derive from lysine polyisocyantes to evaluate mechanical, biodegradation properties in recovery of bone defects such as compressive fracture in the vertebral bodies. The material showed the effect in the osteogenic medium where MC3T3 cells cultured on the material network and cells deposited extracellular matrix [22].

Hydrophillic polyurethane synthesized by reaction among polyethylene glycol, Ɛ-caprolactone and hydroxymethyl propane to fabricate tissue engineered scaffold for hypopharyngeal tissue

engineering. Ferric catalyst showed that significant effect on polyethylene glycol and Ɛ-caprolactone based polyurethane synthesis which also controls the synthesis process. This soft tissue material was examined for fibroblast cell growth to check the potentiality of the material as biocompatible [23].

Polyurethane can be composed of hard, soft or combined both for use as a substitute of tissue. Soft segment of polyurethane are composed by polyester where as hard segment produced by diisocyanate with reacting diol or diamide. Soft segment makes the material as an elastomer while hard segment provides the strength due to urethane linkage involving hydrogen bonding. Biodegradable polyurethane film has been prepared based on diisocyanate with hydrolysable cross-linked bond to form homogeneous structure for biomedical applications [24].

PCL and gelatin based composite nano fibrous scaffold has prepared by electro spinning method for tissue engineering applications. Positive result of DNA quantification on L929 mouse cel has indicated that the membrane prepared by using natural polymer gelatin may serve as a potent biological material. Nano scale feature of nano fibrous scaffold consist of high surface to volume ratio that enhance the cell adhesion and migration including the efficient supply of nutrient in nano fiber architecture scaffold has revealed that it selectively promote the proliferation of osteoblast and differentiat in carbon nanotubes. Study using human corneal epithelial cells on nanogrooved surface can induce the adhesion guidance that causing to align and elongate their cytoskeleton including topological features. Research revealed that electrospun PCL nano-fibrous scaffold can architecturally mimics the extra cellular matrix in living tissue [25].

A tripolymer of gelatin, PCL and collagen type I composite nano-fibrion scaffold has been fabricated using electro spinning techniques for skin tissue engineering including wound healing applications. Collagen type I modified scaffold showed the characteristic cell morphology and high proliferation rate using L929 mouse fibroblast cell [26]. Some studied have shown the modification of surface and internal properties of polymeric nano-fibers to improve cell compatibility and tissue regeneration potential. Research reveals that addition of hydrophilic polymer such as polyvinyl alcohol and polyethylene oxide has increased the cell adhesion and

activity. Moreover, surface coating with collagen or gelatin was reported to enhance the response of endothelial cells [27].

It has been found that prepolymer can provide an additional degree of control over the structure of biodegradable polyurethane. Polyurethane generally prepared by reacting a polyol with diisocyanate. Prepolymer with NCO terminal are oligomeric intermediates shows isocyanate functionally. Catalyst can be used to increase the reaction rate of urethane where NCO:OH plays a vital role in prepolymer processing and chain extension.

$$NCO:OH=Q_{NCO},I / Q_{OH},P= M_I.W_P / M_P.W_I$$

Here, Q is the no of equivalents

M is the each component mass (I denote isocyanate and P denotes polyol)

W is the weight of each component

Prepolyper intermediate have the ability of tailoring with targeted property by varying the NCO:OH ratio that differentiates the polymer and chain extension could be achieved by adding a polyamine or polyol short chain to prepare a high molecular weight polymer [28].

Several studies reveal the polyester based urethane is more susceptible to degradation than the polyether based polyurethane. Polyesterurethane has been synthesized by reaction of ester diol with 2, 4 toluene diisocyanate in NCO:OH molar ratio of 2:1 for biomedical applications. One group of researcher synthesized the polyurethane by reacting 1,4 butanediol chain extender to prepolymer , composed of polybutylene succinate, polyethylene glycol and 2,4 toluene diisocyanate that showed the rapid hydrolytic degradation [29].

2.4 Biological activity:

Polyester urethane urea made from 10% concentrated polymeric solution has shown the ability to support the rat vascular smooth muscle cell adhesion and growth along with evaluation of MTT mitochondrial cell viability assay. Further study reveals that polyester urethane prepared from 5% concentrated polymeric solution, promote higher degradation than 8% or 10% concentrated solution [30].

Biological activity includes the bacterial effect on the tissue engineered material. Study shows that bacterial secretes extracellular polysaccharides forming bio-film which provide protection by various ways like increasing infection rate, substrate accessibility, and metabolic efficacy towards environmental stress. The bio-film inhibits the effect of antimicrobial agents. Chronic wound healing is mostly remarked through prolonged inflammation, erroneous epithelization and also in adequate matrix remodeling. That's why; it is needed to take action for inhibiting the growth of pathogenic microorganisms to eliminate all the possible hurdles that harmful bacteria may cause in tissue regeneration process [31].

Cytocompatibility of polyurethane scaffold to human skeletal muscle cells and primary fibroblasts were separately evaluated. Culture cells on membrane were also examined through morphological observation and immunohistochemistry. Multinuclear cross strained muscle, skeleton muscle are organ specialized towards force rapid production and play an essential role in positional and functional maintenance of hypopharynx tissue. The study also evaluated that gelatin or fibrion grafted polyurethane substrates exhibited sufficient cytocompatibility to human hypopharynx skeletal muscle cells [32].

Polyurethane incorporated with growth factors has been found to have bone repair ability. The bone morphogenic protein into silk fibrion solution and scaffold produced by electro spinning showed more calcim deposition [31]. The bone morphogenetic protein has been used for bone matrix formation along with collagen is generally utilized in bone regeneration for its osteoinductivity. Salt leached porous silk fibrin scaffold was synthesized to improve the characteristics of bone matrix powder and also enhance the adhesion, proliferation and differentiation of bone marrow stem cells [33].

Cross linked polyurethane membranes were showed cytocompatibility to primary hypopharyngeal fibroblast. Primary antibody, antivimentin was used to cells for immunohistochemical staining and exhibiting positive phenotype to confirm human fibroblast origin. The results indicated that fibroblast cells could survive and proliferate well on polyurethane scaffold which indicate the potency of the material on the regeneration of hypopharyngeal tissue with biological function and constitution [30].

Immunofluroscence with primary antibody MyoD1 exhibited in all the cells from hypopharynx and green fluorescence demonstrated that the cells have the capacity to grow on the scaffold surface. The resulting scaffold showed the suitable surface chemistry for cell proliferation, growth along with in vitro cell expansion and differentiation into myofibers using directionally aligned micro channel on the scaffold matrix. This helps in skeleton muscle tissue engineering [34].

Antithrombin heparin incorporation for polyurethane scaffold modification has increased anticoagulant property due to AT portion antithrombin heparin (ATH). Surface functional scaffold were examined for the ability to stimulate cellular and vessel growth under normoxia and chronic hypoxia. PEG, PCL and polydiamine based diol were used to synthesize 3D biodegradable polyurethane scaffold for bone graft substitute. Scaffold porosity and size geometry were maintained for in growth of capillaries and degradability with respect of bone healing. In addition the material has the ability to grow in-vitro and promote osteoblast differentiation [35].

2.5 Physical property and characterization:

Polymeric material is considered for various level of characterization. Proper characterization should be an aim to improve the performance of the polymer. Basically the characterization techniques have linked to the desirable properties of the biomaterial such as strength, thermal stability including optical properties [36]. Characterization techniques are used to understand the molecular structure, surface morphology, thermal property, mechanical properties etc. In research and finding, various types of analytic techniques are considered for the determination of molecular structure of the material. The techniques generally includes in polymer characterization are ultraviolet-visible (UV) spectroscopy, Infrared spectroscopy, nuclear magnetic resonance spectroscopy, X-ray diffraction, electron spin resonance spectroscopy and mass spectroscopy to find and identify the functional groups [37].

Molecular mass of any polymeric material differs from its basic construction molecules, reaction process of polymerization including distributing molecular weight and shapes. Molecular mass

distribution can be evaluated by the weight average molecular weight, number average molecular weight and polydispersity. Few techniques are also taken in consideration to determine this parameter like viscometry, size exclusion chromatography, static light scattering techniques etc [38]. Gel permeation chromatography is a specific type of size exclusion chromatography used in combination with viscometry for determination of molecular weight distribution including the branch ratio and degree of polymerization. This technique helps in the analysis of copolymer molecules mass by UV absorption and differential refractory considering the material composed of two base polymers [39].

Morphology of a polymer depends on the amorphous or crystalline portions of polymeric chain and influence between them. It is basically microscale property which is determined by microscopy techniques such as X-ray diffraction, scanning electron microscopy, transmission electron microscopy, atomic force microscopy etc. Polymer morphology on nanometer scale is important for mechanical and physical properties of many materials. Scanning electron microscopy are an efficient analytical tools to optimize the surface morphology of polymer such as polybutadine-polystyrene, polyurethane and many blend polymers. Transmission electron microscopy gives the internal structural details of polymeric material [10].

Mechanical properties of polymer are generally includes the strength, elasticity, strain, visco elasticity etc. These properties are highly dependent on the vander waals interaction of polymer chain and their ability of chain elongation. It has been found that porosity of polymer can show an impact on mechanical property. In most of the studies tensile strength, young modulus and yield strength are the interest for finding the stress-strain properties of polymeric material. Mechanical strength measurement can indicate the alteration in the molecular weight which could be acheived by manipulating mole ratio and polymerization condition [11].

In the morphological characterization of polyurethane, it shows different domains due to present of different groups. FTIR analysis of the study has recorded the functional group to annotate the particular groups which drive the particular segment such as isocyanate group of hard domain [40].

MDI based biodegradable polyurethane has shown the interaction provided by hydrolysable cross linking bond to check the chain mobility and increase the values of mechanical properties among others. This study has also revealed that mechanical behavior is related to various factors, such as concentration, crystalinity, interconnecting of hard segment and soft segment ability to crystalline with applied stress. The thermal stability of polyurethane is greatly affected by the properties of hard and soft segment of raw material, type of cross-linking bond, chain extender and synthesization method. It has been found that the presence of cross linker elevate the segment aggregation through covalent bonds. Increasing the cross linking density can be important for improving the tensile strength and maximum strain [41].

The cross linking polyurethane have shown the strong absorption from C-H stretching identified by FTIR spectra analysis that suggest the presence of large amount of PEG component. Thermal characteristics like glass transition temperature (Tg), melting point (Tm) at adequate level has made the artificial material to be used as a biomatrix network for soft tissue engineering applications. Several studies were performed in order to know the mechanical nature of natural tissue and compare with the synthesized material to suggest the material potency for tissue engineering [40].

FTIR analysis and SEM were studied to know the morphology of PCL-gelatin composite scaffold to ensure any interaction between PCL and gelatin in fabricated nano-fibrous scaffold. PCL-gelatin composite scaffold also revealed the absorption band shifting towards the lower wave number by using FTIR analysis that also confirms the ester and amide group interactions. FESEM result helps to summarize the fiber morphology and average diameter of nano fibers in the PCL-gelatin composite scaffold. FTIR analysis was performed to further investigate the surface modifications PCL-gelatin composite scaffold by collagen grafting along with pore size calculation for the modified scaffold by FESEM analysis [17].

Dynamic mechanical characterization technique is used to measure storage modules, confirmation of cross-linking, shape and determine the molecular weight. Basically characterization of different properties describes those specificance of the compound to

understand composition and structure including defect that are important for specific preparation, significant use and suffice the production of the material for biomedical research [42].

2.6 Hypopharyngeal tissue on research:

Hypopharynx is the portion between the esophageal inlet and orthopharynx above. Hypopharynx receives the arterial supply from superior and inferior thyroid arteries. It is basically the space from plane perpendicular tip of the epiglottis to superior and lateral aspect of the larynx. It includes the structure of lateral pharyngeal walls, including mucosal membrane and bilateral pyriform sinus [43].

Hypopharyngeal tissue comes on research due to various recent case of hypopharyngeal cancer. Study has shown that high consumption of alcohol and tobacco abuse rapidly increases the chance of hypopharyngeal cancer. This cancer starts in the squamous cell layer refers as squamous cell carcinoma. Carcinoma of hypopharynx represents a clinical significance among the other types of neck cancer. Initially, this cancer starts at hypopharynx and gradually spread in related tissue including thyroid gland, trachea etc. Surgery is the most well known approach for this treatment by removal of effected tissue of the pharynx and replace with other tissue like flap. But this may lead to tissue defects, voice handicap including swallowing in capability. Therefore to overcome such complication tissue engineering comes in this place to synthesis the biomaterial in the form of matrix that could help to repair the tissue defects [44].

Polyester urethane silk fibrion was implanted in rat back, subcutaneously to evaluate the better compatibility towards compatibility and degradation. This kind of finding helps in the artificial matrix for hypopharynx regulation study. Polyurethane scaffold was grafted with gelatin or silk fibrion towards skeleton muscle regeneration for hypopharyngeal tissue engineering. This finding demonstrates that polyurethane scaffold seeded along with mycoblasts to guide hypopharynx muscle regeneration. Cross linking polyurethane was synthesized seeding on fibroblast to check the cyto-compatibility. Biodegradation polyurethane wsa prepared using polyethylene glycol, lactic acid and diisocyanate [45].

Ferric catalyst driven synthesized polyurethane processed good wet ability, fast degradability and good mechanical parameters which made excellent matrix material for soft tissue like hypopharynx. Biological property of this matrix can support hypopharyngeal fibroblast growth and low inflammatory reaction in subcutaneous implantation. 3D muscle grafted being polyurethane composite scaffold to seed with myoblast which can be used for muscle tissue defect. The study has been revealed that protein grafted scaffold surface promote human hypopharyngeal cell proliferation and differentiation [46].

Artificial biological material like a cellular dermal matrix (ADM) along with pectoralis major myocutaneous flap (PMMFs) is used to reconstruct. The large circumferential shows defect involving the oral cavity and hypopharynx. Another study was revealed the synthesis of poster pharyngeal wall by using a cellular dermal matrix. Synthesized polyurethane membrane was efficiently recovered the wound and trauma area with covering and by growing epithelium. The study was found that ADM grows well with the surrounding mucosa which helps in the pharyngeal cavity reconstruction; consequently ADM may be as a safe along with PMMF for a large defect recovery of the hypopharynx [5].

Hyperbranched polyurethane scaffold are synthesized using TDI, PCL and butanediol shows the cell adhesion and proliferation which may refer as a prospective scaffold material in hypopharyngeal tissue engineering. Study showed that flap of hyper branched polyurethane can be used in reconstruction of the pharyngoesophageal segment for advanced hypopharyngeal cancer. Porus polymeric scaffold have been prepared using polyester urethane to engineer hypopharynx. Culture of epithelium cells and fibroblast was conducted on polyester urethane scaffold to evaluate cytocompatibility in both in-vitro and in-vivo to support hypopharynx regeneration [47].

2.7 In-vivo finding:

Polyurethane has prepared by using ferric catalyst that was implanted in female SD rats with assign groups as per the in-vivo experimental model to check the biocompatibility and degradability within tissue regeneration. In the study, the scaffold with surround tissue was

explanted and histologically analyzed to investigate biocompatibility and tissue infiltration. Investigation showed that the material can promote tissue infiltration and also better cytocompatibility [48].

Cross link polyurethane synthesized by HDI and PEG shows the cytocompatibility to primary hypopharyngeal fibroblast and low inflammatory reaction in in-vivo finding. Further real time PCR analysis of cytokine gene expression has revealed the bio-security of the material. Polyurethane scaffold has been synthesized to check the in-vivo degradation and compatibility by subcutaneous implant into SD rats. Finding shows, the material can be believed to a biocompatible and biodegradable material for soft tissue regeneration [49].

3D protein grafted micro channel patterned scaffold was used in vivo muscle repair by subcutaneous implanting into mice model. The scaffold was examined to evaluate the slow degradation over time and healing of muscle was improved by an increased quantity of vascularized muscle fiber. This finding suggested that 3D grafted scaffold created from collagen composite material can be used for muscle tissue repair [23].

Porous polymeric scaffold have been prepared to check the cytocompatibility of primary epithelial cells and in-vivo biocompatibility of the material. Porous polymeric scaffold was grafted by silk fibrin and subcutaneously implanted into wistar rats. Cell free polyurethane silk fibrin scaffold was shown homologous with the surrounding tissues and accompanied with sufficient angiogenesis. Further histopathological analysis was revealed the tissue infiltration into the scaffold [50].

Biodegradable cross-linked polyurethane was synthesized using PEG and TDI to evaluate in-vivo cytocompatibility and degradation in SD rats. Cytocompatibility of the synthesized polyurethane was investigated in hypopharyngeal fibroblast seeding. Further this material was implanted subcutaneously into the back of female SD rats. Further finding shows the angiogenesis and dehydration of fragments followed by visual and histopathological analysis. This investigation may be considered as the material for tissue engineering [51].

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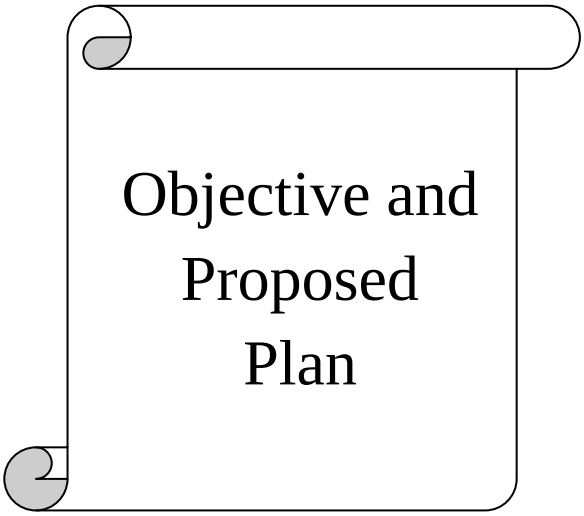
Chapter 3



3.1 Objective:

Since long polyurethane is rapidly used in tissue engineering research. Tissue engineering has been applied in hypopharyngeal research as the rate of hypopharyngeal cancer is increasing and the treatment using surgical flap couldn't able to restore the normal function in most of the cases. Moreover this type of cancer is generally diagnosis at advanced stages so, the treatment should use specialized technology design by tissue engineering to achieve the overall treatment goal, the objectives of this project are listed below-

- To design a biomaterial for tissue engineering that posses effective degradation, cytocompatibility and mechanical property which will make it one of the best preferred material for soft tissue engineering specially for hypopharynx.
- To evaluate the material ability in angiogenesis and biocompatibility using in-vivo model.

Here, we have chosen ester diol based polyurethane as it is more prone to biodegradable. Polyethylene glycol is incorporated in polyurethane synthesis as of its fantastic biodegradability and biocompatibility. Physical property analysis has confirmed the molecular level structural and functional characterization. Degradation ability, hemo-compatibility, antimicrobial activity and cytocompatibility analysis help to explore the efficacy of the candidate biomaterial for tissue research. Animal model study has provided the tunable in-vivo characteristics of the material for further exploration including clinical trials.

3.2 Relevant and priorities:

In the Eastern and North Eastern Part of India, tobacco related cancer tops the list. According to cancer foundation of India, maximum number of tobacco causing cancers was reported in West Bengal, its surrounding Bihar and Jharkhand, as more than 75% of the population consumes some form of tobacco. As per the further report, more than 57% of the students are smoke and abuse tobacco. The younger the age at which a person starts smoking, the greater is the possibility of developing hypopharyngeal or lung cancer in early stage of life. Similarly alcoholism has become a social disease in various states. This rate of increasing alcohol and

tobacco abuse resulted in the upsurge of oral and lung cancer cases. The cases of passive smoking leading to cancer in non-smokers were also noted in the several cancer hospitals in Kolkata, West Bengal [1].

This rapid outbreak/surge of cancer development demands better and economical ways to perform onco-surgery and chemo therapy. At the same time, the health care infrastructure related to cancer treatment should also be developed. West Bengal government along with public-private initiatives supports the cancer related research but there is a dire need to focus on the problems arising due to the tissue rejection because of insufficient availability of suitable tissue engineering materials.

Initially, this initiative will bring a change in the layout of oncological treatment procedure for oral & pharyngeal tissue recovery in those states which will gradually expand all over the country. Therefore, this proposal holds a strong relevance and purpose for cancer treatment and research in India and will surely improve the state of cancer patients undergoing surgery [2,3].

3.3 Background of hypopharyngeal tissue research:

The rapid development of tissue engineering brings new possibilities for laryngeal and hypopharyngeal reconstruction. Biodegradable materials with good mechanical strength, elasticity, and nontoxicity are good potential candidates as biological substitutes. Lactic acid is a well known monomer used extensively for the synthesis of biodegradable polymer for biomedical application. Lactic acid-based telechelic pre-polymer with hydroxyl groups at the ends was prepared, which can be used as a diol for polyurethane preparation [4]. Polyurethane is a widely used material in tissue engineering because of its good mechanical properties, biocompatibility, and biodegradability. It is usually synthesized via a cross linking reaction between diisocyanate and polyhydric hydroxyl with polyol or diamine as the extender. Some components such as caprolactone, poly (ethyleneoxide) lactic acid, and ricinoleic acid have been experimented with in the production of thermoplastic and biodegradable polyurethane. Biodegradable polyurethane is an ideal candidate material to fabricate tissue engineered hypopharynx for its good mechanical properties and biodegradability [5].

3.4 Proposed plan of research:

- The work was initiated by preparation of ester diol in presence of suitable catalyst. The reaction mixture was left untouched at particular temperature for the fixed duration of reaction completion and collected by vacuum distillation.
- In Next stage, Polyurethane was synthesized by adding that ester diol with Hexamethylenediisocianate (HDI) in proper molar proportions of NCO: OH. This reaction was performed in presence of a suitable catalyst to increase the reaction rate.
- The physical and mechanical property of the material was characterized by using following study.
    - SEM study: The surface morphology of polyurethane was investigated by field emission scanning electron microscopy.
    - FTIR Study: FTIR spectrum of cross-linked polyurethane was taken for structural analysis using FTIR machine with suitable frequency range.
    - Mechanical Property: Polyurethane was subjected to test various mechanical properties such as tensile strength elongation at break and modulus using tensile testing machine.
- In vitro degradation property was analyzed by weight loss percentage with time intervals.
- Hemolysis assay was performed using fresh blood sample and compare with the percentage of hemo-compatible material defined by American standard testing material.
- Antimicrobial activity was performed against clinical pathogenic bacteria, E. coli and S. aureus.
- Cytotoxicity assessment of the material was examined by MTT assay on PBMC (which was separated from whole blood).
- Primary hypopharyngeal fibroblast culture on the membrane, Immunofluroscence staining for analysis of fibroblast growth and cytocompatibility analysis through mitochondrial activity check by MTT assay.
- The material was subcutaneously implanted into the back of Wister rats in order to assay the material's *in vivo* biocompatibility. (*In accordance with the animal ethical committee)

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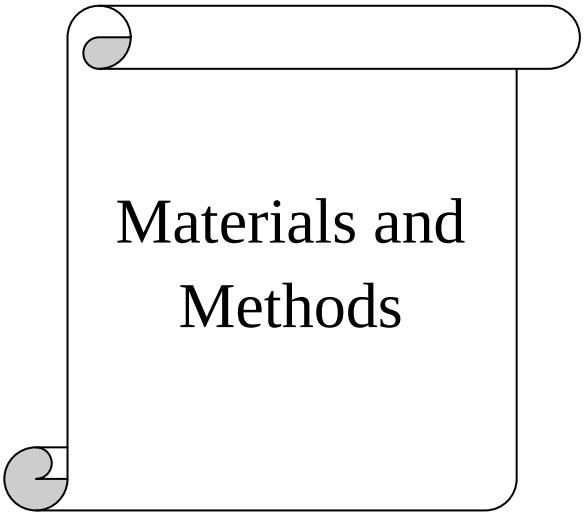
Chapter 4



4.1 Preparation of ester diol:

Materials required- Polyethylene glycol (PEG 400); lactic acid; sulfuric acid; benzene.

Instruments required- Round bottom flask, dean stark apparatus, heater with temperature control facility, vacuum distillation facility.

Ester diol was prepared by reacting polyethylene glycol 400 (PEG 400) with lactic acid (LA) in presence of catalyst, sulfuric acid. 1.126 gm/ml of PEG 400 (78.15ml) was reacted with 1.209 gm/ml of lactic acid (55.8ml) in a round bottom flask. 0.878 gm/ml (55.81ml) of benzene was added in the reaction mixture as a solvent which is maintained at 50 wt% of the reactants. The reaction was refluxed for 14hrs at maintain temperature around 80ºC. This reaction was continued with a vacuum distillation column to collect the final product. Finally light yellowish viscous ester diol (polyethylene lactate ester diol) was collected by vacuum distillation.

4.2 Synthesis of polyurethane:

Materials required- Hexamehylene diisocyanate (HDI), Dibutylene dilaurate (DBTDL), Tetrahydrofuran

Instruments required- Round bottom flask, Magnetic stirrer, vacuum oven, desiccators.

Polyurethane was synthesized by reacting hexamethylene diisocyanate with ester diol in NCO:OH, 2:1 molar properties. 1.4252g/mL hexamethylene diisocyanate was reacted with 2gm/ml ester diol in a round bottom flask. Tetrahydrofuran (THF) was added in the reaction mixture as a solvent, 50 wt% of reactants and dibutyl dilaurate (0.05%) was used as a catalyst in this reaction. The reaction was maintained at room temperature around 29-30ºC for 2 h with control stirring. After 2 h of synthesis, viscous polyurethane was casted onto a glass petri dish. The casted film of polyurethane was moisture cured for 24 h at room temperature in vacuum desiccators. Then it was undergoes a heat treatment of 80ºC in an oven for 5 h.

4.3 FESEM study:

Materials required- Gold powder (for coating purpose)

Instruments required- Scanning electron microscope

The surface morphology of synthesized polyurethane membrane was investigated by field mission scanning electron microscopy (FESEM). The mature was cut into small thin piece and coated with gold powder [1]. After that it was placed in the FESEM sample loading unit. The image was detected at 5000X magnification with an accelerating voltage of 15KV. The final image was taken using the various adjustments at detection recording panel. Detailed surface property and pore size were efficiently detected using the photograph analysis of FESEM.

4.4 FTIR Study:

Materials required- Testing material (synthesized polyurethane), KBr

Instruments required- Infrared spectroscopy and molar-pestle.

Structural and functional group of polyurethane membrane were studied by using FTIR spectrum. Small piece of solid sample was taken on a microspatula and small amount of KBr, mix well in a motar while grinding with the pestle. In case of large crystalline sample, grind the sample, separately and then added KBr. Enough amount of sample was placed to cover the bottom in pellet die. Then it was placed and pressed at 5000-10000 psi [2,3]. After that the sample was carefully removed from die and placed in the FTIR sample holder. Finally launch the FTIR progress in the computer and record the data with plots.

4.5 Mechanical property study:

Materials required- Testing material (synthesized polyurethane membrane)

Instruments required- Universal tensile testing machine

Before the study, the solvent cured polyurethane membrane was dried and kept in vacuum desiccators for at least 24 hrs. Then the membrane was cut into dumbbell shaped form [4]. This dumbbell shaped polyurethane membrane was placed in the UTM sampling holding area and it was subject to test. This test finally had given the several mechanical properties of polyurethane membrane like tensile strength, specific tensile strength, and elongation at break etc.

4.6 Degradation Study:

Materials required- Simulated Body Fluid

Instruments required- Electronic balance and Hot air oven.

SBF preparation- For SBF preparation, Components were NaCl (800mg/l), KCL (400mg/l), KH_2Po_4 (60mg/l), Na_2HPo_4 (48mg/l), $MgSo_4$ (98mg/l), $CaCl_2$ (140mg/l) and $NaHCo_3$ (350mg/l) [5]. At first 1 litre of distilled water was taken in a glass contains with stoppers. Then the above component was weighted properly using weighing balance. Each component was mixed separately with distilled water to solublize them individually. After that each solution was added to a volumetric flask. The pH value was checked and maintained to 7.5-7.7 at room temperature (32-33ºC). Finally the volumes make up with distilled water and maintain the pH value.

Solvent cure membrane was cut into small pieces (2 mm diameter) and incubated for 0, 5, 10, 20, 40, 60 days in SBF solution. After swollen, the piece were transferred into an oven and dried for a period of 24 hrs at 37ºC. Final sample weight was measured using an electronic weighing balance. The percentage of degradation was calculated by using following formula [6]-

$$\% \text{ of degradation} = \frac{W_0 - W_l}{W_0} \times 100$$

Here, W_0 denotes the initial weight of the sample (mg), W_l denotes the final weight (after degradation) of the sample (mg). The test was performed in triplicate for all the time intervals.

4.7 Hemo-compatibility study:

Materials required- normal saline, sodium carbonate

Instruments required- Incubator, UV- spectrophotometer, centrifuge machine.

To determine the hemo-compatibility, the blood sample was collected in citrated breaker; containing sodium citrate was diluted in normal saline solution (8ml blood in 10ml of n-saline) [7]. The solvent cure sample were cut in normal shape (1 mm diameter) and taken in a standard

test tube (16 mm×150 mm) containing 10ml of n-saline and kept it in incubator at 37ºC for 30 min to provide temperature equilibrium. Then 0.2ml of diluted blood was added to the test tube and mix gently to place for 60 min incubation. For positive control 0.2ml of diluted blood was taken in 10ml of 10% Na_2Co_3 solution and similarly for negative control 0.2ml of diluted blood was taken in 10ml of n-saline solution followed by 60 min incubation at 37ºC. After 60 minutes of incubation, all test tubes were centrifuged for 3 min at 1000 rpm and the supernatant was carefully removed to transfer in square section glass cell of the UV spectrophotometer. The absorbance was taken in 545 nm. The UV spectrophotometer should be put on and the cell (filled with fluid) be placed in the square slot with ground faces touching the slot and the transparent side should be kept free for light transmission. The percentage hemolysis was calculated using the following formula [8]-

$$\% \text{ of hemolysis} = \frac{\text{Abs (Test)} - \text{Abs(Negative)}}{\text{Abs (Positive)} - \text{Abs(Negative)}} \times 100$$

Here, Abs (Test) denotes the sample, Abs (Positive) denotes the positive control, and Abs (Negative) denotes the negative control. The experiment was performed in triplicate to get the significance result.

4.8 Antimicrobial assay:

Materials required- Nutrient broth, agar-agar.

Test microorganisms- Standard strain of E. coli and S. aureus

Instruments required- Incubator

Small pieces of membrane were studies for antimicrobial activity against pathogenic bacteria. Small pieces of membrane were sized as disc. Bacterial culture was prepared using nutrient broth containing agar and plated on glass petridish [9]. The membrane was placed on the culture plate containing strain of bacteria and subject to incubation at 37ºC for overnight. Next day, the result (zone of inhibition) was measured to check the potency of material.

4.9 Cytotoxicity study on PBMC using MTT assay:

Cells required- PBMC cell

Materials required- MTT solution, DMSO, RPMI1640, DMEM, FBS, Penicillin, streptomycin, PBS

Instruments required- CO_2 incubator, centrifuge machine, well plate reader

Separation of PBMC- Fresh blood was collected in 3ml EDTA vial. It was diluted in 1:3 ratio using chilled normal saline solution. In another falcon tube 2.5ml of histopaque (HiSep LSM 1077), a density gradient medium was taken and 7.5ml of diluted blood was poured carefully to form a layer over it. The tube was centrifuge at 2000 rpm for 15 minutes. The density gradient centrifugation causes to form four layers. The top most layer was plasma thrombolysis followed by a white colour ring layer containing PBMC [10]. This layer was aspirated out carefully without any RBCs and thrombocytes, taken in a fresh falcon tube. It was washed three times using RPMI1640 media and suspended in the same complete media containing 88% RPMI1640, 10% FBS and 2% antibiotic solution (penicillin-streptomycin). Then it was kept in 5% CO_2 incubator at 37ºC for 3-5 days for suitable growth.

Cytotoxicity test- PBMC was cultured in RPMI1640 media. The cells were added on 96 well Plates contacting the biomaterials in the well. Each well must be containing equal no of cell, including blank with water. Then the well plate was incubated at 37ºC for 24 hrs in 5% CO_2 incubator. Next day, 10µl of MTT (5mg/ml) was prepared in PBS solution and added into each well followed by 4 hours of incubation. After that, media along with MTT was pipette out carefully and 100µl of DMSO was added in each well to dissolve the formed formagen [11]. Finally the plate was placed in the microplate reader to take the absorbance at 490 nm.

4.10 Primary hypopharyngeal fibroblast culture:

Cells required- primary hypopharyngeal fibroblast cell

Materials required- PBS, DMEM, FBS, Penicillin, streptomycin, glutaraldehyde, and ethanol.

Instruments required- CO_2 incubator

Fibroblasts were obtained from goat hypopharynx connective tissue. The tissue was placed on a petridish and was thoroughly washed with sterile PBS containing antibiotic (penicillin and streptomycin, 100µg/ml) followed by sterilized in 75% ethanol for few seconds. After proper washing, the tissues was cut into small cubes (1×1×1mm dimensions) and attached to the culture flask containing culture media. The culture media was prepared by using dulbecco's modified eagles medium (DMEM-88%), fetal bovine serum (FBS-10%) and antibiotics (Penicillin and streptomycin-2%). The culture flask was incubated at 37°C for 24 hours in a CO_2 incubator (5%). After few days, fibroblasts extended from the tissue cubes and attached to the culture plate which was collected and sub cultured for passage to achieve 80% confluency [12]. After 4^{th} passage, the cells were seeded on polyurethane membrane surface at the density of 5×10^5 cells/ml. Before the cell seeding, the polyurethane membrane was sterilized in 75% ethanol for 2 hours followed by 2 hours rinsed in PBS before cell seeding. The culture was maintained in predefined media composition and incubated at 37°C in 5% CO_2 incubator. The culture media was changed in every two days.

For the morphological observation, cells were fixed in 2.5% glutaraldehyde for 60 minutes, the rinsed in PBS and dehydrated through series of graded ethanol (generally from 50 to 100% with 10% increasing in each steps).

4.11 Immuno-fluorescence staining:

Cells required- primary hypopharyngeal fibroblast cell

Materials required-. Paraformaldehyde, PBS, Triton X-100, mouse antivimentintin antibody, FITC conjugated goat anti mouse IgG, DAPI.

Instruments required- CO_2 incubator, confocal laser scanning microscope.

Fibroblast cells seeded on polyurethane membrane were fixed in 4% paraformaldehyde solution for 10 minutes at room temperature followed by washed in PBS 3 times with 5 minutes for each. Then it was soaked in 0.2% Triton X-100 at 37°C for 15 minutes followed by 3 times wash for 5

minutes each. After that the material were blocked in 10% goat serum at 37°C for 20 minutes and placed for overnight incubation in mouse antivimentintin antibody at 4°C. After overnight incubation the material (diluted in PBS, 1:200) was washed in PBS for 3 times with 5 minutes for each and was incubated in fluorescein isothiocyanate (FITC) conjugated goat anti mouse IgG (diluted in PBS, 1:50) at 37°C for 2 hours in completely dark room. Then it was washed with PBS and was deepen in 4, 6-diamidino 2- pherylindole dihydrochloride (DAPI) solution (prepared in PBS, 3µg/ml) for 5 minutes to stain the nuclei (blue fluorescence) [13]. Immuno-florescence was observed under confocal laser scanning microscope (CLSM, Olympus fluo view-800).

4.12 Mitochondrial activity of hypopharyngeal fibroblast using MTT assay:

Cells required- hypopharyngeal fibroblast cell

Materials required- MTT solution, DMSO, DMEM, FBS, Penicillin, streptomycin, PBS

Instruments required- CO_2 incubator, centrifuge machine, well plate reader

Mitochondrial activity was evaluated using MTT method at 5^{th} and 10^{th} day. 100µl of methylthiazolyldiphenyl tetrazodium (MTT solution 0.5mg/ml) was added into each well of 96 well plate and culture placed for incubation at 37°C for 4 hours in 5% CO_2 incubator. After that DMSO was added to the well to dissolve the purple formazan and OD was taken using well plate reader (Instrument details) at 490nm. The absorbance was measured for blank where same procedure was performed without biomaterial. Similarly, the cells also cultured on the Mesh (polystyrene mesh) were referred as a positive control. The experiment was performed in triplicate and was averaged the data for analysis.

4.13 In-Vivo biocompatibility assay:

Experimental animal- Healthy wistar female rat weight between (150-180grams) was used here. Before stating the experiment animal was acclimatized to laboratory conditions for 7days. The animals were resided in polypropylene cage with laboratory conditions, such as temperature 20-

24ºC and light dark cycle of 12 hours. Standard diet was provided to the Rats with water ad libitum entire the experiment.

Tissue grafting experiment protocol:

Materials required- Ethanol, diethyl ether, formal dehyde, Polypropylene tissue reconstruction mesh (LME 66-1).

Instruments required- Surgical blade (no 10), surgical niddle, suture no 1/10, forcep, 3cissors.

The dorsal far of rat was removed and the area was cleaned with 70% ethanol. The animals were divided into four groups. Each group was contains 3 rats. Group I was controlled with normal physiological conditions. Group II was challenged with no treatment. Group III and Group IV were treated with polypropylene mesh and synthesized polyurethane biomaterial respectively. Prior to the incision wound model for tissue grafting the rats were anesthetized with Ketamine (80mg/kg body weight) [14]. An incision of 1cm long tissue wound was made using surgical blade (no 10). After the incision, the polyurethane biomaterial and polypropylene mesh were grafted in the tissue wound area a surgical sutures (no1/10) was applied to close the skin. After the predetermined time (14 days) the material was explanted with small amount of surrounding tissue. The rats used in this experiment were treated in accordance with the ethical committee of Bengal School of Technology, chuchura, West Bengal and the NIH's principles of laboratory animal care.

4.14 Pathological examination:

Instruments required: Microtome sectioning machine (Leica RM), Microscope (Olympus cx21i).

Tissue specimens with the biomaterial were fixed in 10% formalin solution for 1 hour. Then the samples were freeze embedded and performed microtome slicing into 4µm section. It was stained with hematoxylin - eosin stain and finally analyzed under light microscope [14]. The images were taken by Olympus camera combined with microscope.

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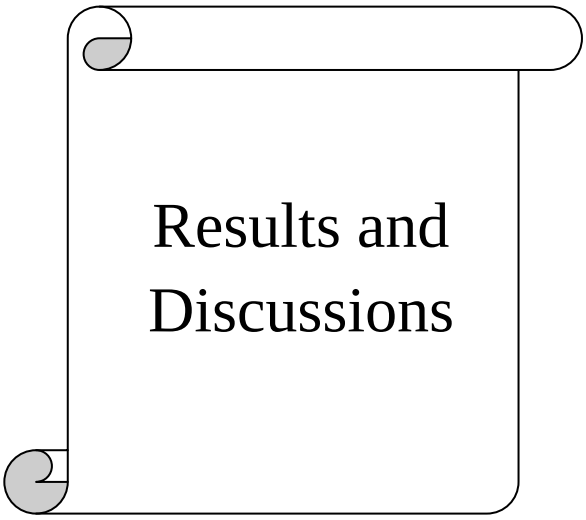
Chapter 5



5.1 Polyester urethane synthesis:

In this study, Polyurethane was synthesized by incorporating the ester linkage in the backbone. It manifest that the rate of biodegradation would be controlled by the frequency of ester linkage on the polyurethane backbone [1]. To design polyester urethane, firstly polyurethane glycol 400 (PEG 400) and lactic acid (LA) was reacted in presence of sulfuric acid as a catalyst. Ester diol was synthesized by continuing the reaction for 14hours and collected by vacuum distillation. This ester diol was proceed for the polyester urethane synthesis by reacting with hexamethylene diisocyanate (HDI) with NCO:OH ratio 2:1

In this first step, ester diol preparation, carboxyl group (COOH) of lactic acid was reacted with hydroxyl group (OH) of polyethylene glycol 400 in presence of pinch of sulfuric acid (H_2So_4). Self condensation of lactic acid was significantly reduced by providing the higher amount of polyurethane glycol 400 [2]. Esterification reaction was continued with benzene as a solvent (50wt% of the reactant) for 14hrs followed by removal of water as azeotropic mixture with benzene. Vacuum distillation was facilitated to obtain the pure ester diol from the reaction mixture of not reacted lactic acid and polyethylene glycol 400, remaining in flask. Light yellowish colour ester diol was collected from the reaction flask, depicts in figure 5.1.

Figure 5.1: Preparation of ester diol

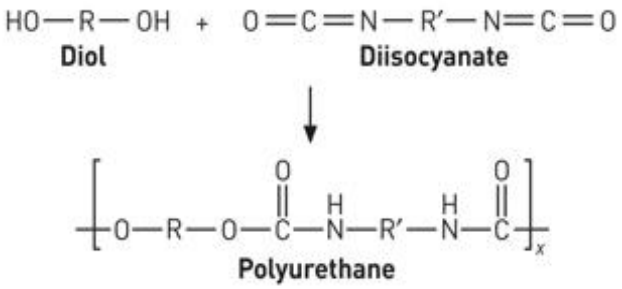


Figure 5.2: Polyesterurethane preparation reaction

In the second step, Hexamethylene diisocyanate (HDI) was reacted with first step prepared ester diol at room temperature in presence of dibutylene dilaurate (DBTDL) as a catalyst. The reaction was continued in Tetrahydrofuran (THF), solvent medium for 2 hours to get the viscous polyurethane (shown in figure 5.3). Viscous polymer was efficiently casted on glass petri plate under vacuum condition to avoid the bubble formation during casting (shown in figure 5.4) and solvent evaporation after casting.

Figure 5.3: Preparation process of polyesterurethane membrane

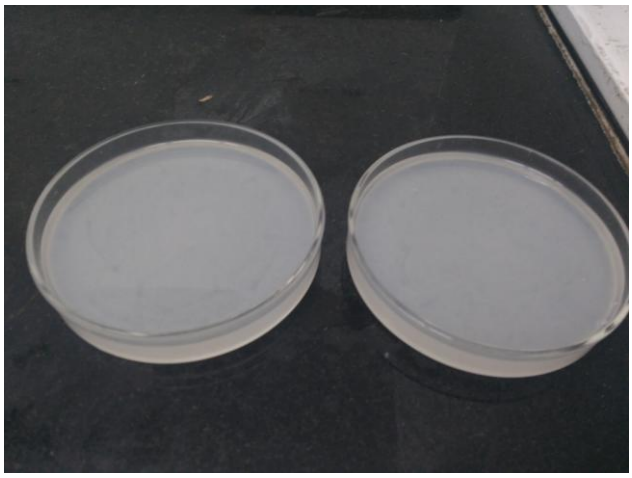


Figure 5.4: Casted polyurethane membrane on glass petri dish

5.2 Proposed steps for curing of polyurethane:

Solvent curing of polyurethane film was facilitated by gradual mild evaporation of residual solvent in the membrane at atmospheric moisture for 24 hours. In next step, synthesized polyurethane membrane could be peeled off carefully and was subjected to boil in normal distilled water for the removal of residual solvent, if presented in very low quantity. Finally, the film was dried and kept in vacuum desiccators for 24 hours.

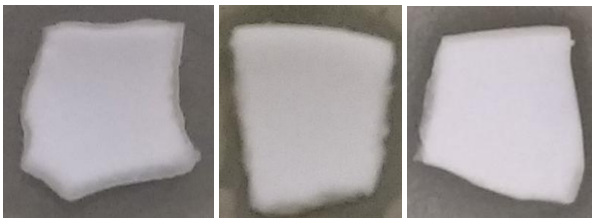


Figure 5.5: Pieces of synthesized polyurethane membrane after solvent curing.

5.3 FESEM analysis of polyurethane membrane:

Surface topology can effectively influence the cellular activity on the membrane [3]. Field emission scanning electron micrograph of polyurethane membrane is shown in the Figure 5.6, which depicts the dense structure. It is clear that highly condensed cross linking decreases the pore size. From the FESEM results, the expected pore size may be in approx of 1 μm. This

structure may facilitate the cellular migration, proliferation and nutrient supply as the porosity controls the transfer of water and cellular metabolites [4]

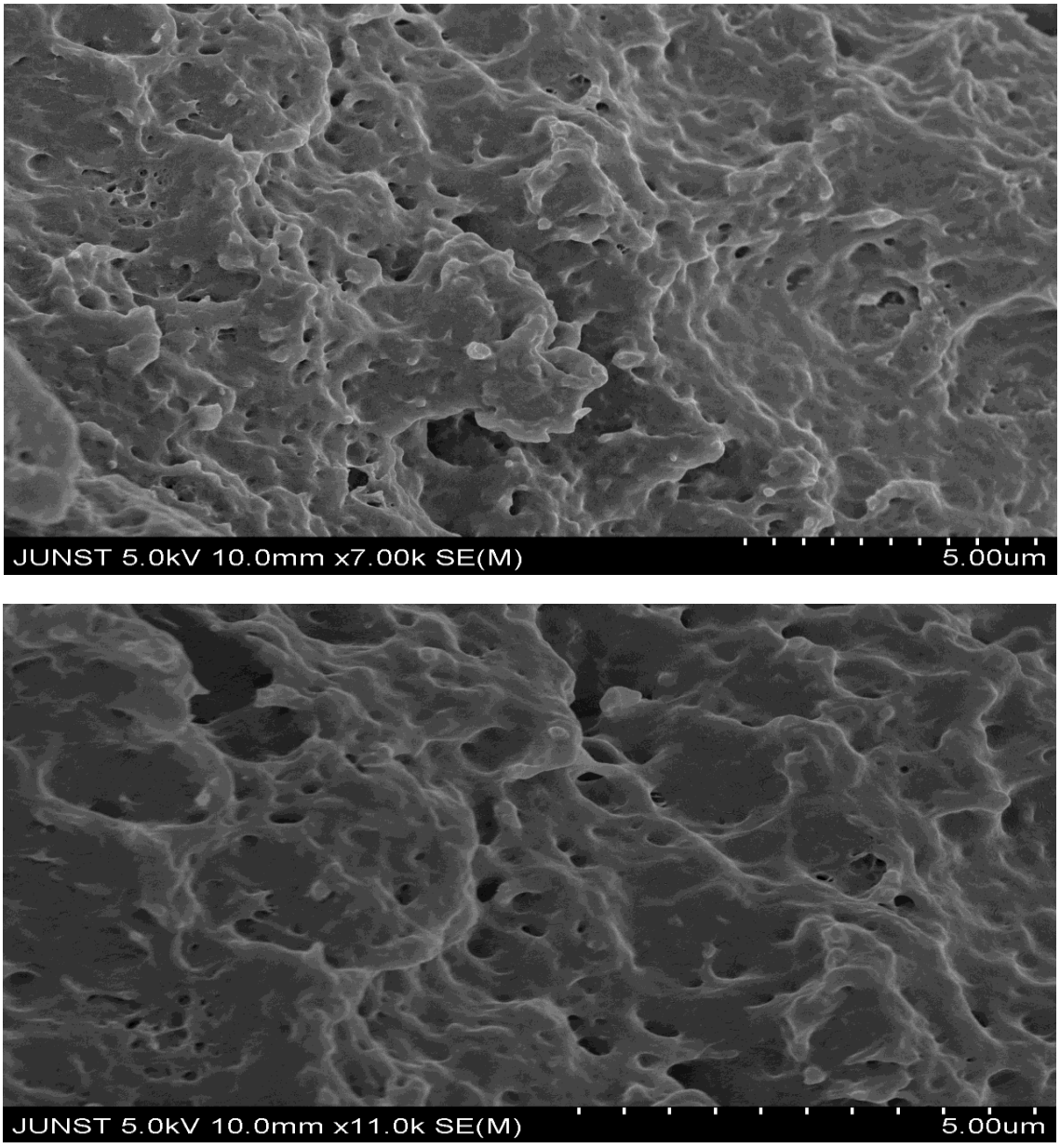


Figure 5.6: (a), (b) FE-SEM micrograph of different position on ester diol based polyurethane membrane.

5.4 FTIR analysis of polyurethane membrane:

The Fourier Transform infrared spectroscopy of ester diol based polyurethane is shown in Figure 5.7. The important peaks are assigned with details in the Table 5.1. Some typical functional groups of polyester polyurethane were observed in between the frequency range 4500-500cm^{-1}. Symmetrical stretching of NCO and COC group was observed at 940 cm^{-1} [5]. The band at 1752 cm^{-1}, 1247 cm^{-1} and 1126 cm^{-1} may confirm the ester linkage [6]. A small shoulder was seen near 3507 cm^{-1} which is related to N-H stretching [7]. The peak at 2864 cm^{-1} and 2948 cm^{-1} are assigned to C-H stretching and asymmetric CH_2 group respectively [8].

Table 5.1: FTIR peak assignment with wave number for polyurethane membrane.

Wave number (cm^{-1})	Peak assignment
940	Symmetric stretching of N-CO and C-O-C bonds in polyurethane
1126	Ester linkage
1247	Ester linkage
1700	C=O
1752	Ester linkage
2864	C-H stretching
2948	Asymmetric CH_2 stretching
3507	N-H stretching

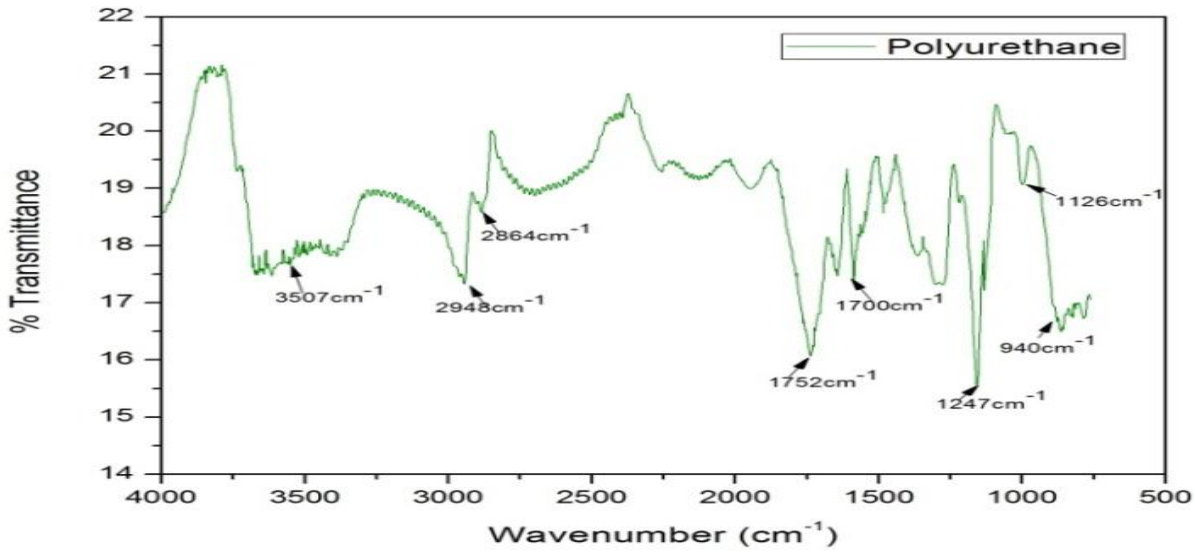


Figure 5.7: FTIR spectra of ester diol based polyurethane with typical bands

5.5 Mechanical property analysis:

Mechanical characterization clearly explains the adequate strength and stability of polyester diol based polyurethane. Mechanical behavior of material generally depends on the molecular weight of synthesized biomaterials [9]. Thus the modification of molecular weight can greatly alter its mechanical properties. The stress-strain curve which depicts the essential mechanical behavior is shown in Figure 5.8 and detailed in the Table 5.2. The tensile strength and strain were marked at 5.15±0.58 Mpa and 124±16.4% respectively. This finding can explain that the material was stronger than natural polymer collagen (0.31Mpa) [10]. Moreover the analysis showed that, this synthesized polyurethane was somewhat soft and less brittle.

Table 5.2: Mechanical property details of ester diol based polyurethane membrane

Sample details	Density (gm/cc)	Tensile strength (MPa)	Strain %	Elastic modulus
Polyesterurethane	1.09	5.15 ± 0.58	124 ± 16.4	2.9 ± 0.2

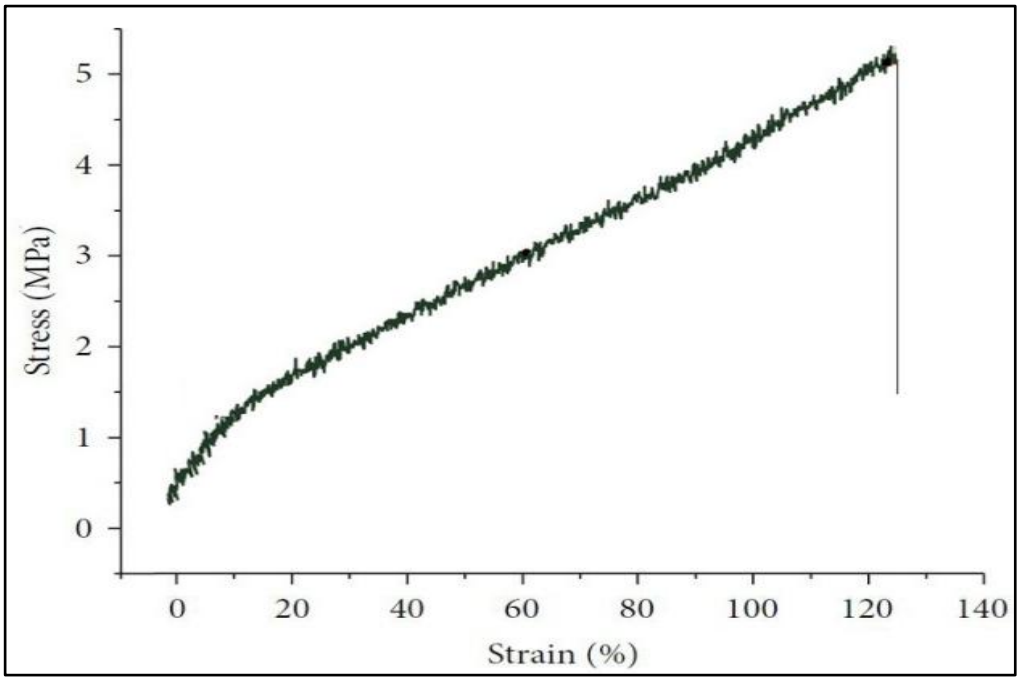


Figure 5.8: Tensile stress-strain curve of ester diol based polyurethane membrane

5.6 Degradation property analysis:

Degradation property analysis is a significant benchmark for synthesized polyurethane to estimate the weight loss [11]. The property analysis was performed by plotting the weight loss percentage vs time. Weight loss percentage was calculated by the change of weight to initial weight of the material. The test was performed in vitro over a period of 60 days. Result of degradation is shown in Figure 5.9. The results depicts that initially the polyurethane membrane was slowly reduced weight up to 10 days (5% approx), then the rate of degradation was increased slightly up to 40 days (17% approx). Finally at 60th day the degradation rate was marked at 22%, which denotes that the material shows significant biodegradability. As the synthesized polyurethane is not a hydrophilic material so it is expected the weight loss is due to degradation and not due to erosion of polymer matrices or dissolving of matrix in the solvent PBS.

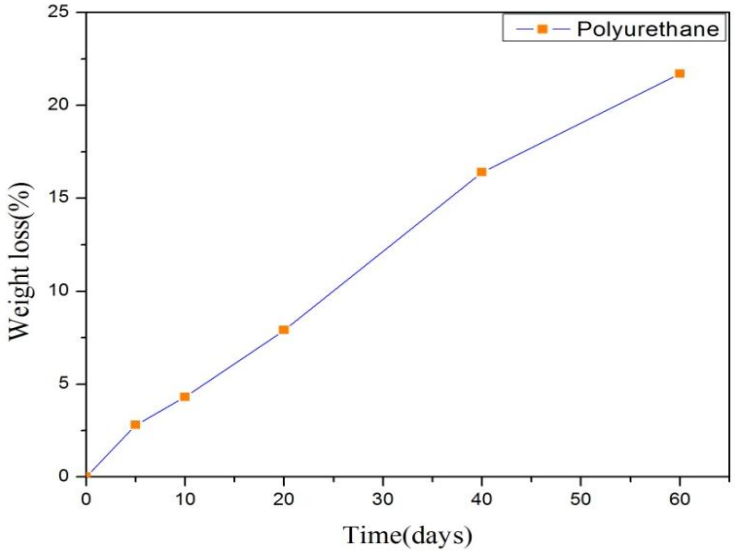


Figure 5.9: Degradation plot of polyurethane membrane

5.7 Hemocompatibility and antimicrobial activity:

Hemolysis assay was performed using polyester diol based polyurethane membrane to check the blood compatibility of synthesized material. Percentage of hemolysis was calculated between difference of test absorbance and negative control absorbance to difference between positive and negative control absorbance [12]. The result analysis of this material denotes 0.83% hemolysis (shown in figure 5.10) which signify the material as highly hemocompatible because as per

ASTM (American Society for Testing and Materials), a highly hemocompatible material should show less than 5% hemolysis [13].

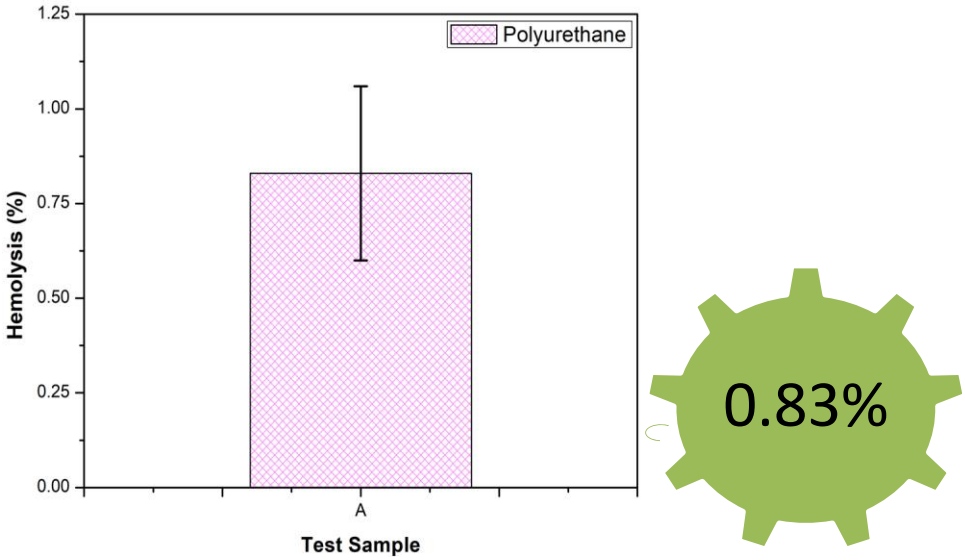


Figure 5.10: Showing the percentage of hemolyisis of the test polyurethane sample

Antimicrobial activity result explains the materials potentiality to inhibit the bacterial growth on the biomaterial [14]. Result of antimicrobial assay is shown in Figure 5.11 for E. coli and S. aureus. The result analysis showed that a clear zone surrounding the membrane and no growth on the membrane surface. This finding may conclude that this synthesized material doesn't promotes pathogenic bacterial growth and also inhibit them.

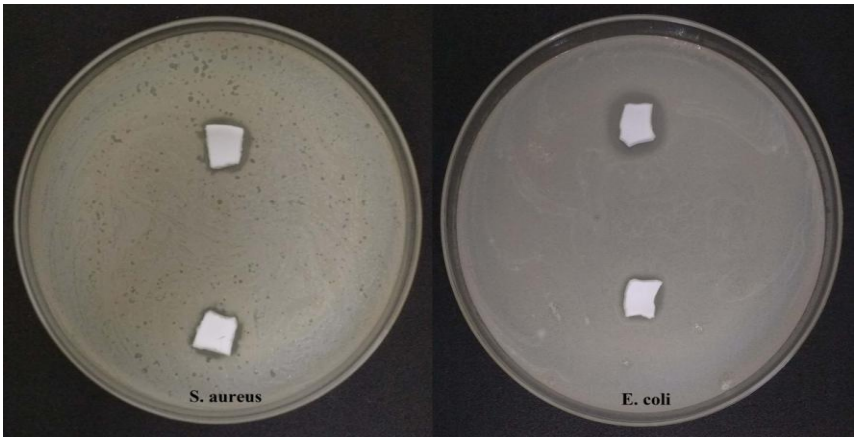


Figure 5.11: Antimicrobial assay through zone of inhibition of polyurethane membrane.

5.8 Analysis of cytotoxicity test on PBMC:

Cytotoxicity assay was performed to check whether synthesized polyurethane membrane shows any kind of toxicity towards cells. Cytotoxicity assay of polyester diol based polyurethane membrane was done against isolated PBMC (Peripheral Blood Mononuclear Cell). The result of cytotoxic assay for control and treated membrane is shown in Figure 5.12. One way ANOVA was performed to check the statistical significance level which reveals the computed P value 0.17897 at 0.05 level significance and the population mean are not significantly different. It implies that when small piece of polyurethane membrane was placed in PBMC cell culture, the cells went to significant proliferation.

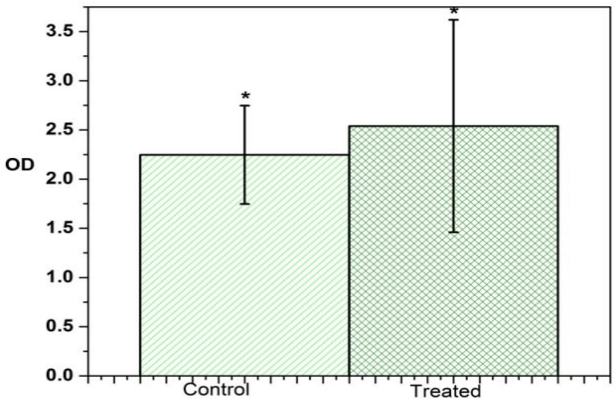


Figure 5.12: Cytotoxicity analysis result with statistical significance

5.9 In vitro cytocompatibility:

Cytocompatibility of synthesized polyurethane membrane was measured via goat hypopharyngeal fibroblast seeding where commercial mesh was used as a control. Cells were stained using immune-fluorescent staining in order to visualize them on the membrane. MTT assay showed that cells grew and proliferate on the membrane. Vimentin is the reliable fibroblast marker that frequently found intermediate filament in fibroblast [15]. Primary antibody antivimentin was used in which cells on the membrane were immune stained **(**here green fluorescence**)** to confirm the fibroblast after 5^{th} and 10^{th} day using in-vitro culture respectively. The fluorescence stained cells were shown in figure 5.13 (a) and (b) for 5^{th} and 10 days respectively. These finding indicated that fibroblast cells could grow onto the polyurethane

membrane using the in-vitro cell culture methodologies. MTT assay on polyurethane membrane was measured against fibroblast cell for 5^{th} day and 10^{th} day where increased OD value from 5^{th} day to 10^{th} day, indicate the cell proliferation capacity on the membrane. The result of MTT assay is shown in figure 5.14. The cells were seeded at a density of 5×10^5cells/ml and culture for 5 and 10 days. One way ANOVA was performed using the value of OD to check the statistical significance, where the P value was 0.1452 and 0.2109 at 0.05 significance level and no significant difference with population mean. This result indicates that fibroblast cell can effectively grow on polyurethane membrane compare to commercial mesh so the material is cytocompatible.

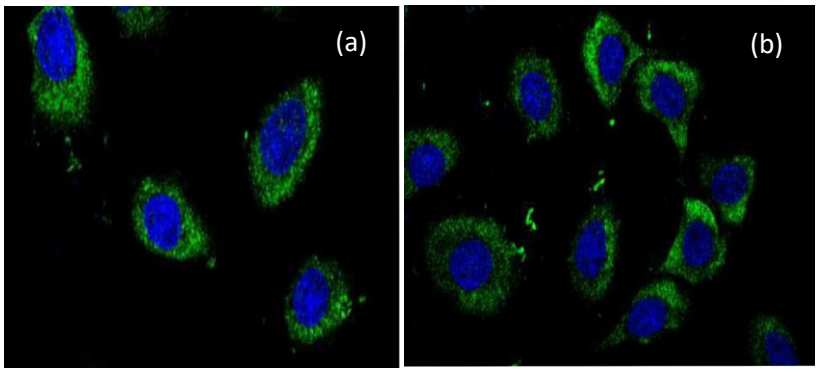


Figure 5.13: Represents the primary hypopharyngeal fibroblast cell growth (a) after 5 days and (b) after 10 days.

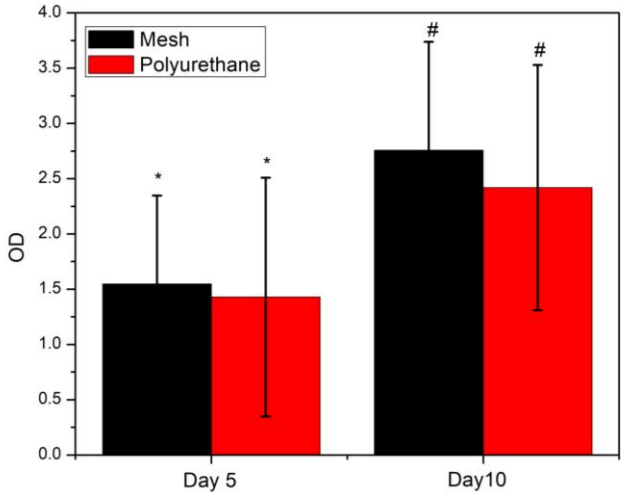
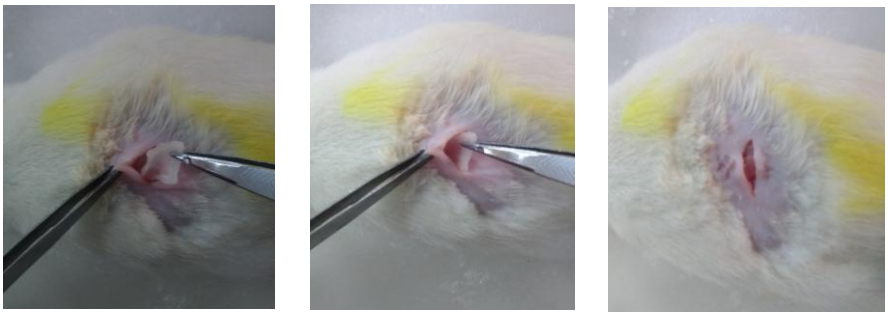


Figure 5.14: Statistical significance of cytotoxicity check on hypopharyngeal fibroblast cell using MTT assay for synthesized polyurethane membrane and commercialized mesh.

5.10 In vivo efficiency analysis:

On 14th day animal was observed and found that treated one with biomaterial (polyurethane) showed excellent biocompatibility and degradation than the other groups. The tissue healing was also improved where biomaterial was used. The result showed that synthesized polyurethane has similar kind of biocompatibility and degradation as commercially available mesh.



(a) (b) (c)

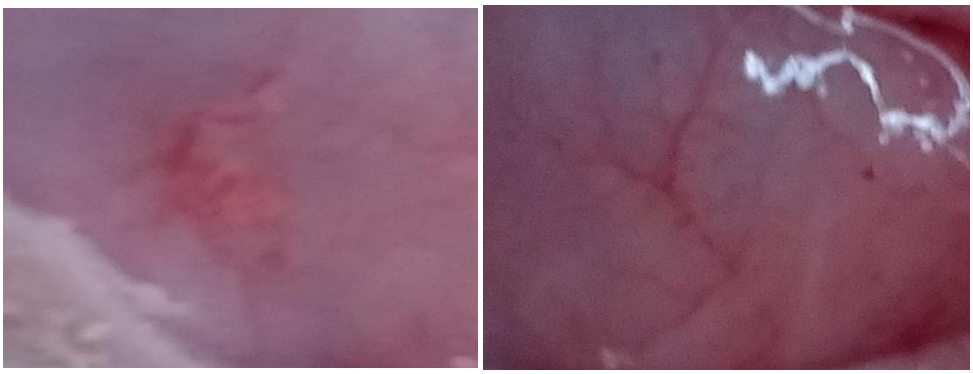
Figure 5.15: (a), (b), (c) Biomaterial was efficiently implanted to the back of wistar rat.

(a) (b)

Figure 5.16: (a) Post surgery anesthetic recovery. (b) Visual observation after 24 hours shows the normal activity

In figure 5.16 (a) showed that the anesthetic recovery after surgery. This result indicated the anesthetic dose efficiency as per regulation of animal ethical committee. After 24 hours of the surgery, it was found that the rats are behaves normally and shows the general physiological

activity (shown in figure 5.16 (b)). After 7th day it was examined that the material has been degrading and no such tissue level infection in the implanted area, depicts in figure 5.17(a). In figure 5.17 (b) shows the explanted tissue after 14th day and examined that the material was almost completely degraded and proper angiogenesis.



(a) (b)

Figure 5.17: After 7th day the degradation of polyurethane and no infections, (b) Almost complete degradation and proper angiogenesis

Complete degradation revealed the biocompatibility and significant degradation of the synthesized polyurethane membrane. The result of tissue repair was quite impressive and excellent comparable with standard mesh used in surgery. The material was promoting the angiogenesis as per the final observation.

5.11 Histopathological examination:

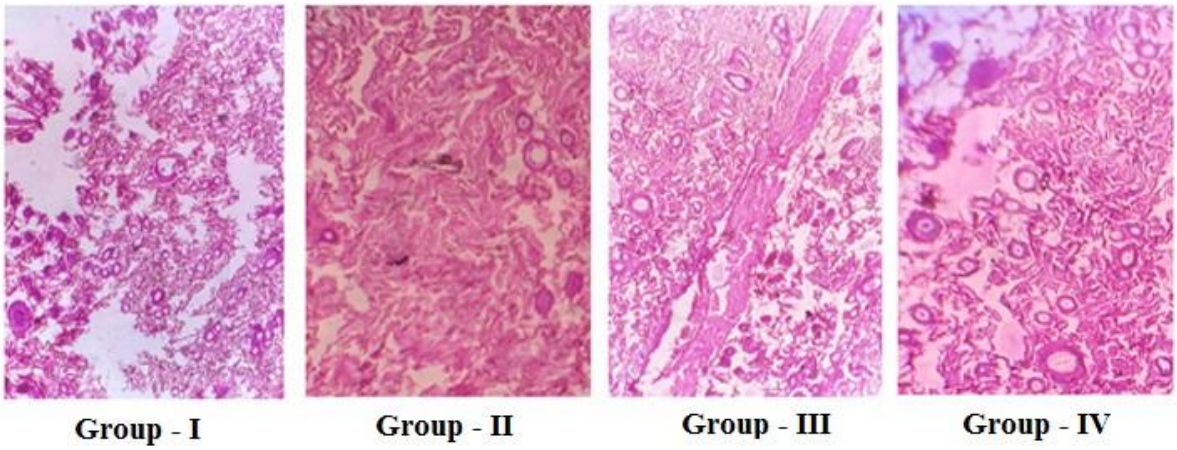


Figure 5.18: Histopathological examination of excised tissue with H and E staining

Histopathological test of explanted tissue was stained with H & E. The examination result was shown in figure 5.18. In group-I the no of cells was highest as no treatment took place in these group. Similarly challenged group (Group-II) was shown the least no of cells among others. Group-III and Group IV were shown almost equal no of fibroblast cells and collagen content in both groups (polyurethane treated and mesh treated) had shown almost similar in tissue healing process. This examination reveals that synthesize polyurethane membrane has the similar kind of cell proliferation capacity in comparison with commercially available mesh.

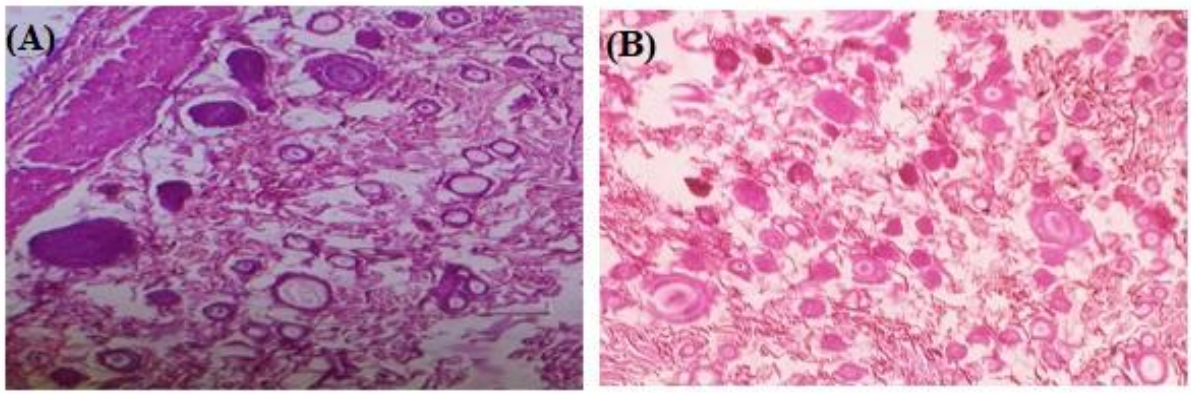


Figure 5.19: Shows the collagen (A) and fibroblast (B) in polyurethane treated group

In figure 5.19 (A) collagen was observed in biomaterial (polyurethane) treated group. Similarly, fibroblast cells in the dermal layer were more in numbers in case of treated animals than the challenged group. Figure 5.19 (B) shows huge no of fibroblast cells were shown in polyurethane treated group. Sufficient no of cell proliferation in polyurethane treated group was clearly denoted the material effectiveness towards in vivo treatment.

5.12 References:

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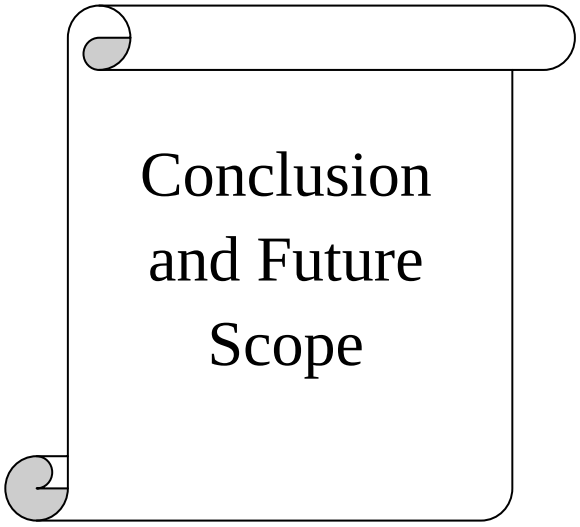
Chapter 6



6. 1 Conclusion:

This study revealed that ester diol based polyurethane synthesis by reaction of polyethylene glycol, lactic acid and hexamethylene diisocyanate. SEM analysis helps to analyze the surface morphology which showed the adequate pores that reveals the membrane as a porous material. FTIR spectrum analysis expressed the reaction chemistry like symmetric and asymmetric bond stretching including ester linkage. Mechanical property effectively analyzed through the tensile testing which stress-strain curve explained the material as a soft and less brittle. Synthesized membrane degraded in SBF solution due to ester linkage in the backbone of polyurethane membrane that signifies the material as a biodegradable. Less hemolysis percentage indicated the material as a high hemocompatible. PBMC proliferates over the surface of polyurethane membrane that exhibits no cytotoxicity. Primary hypopharyngeal fibroblast culture undergoes FITC conjugated immunofluorescence staining to express the sufficient growth on the polyurethane membrane followed by mitochondrial activity to access the cytocompatibility of the material. Antimicrobial assay ensured that the material didn't promote the bacterial growth and also inhibit the surrounding bacterial growth. Subcutaneous implantation of the polyurethane membrane in rats suggests that the material has good biocompatibility and effective biodegradation property. This fact was justified by histopathological examination that showed sufficient collagen and fibroblast cell proliferation in explanted tissue.

From the overall study, it may assume that the product chemistry, biodegradation and biocompatible property confirm the synthesized polyurethane membrane as a potent candidate for hypopharyngeal tissue engineering.

6.2 Future Scope:

- Synthesis of scaffold through electro-spinning method and incorporate the synthesized collagen from organic waste concerning cost effectiveness.
- In vitro primary human hypopharyngeal fibroblast culture on the scaffold.
- In vivo efficacy test by hypopharyngeal tissue replacement through animal model.
- In silico comparative study of gene and protein expression (genomics and proteomics) among natural and repair tissue using biomaterial.
- Investigate the material characteristics and property for post surgery relapse of carcinoma.
- Draw the experimental plan for FDA approval towards human trial.
- FDA approval and patent licensing for human use.
- Commercialize the material as an entrepreneur aspect.