1.0 ABSTRACT

Tissue engineering is area which is now witnessing a major role in the field of regeneration of body parts lost due to accidents. There are a number of ways to fabricate the scaffold and there are various scaffolds which are available in the market but yet a scaffold which is very reliable in the tissue engineering is lacking. The parameters which are required by the scaffold replacing the tissue make it very challenging task to fabricate scaffold which can mimic the function of tissue. Here we are concerned about the porous nature of the scaffold, surface structure, what affect it has on the cells of the body and many more things. Porosity plays very important role in the process of regeneration. In the course of regeneration the nutrients and oxygen which is needed should pass through the tissue to reach the nearby cells. The surface of the scaffold is also very important, if the surface is even cells will not be able to anchor on the surface and it will hinder the regeneration process. In case of bone and cartilage one thing is very important that is mechanical strength, due to load bearing function of bone tissue which is an integral part of function of bone tissue. Mechanical strength is required in the case of cartilage but right blend of right blend of strength and with elasticity, biocompatibility and biodegradability is a major task ahead. We are here trying to fabricate three types of scaffold with the help of alginate chitosan and gelatin which has many advantages. We want to make it more suitable with the manipulation of some parameters of formation of scaffold.

We are her trying to characterize the finally prepared scaffold to study all the properties which it possesses. We will see its surface structure, biocompatibility and how it behave at different pH, as a scaffold will come in contact with body fluids of different pH. Instrument we are going to use are, Scanning Electron Microscope (SEM) which is now a day a very important instrument to have an insight in any materials structure, we are also using here X-ray diffraction for determination of structure of the scaffold.

2.0 INTRODUCTION

Tissue engineering is a field in which we can use knowledge of biology and technology for the exploration of working of the human tissue, it is actually have little to do with the exploration it mainly provide us the alternative materials to take place of the damaged tissue and let the tissues to regenerate [1]. Tissue engineering is used for regeneration of many tissues but we had talked here mainly about cartilage and bone tissue engineering. This field has seen much advancement one of the advancing area is to use scaffold material with pores in it, to promote the formation of bone tissue. These materials which we have developed act as a platform, so that cells can grow on it [2]. These materials have some positive points on the tissues which are grafted. Tissue which are grafted are categorised in three groups i.e. auto graft, allograft and xenograft. In auto graft the tissues are taken from the other organism but from the same body but from different part of the body [3]. When the tissues are taken from the same species such as one human give tissue for another human that is known as allograft and when the tissue cadaver is taken from different species such as porcine etc, then that is known as xenograft, but these grafting techniques have many problems such infection and scarcity of donor [4]. Tissue engineering is now a developing field which is answer for all the above listed problems. It is emerging as substitute to induce the healing in the diseased tissue, but to make a scaffold material with desirable pore structure, porosity and other necessary qualities is a tough task. Porosity, structure and size of the pores and their connectivity, load bearing properties, biodegradability they all are very important factors in the scaffold designing. Environment is a big factor for the tissue regeneration; we are trying to copy the properties of natural tissues so that we can provide them the best possible natural conditions [5]. The advancements in the field of tissue engineering have opened the gates of a better and safe outlook towards the treatment of damaged tissues. Tissues like bones and cartilage are vital tissues of our body which help in keeping the body organs safe and performing body movements [6]. Bone and cartilage are some of the tissues which once damaged are not easily repaired. Here comes the role of tissue engineering which provides an ingenuous solution for repairing the damaged tissues of our body [7]. There has been quite a significant advancement in the field of articular cartilage tissue engineering. Tissue engineering involves proper scaffold designing and maintaining all the required conditions which are important for proper growth of cells within the scaffold [8].

There are many factors and issues about which we are concerned, which should be taken account of and which need to be properly addressed while designing the apt scaffold for growth of cells. Some of such factors are:-

Porosity:- Porosity is a very important aspect in scaffold designing as porosity of the scaffolds have an important role to play in directing tissue formation and function [9]. A sound amount of scaffold porosity is often needed to allow for even distribution of cells and connection between the pores throughout engineered tissues [10][11]. In addition, increased porosity can have a helpful effect on the transportation of nutrients and oxygen, especially in the absence of a functional vascular system [12] [13].

Mechanical strength: - Mechanical properties should be taken care when we searching for the materials for load-bearing structures. For bone and cartilage tissue engineering mechanical properties should also be looked at, especially in the case of bone tissue where load bearing is a necessary function we should have in vivo data of mechanical threshold so that we have an idea at which point scaffold can collapse. It will help us to design scaffold of same mechanical properties. Studies show that mechanical stress and other physical factors can help in the cell seeding and better proliferation of cells in scaffold matrices [14].

Bio-degradability: - Biodegradable polymers have their need in those tissue-engineering products in which tissue repair or remodeling is the goal, but not where long-term materials stability is required [15]. It becomes a key concern when tissue engineering is aimed for bone tissue engineering. In bone tissue engineering more important is duration in which the scaffold should degrade, we have to customize it in such way that it should degrade in the same duration in which new bone tissue in regenerating and going to replace the implanted artificial tissue. One more area of concern is the end product of the degradation, the end product in any way should not be toxic for the body [16].

Biocompatibility: - Biocompatibility is a wide concept, which escapes easy definition. In general, the scaffold material should not generate inflammatory response, it would not let the bacteria to grow and colonize, and would help in proliferation of cells in the nearby tissues [17]. Biocompatibility is dependent on the site of implantation and where we are going to apply this concept, it is context dependent [19].

With the advancement in this field, a huge number of materials have come forth which provide us with better alternatives for choosing the best possible material for scaffold development. In

this article we have mainly focused on development of better scaffold and discussing its various characteristics which help in ensuring its use for the important process [20].

Swelling properties: - swelling behaviour of the scaffold is also tested and that too at different pH because after implantation scaffold will always in constant contact with the fluids. So it must be tested that the scaffold should not deform while in long contact with the fluids. It is also needed because initial swelling gives the space for the blood capillaries to intrude into the tissue and supply nutrients and other vital things such as oxygen [21].

1.0. LITERATURE REVIEW

3.1.Materials for Scaffold Fabrication

Depending on the need of scaffold for treatment of different types of tissues various types of materials are used for scaffold fabrication. These materials fall in any of the three categories which are synthetic polymers, natural polymers and bio-ceramics [23].

3.1.1Synthetic Polymers

There has been tremendous increase in the use of synthetic polymers for scaffold development due to their various desirable features. Synthetic polymers have been used as surgical sutures worldwide in the last two decades, with proven track record of clinical success and many are approved for implantation in humans by the Food and Drug Administration (FDA) USA. PCL, PLLA, PGA, PEG and PLGA are some of the polymers which are given a green signal by FDA for implants but the problem with these synthetic polymers is that they do not have surface chemistry for which the cells are used to; the cells mainly have interaction with natural polymer that is Fibronectin, laminin, proteoglycans, glycoprotein, ealstin and collagen. These materials have their own benefits as a scaffold material. Synthetic polymers have their own advantage as they can be easily manipulated for desired degradation rates and mechanical properties. Raw material for synthetic polymer can be easily obtained but they always have a possibility of generation of immune response. The rate of degradation can be governed by manipulation of the amount of monomer [24]. Synthetic polymers can be further categorised into two types of polymers which are:

a) Bulk eroding polymer: In these types of polymer the rate of erosion is proportion to the total volume. Examples are: poly-α-hydroxy esters, polypropylene fumrate, etc.
b) Surface eroding polymer: In these types of polymers the rate of erosion is directly proportional to the surface area. Examples are: polyortho esters, polyhydrazene, etc.

Synthetic polymers can be reacting together to combine their unique properties. Here some of the synthetic polymers applied in tissue engineering are described briefly.

3.1.1.1. Poly (D, L-lactic acid)

Poly lactic acid (PLA)is a condensation product of monomeric units of lactic acid which is found in nature but the polyester is not a natural product. Lactic acid is a chiral molecule available in the L and D stereoisomer forms. L-lactic acid is a metabolite that is found in almost all microorganism and animals its precursor is pyruvate which end product of glycolysis, this polylactide is non-toxic and can be used without any glitch of toxicity [25]. This material is having a proven track record as scaffold material implanted successfully in tissue regeneration and cell transplantation. This material has all the qualities for a good scaffold material such as biocompatibility, biodegradability and solubility in common solvents for manipulation [26].

3.1.1.2Poly (lactic-co-glycolic acid)

Poly (glycolic acid) (PGA) and poly (lactic acid) (PLA) are synthetic polymers with biodegradable property, which can react to form the copolymer poly (lactic-co-glycolic acid) (PLGA) [27]. PGA is highly crystalline and has a high melting point and low solubility in organic solvents. Research is going on preparation of PGA combination polymers with PLA which is extra hydrophilic. The ester bond can be hydrolyzed which form backbone of the polymer, after implantation, causing the polymer to degrade into metabolite by-products. pH changes around the implantation site might be caused by the absorption of these by-product by the body. By the alteration in ratio of PGA to PLA the degradation can be changed in copolymer [28]. The hydrophobicity of the lactic acid is increased by the availability of an extra ethyl group. The water uptake of films can be limited to 2% by the hydrophobicity of PLA, and the backbone hydrolysis rate can be decreased in respect to the one of the PGA homopolymer. Moreover, PGA has less solubility in organic solvents than PLA. Studies have suggested that ratio of lactic acid to glycolic acid has to do nothing with their properties when it comes to their linear concentration [29]. When PGA/PLA copolymers are formed crystallinity of PGA decreases steeply. These morphological changes lead to an increase in the hydration and hydrolysis rate and copolymers tend to degrade more rapidly than the homopolymers of PGA or PLA do [30].

3.1.1.3. Poly-β-hydroxybutyrate

Poly-β-hydroxybutyrate (PHB) is a linear head-to-tail homopolymer of (R)-β hydroxybutiric acid, which forms crystalline cytoplasmic granules in the wide variety of bacteria. This material is biocompatible and biodegradable polyester produced by microorganisms, which after implantation have slow degradation at body temperature and is secreted in urine as a non-toxic metabolite. This metabolite which is also used as wound scaffolding device is known as PHB,

designed to protect and support a wound against more damage while helping in healing by promoting cellular growth within and on the device from the surface of wound.

3.1.1.4. Poly-e-caprolactone

Poly-e-caprolactone (PCL), an aliphatic polyester which is bioresorbable and biocompatible, is generally used in pharmaceutical products and wound dressings [31]. This polymer has low melting point of around 6o°C and a glass transition temperature of about -60°C [32]. Ester linkage are hydrolyzed at physiological condition (e.g. in the human body) hence degraded and therefore, it is supposed to be the most suitable implantable material and therefore it is in the main stream of the research [33].

3.1.1.5. Poly (ethylene glycol)

Poly ethylene glycol (PEG), also known as poly ethylene oxide (PEO) or polyoxyethylene (POE) is ethylene oxide polymer which can resist protein adsorption and adhesion of cell, commercial value of this polymer is very high. These characteristics help minimize the immune response after implantation. Additionally, this polymer is used to cementing cell membrane after injury, which makes it helpful in reducing cell death. Hydrophilic PEG hydro gels can be made through a variety of cross -linking schemes to create scaffolds with varying degradation as well as release rates. Further chemistry can be helpful to manipulate these gels to add sites for cell adhesion or extracellular matrix (ECM) molecules to allow cells to infiltrate into these scaffolds, extending their potential applications PEG can be converted into a hydro gel in mild condition by photo polymerization that is biocompatible and non-toxic. Bioactive molecules that are growth factors, cell adhesion ligands and proteolytic degradation sites have been previously inserted into PEG hydro gels and they had their effect on adhesion, proliferation, migration, and extracellular matrix production of vascular smooth muscle cells.

3.1.1.6Poly (glycerol sebacic acid)

Poly glycerol sebacic acid (PGS), is a hard elastomer whose monomers are biodegradable, it is also known as bio-rubber. PGS has that fine blend of toughness and elasticity. It is also degradable biologically. Studies suggest that it has biocompatibility as high as PLGA, a widely used polymer which is biodegradable [34].

3.1.1.6. Poly (2-hydroxyethyl methacrylate)

Hydroxyethyl methacrylate (HEMA) is a hydro-soluble monomer, which can be polymerized (under various circumstances) at low temperatures (from -20°C to +10°C). It can be used for the immobilization of the cells and in preparation of hydro gels. It has many applications in medicine as an appropriate biomaterial. Poly (2-hydroxyethyl methacrylate) (pHEMA) is particularly attractive for biomedical engineering applications. This polymer posses very good biocompatibility and physical properties too and with the help of formulated chemistry it can be manipulated as desired, which opens the gates of its application in medical for e.g. orbital implants contact lenses and prostheses. Furthermore, incorporation of this scaffold into nerve tubes can be easily done [35].

3.1.2. Natural polymer

There are several natural polymers which have come in the lime light for scaffold fabrication. Collagen and chitosan are most commonly used natural polymer.[36]

3.1.2.1. Collagen

Many studies suggest that collagen is a good scaffold material as it is found in our body extra cellular protein. It provides mechanical support to connective tissues such as tendons, bones, skin, cartilage, blood vessels, and ligaments in its native microenvironment, and shows interaction with cells in connective tissue and helps in transduction of signal for cellular anchorage, migration, proliferation, differentiation, and survival [37]. This bio-scaffold material collagen has all the positive factors on its side i.e. biodegradability, fine biocompatibility, low antigenicity and it can be easily altered for desired results; collagen type 1 is most frequently occurring type out of the twenty seven types of this protein which reported so far [38]. The processing of collagen is also easy which gives researchers the luxury to have it in different forms like gels, sheets and sponges. Its degradation rate can be altered by the cross linking agents which gives it the desired strength [39]. However, for medical applications, the implantation of foreign cells causes immunological problems. Collagen has potential uses as follows:

- This biopolymer can maintain it shape according to the culture and cell seeding
- Scaffold design has very high permeability [40].
- Production of tissue implants for reconstructive/cosmetic surgery applications
- Generation of spinal cord repair implants [41].

3.1.2.2Chitosan

Chitosan is a cationic polymer obtained from chitin comprising copolymers of β (1→4)-glucosamine and N-acetyl -D-glucosamine. Chitin is a natural polysaccharide found particularly in the shell of crustacean, cuticles of insects and cell walls of fungi and is the second most abundant polymerized carbon found in nature [42]. This polymer has many suitable properties. It can be used for wound dressing, drug delivery, and tissue engineering (cartilage, nerve and liver tissue) applications [43]. These properties include:

- Reaction to foreign body is minimum,
- Minimal processing conditions (chitosan will dissolve in water based on pH other synthetic polymer needs hard chemicals for their solubility)
- Controllable mechanical/biodegradation properties (such as scaffold porosity or Polymer length)
- Chemical side groups are available for attachment to other molecules.

Chitosan has already been established for application in the engineering of nerve, liver tissue and cartilage. There are few problems in using chitosan as a polymer scaffold in tissue engineering some of them are inconsistent behavior with seeded cells and low strength. Its strength and cell anchoring capacity can be increased by the combination of this material with other materials of high caliber. Their combinations with polymers which are synthetic such as poly (vinyl alcohol) and poly (ethylene glycol), or polymers which are natural such as collagen, have already been produced. These combinations surely improve the performance of the combination product over the behavior of either component alone.

3.1.2.3. Fibronectin

Fibronectin is a glycoprotein which exists outside cells and on the cell surface. It also exists in blood, other body fluids and on the cell surfaces of connective tissue. This protein has interaction with other extra cellular matrix (ECM) proteins like collagen, glycosaminoglycans and fibrinogen and with suitable receptors which are present in the cell membrane. Fibronectin contains of repeats of three different types (I, II and III) of individually folded subunits.

3.1.2.4. Gelatin

Gelatin is the primary protein component of animal connective tissues, such as hide, bone, tendon and skin. Collagen is the derivative of this protein known as gelatin and its molecular weight is very high. It is prepared by the thermal denaturation and physical and chemical

degradation of collagen. Gelatin in a dry form consists of 98-99 % protein [44]. The molecular weight of these large protein structures typically ranges between 20,000 and 250,000 g/mol, with some aggregates weighting in the millions. The chemical structure of gelatin is described by a linear sequence of amino acids. It is always written from the -NH2 end to the -COOH end. The predominant amino acids are glycine, proline and hydroxyproline [45]. Due to this, comparatively high levels of these amino acids are found in gelatin: proline 10-18 %, hydroxyproline 7-15 % and glycine 26-34 %. Other amino acids which are in considerable amount include: alanine 8-11 %; arginine 8-9 %; aspartic acid 6-7 % and glutamic acid 10-12 %. The water content will vary between 6-9 %. This polymer is used in drug delivery systems, wound healing materials and scaffolds material for tissue engineering, since it has good biocompatibility, high water adsorbing ability and biodegradability in vivo. Here, gelatin was chosen as the raw material for preparing gelatin scaffolds with microtubules orientation structure by unidirectional freeze-drying, and the structure and properties of the gelatin scaffolds were studied. The results show that gelatin is an ideal biomaterial, which can be used to prepare aligned porous scaffolds for tissue engineering.

3.1.2.5. Alginate

This is the material we have selected for our project so will discuss this in detail about its structure and all other thing. Alginate and agarose are linear polysaccharides obtained from algae and seaweed, respectively. To prevent generation of immune response these oligosaccharides must be extensively purified. Moreover, some types of cells if encapsulated into the alginate beads will certainly enhance growth and cell survival. In addition, application of alginate is as diverse as from cartilage, bone, heart and liver. There are some limitations while using alginate as scaffold material which include poor cell adhesion and mechanical weakness. In order to overcome these limitations, flaws, the strength and cell behavior of alginate have been enhanced by combination with other materials, which include the natural polymers Chitosan and agarose. Mohan et al. described the preparation and characterization of alginate sponges to be used as scaffolds in tissue engineering. They designed three dimensional scaffolds from cheaply available sodium alginate with nice porous structure, which shows very good biocompatibility. The scaffold designed by a mixture of particulate leaching and freeze drying, showed increased pore size and porosity. Better swelling properties and pore characteristics may permit more nutrient supply and cell invasion. Moreover alginate is non-cytotoxic, thermally stable and

biodegradable. Alginic acid is a linear copolymer composed of homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronte (G) residues, respectively, covalently linked together in different sequences or blocks. The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks) or alternating M and G-residues (MG-blocks).

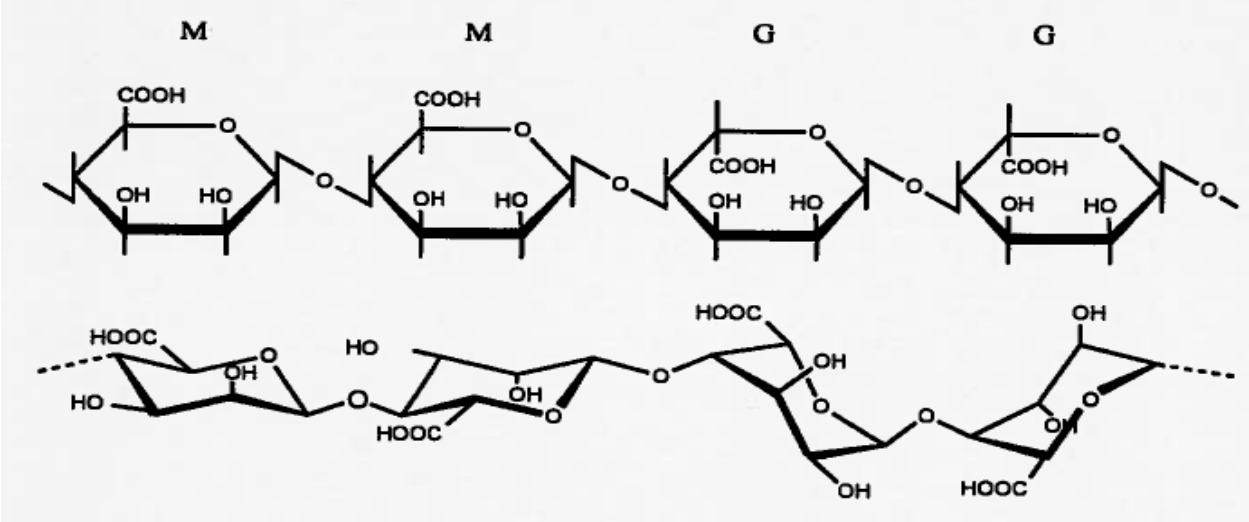

Fig1. Structure of alginate illustrated by the MMGG fragment. Upon C5 epimerization the sugar ring flips to the C4 conformation.

And due the below listed advantages of alginate over other scaffold material we chose this for our scaffold preparation

- Thermally stable
- Non-cytotoxic
- Biodegradable

3.2. Scaffold Fabrication techniques

With the advancement in the field of science and technology there has been significant advancement in the field of scaffold fabrication techniques as well [46]. We will be discussing about few of these scaffold techniques here.

3.2.1. Solvent casting and particulate leaching

It is one of the most common and simple most techniques used for scaffold fabrication. In this technique the polymer solution is poured into the mould which already contains any water soluble salt. Followed by evaporation or lyophilisation polymer solution dries up and the salt particles sweep inside the polymer. This mixture is allowed to stand in water for enough time to ensure that the salt particles are properly dissolved in water leaving behind porous polymer scaffold. The size of pores and porosity can be controlled by size of salt and polymer/salt concentration respectively

3.2.2. Electrospinning

This is also commonly used technique for scaffold preparation. In the past 10 years the electro spinning has become a widely used technique for the formation of extra cellular membrane (ECM) substitute structures for tissue engineering applications, in part because of its capacity to meet the three previously mentioned criteria [47]. With its competence to quickly fabricate scaffolds closely resembling the architecture of the native ECM that too of nanoscale fibers, this technique has witnessed a coming of new dawn in recent years and continues to find new dimensions in the field of tissue engineering [48]. This comparatively simple technique has proved its mark as it is highly adaptable, with the quality for use with numerous synthetic and natural polymers, cost effectiveness, production on large scale as and when required, and the ability to provide users control over a number of processing parameters which can be modified to elicit fine control over the end-product's physical features [49].

Electrospinning is not a specialized process and not require much specialized equipment if we have to perform simple electrospinning; all that is required is a a grounded target, high voltage power supply, and a conductive capillary of small radius to release a solution of polymer in a controlled fashion. Solution of polymers is prepared by dissolving them in solvents as and when required for different polymers, ranging from organic solvents to water. For the polymer chain to quagmire the concentration is kept quit high. As polymer concentration increases the viscosity of the solution will increase, the diameter of the resulting fiber increases in linear fashion, with the solution of polymer inside the capillary charged to a high voltage potential opposite a target on ground to generate a static electric field. A Taylor cone is formed when polymer solution aggregates at the tip of capillary and a strong electric field is generated to surmount the force of

surface tension created by solution of the polymer. A jet of polymer is produced by the stretching Taylor cone. Polymer solution is pulled towards the target on ground from Taylor cones tip as the jet travels through the air towards the collecting target the solvent gradually evaporates, resulting in the collection of nano to micron scale fiber on the target. The charge from the fibers dissipates into the surrounding environment, and a non-woven fiber mat comprising of fibers 10 µm to 50 nm in diameter is formed. The polymer solution is fed through a capillary (typically a needle with a blunt end) at a constant flow Scaffolds consist of several fibres with varying diameters. In this process the polymer solution id filled into the capillary tube which is connected to a high voltage and collector over which fibres are collected is kept at ground voltage. When the voltage difference overcomes the surface tension of the polymer solution, then a steam of jet is ejected. The solvent evaporates in the air and the resulting fibres are collected forming the scaffold.

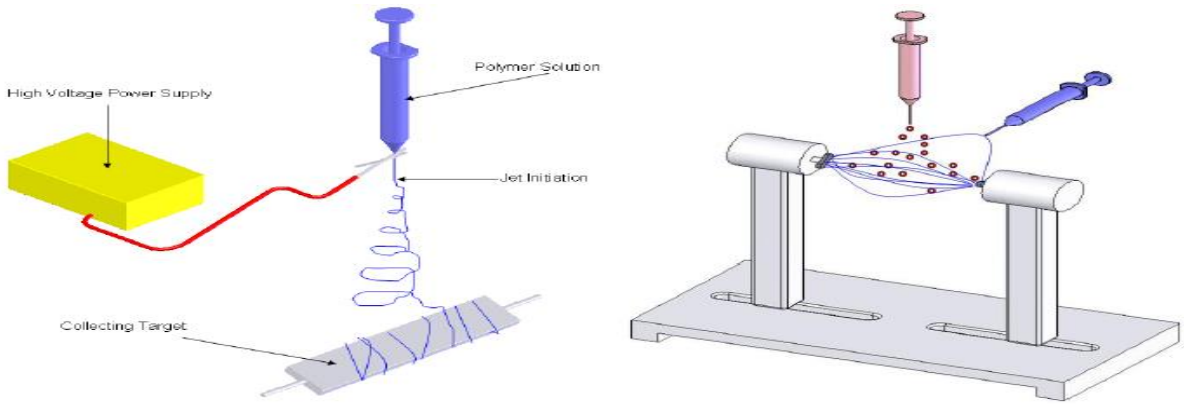

Fig2. Diagram showing a traditional horizontal orientation setup of electrospinning (Left), and the setup for micro integration of the electro spun polymer and cells simultaneously using an a electrospinning setup with air gap (Right)

3.2.3.Rapid prototyping

This is the advance most technique taken into use for 3D scaffold fabrication. In this technique every data is fed into the computer and monitoring each and every detail like porosity, interconnectivity of pores, etc [50]. the desired scaffold is prepared. In one such process the scaffold design is prepared through computer aided design (CAD) and the molten polymer

solution comes out of the nozzle. The movement of nozzle is governed by the algorithm and the scaffold is prepared layer by layer.

3.2.4.Freeze drying

Freeze-drying**,** which is also known as cryodesiccation or lyophilisation, is a dehydration process generally used to conserve a material which spoils early. Particulate-leaching processes, extrusion and heat compression are basis of many technologies which are used for the designing of polymers. However, the conditions in which these processes are performed used to be very harsh which in turn may restrict the incorporation of cells; bioactive proteins chemical solvents remaining in the fabricated scaffold required can cause toxicity in vivo. Freeze-drying is a method which does not require additional chemicals having its thrust mainly on the water and by this highly porous scaffold can be fabricated. This method, used in hydro gels, forms crystals of ice and sublimation of these ice crystals is possible from the polymer, providing peculiar micro-architecture. Because the direction of growth and size of the ice crystals are a function of the temperature gradient, linear, radial, and/or random pore directions and diverse sizes can be produced with this methodology.

Process of complete drying comprises of four steps as listed: pre-treatment, freezing, primary drying, and secondary drying

3.2.4.1. Pretreatment

Pre-treatment means any method of product treatment before freezing or further processing. This may include formulation revision (i.e., increment in stability and/or improve processing by the combination of other materials), concentrating the product, lowering a high vapour pressure solvent or surface area increment. In many cases the judgement to treat a product is not based on practical knowledge of freeze-drying and its requirements, rather it is demanded by circumstances or product considerations that we precisely wants. Pre-treatment methods include: freeze concentration, solution phase concentration, formulation to preserve product appearance, , formulation to increase the surface area, formulation to stabilize reactive products and lowering solvents of high vapour pressure.

3.2.4.2. Freezing

Freezing is a process which is performed in the lab by placing the material in a mechanical refrigerator which is cooled by liquid nitrogen, or dry ice. The material in the refrigerator is kept in a shell freezer which has rotating and freeze drying flask for the purpose. Freeze drying is a process in which sublimation takes place that is solid directly transfer into gaseous form so the scaffold should be sufficiently prefreezed before going for freeze drying. The final temperature of product to be frozen and the process of prefreezing can interfere in the ability to successfully freeze dry the material successfully. Speedy cooling ends up in ice crystals of small size, useful in conserving structures that are microscopically examined, but the product that is formed by this process comparatively difficult to freeze dry. Slower cooling results in less restrictive channels and larger ice crystals in the matrix during process of drying. When we have to do freezing on large scale it is done by freeze drying machine. In this step, the material is cooled lower than its its triple point and is a important step, it is the lowest temperature at which the liquid and solid phases of the material can exist together. This will guarantee sublimation and prevent melting. It is easier to freeze-dry the larger crystals. The product should be frozen slowly or temperature can be fluctuated, to produce larger crystals. This cycling process is known as annealing. However, when it comes to objects with formerly-living cells and food, crystals of ice with larger size will break the cell walls; consequently annihilation of cell will take place which can end-up in acceleration of nutritive content and poor texture. In this case, the freezing is done fast, so that the temperature of the material reaches to its eutectic point rapidly, thus avoiding the formation of ice crystals. Usually, the freezing temperatures are between −50°C and −80°C. Most important part in whole process of freeze drying is freeze if not performed properly it will ruin the whole process. Amorphous materials do not have a eutectic point, but they do have a critical point, below which the product must be sustained so that they do not melt-back or collapse while performing primary and secondary drying.

Primary drying: Several factors can affect the ability to freeze dry a frozen suspension. While these factors can be discussed independently, it must be remembered that they interact in a dynamic system, and it is this fine interplay between these factors that as a resultant fine freeze dried product is obtained. To obtain dry and structurally intact product sublimation is done to remove the ice from the frozen product and the parameters for this determined very carefully. There are two very important parameters to watch for in this process of freeze drying and they

are temperature and pressure. The difference in vapor pressure of product with the ice collector decides the sublimation rate of ice from a frozen product. Molecules migrate from the higher pressure sample to a lower pressure area. Since vapor pressure is related to temperature, it is mandatory that the cold net (for containment of ice) temperature is cooler than the product temperature. It has to be seen that the temperature at which a product is freeze dried is balanced between the temperature that maximizes the vapor pressure of the product and the temperature that sustains the frozen architecture of the product. This balance is crucial for optimum drying. The temperature is kept lower than the glass transition temperature and after some time it is raised to catch up with eutectic temperature but still kept lower than that. This is beginning of freeze drying process

Secondary drying:

The secondary drying facet of this process involves removal of unfrozen water molecules, since primary drying phase has removed all the ice crystals. This part of the freeze-drying process is dependent on the material's adsorption isotherms. In this phase, the temperature is elevated from the level of the primary drying phase, and it can be 0°C, to demolish any bonding that is developed during the process, between the frozen material and water. Usually the pressure is also decreased in this phase to promote desorption. However, there are products that benefit from increased pressure as well. After the freeze-drying process is complete, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed.

4.0. MATERIALS AND METHODS

4.1. Materials

Sodium alginate **(**Sigma-Aldrich)

Calcium chloride (Sigma-Aldrich)

Gelatin (Sigma- Aldrich)

Chitosan (Sigma-Aldrich)

Doubled-distilled water

Sodium chloride (Sigma-Aldrich)

Potassium chloride (Sigma-Aldrich)

Sodium dihydrogen phosphate (Sigma-Aldrich)

Potassium hydrogen phosphate (Sigma-Aldrich)

Lysozyme powder from chicken egg white (Sigma-Aldrich)

Hydrochloric acid (Sigma-Aldrich)

Centrifuge

Calorimeter

Microscope

Hemocytometer

Lyophilizer

Ultra sonicator

Glass ware, Petri plate, measuring cylinders, glass pipettes, glass jars etc

4.2. Methodology

4.2.1. Prepration of scaffold

Scaffold was prepared by using sodium alginate, chitosan and gelatin. Following steps and processes are involved in the preparation of scaffold and their composites with biopolymers: 1. For the scaffold formation we had taken 4% sodium alginate and 4% gelatin which was dissolve in ionized water slowly to avoid any air bubble formation. We have to make sure on this step to use only ionized water. Chitosan was dissolved in 1% acetic acid.

2. Then we had used ultra sonicator so that the alginate, chitosan and gelatin slurry which we had prepared earlier must be free from the air bubble. The air bubble will deform the structure, have adverse affect on the porous structure and will make some pores very wide

3. Then we had Prepared 2% solution of calcium chloride.

4. Now after the preparation bubble free alginate slurry and calcium chloride solution we had treated alginate slurry with the calcium chloride so that the gelation process takes place. To insure complete gelation treatment of alginate slurry with calcium chloride we had to place this mixture for whole night so that the reaction take lace quit well.

5. Chitosan and sodium alginate slurry now taken in 1:1 proportion and mixed thoroughly. Similarly gelatin and sodium alginate slurry taken in 1: 1 proportion and mixed.

5. Now comes the main step of our experiment variation of temperature to do this we had first deep-freezed calcium chloride treated alginate slurry at -20^{0}C for 48 hours. While keeping this calcium chloride treated alginate slurry for the deep-freezing we had to ensure that there should be no spilling.

6**.** Now we had to do the drying process which is also known as primary drying it is necessary to ensure the removal of the ice crystal from the frozen product via sublimation which results in the dried product. This requires very carefully control of the two parameters. Temperature and Pressure we had here fluctuated the temperature form -5°C to 0°C.

7. Then we had done secondary drying, the aim is to remove the unfrozen water molecule from the product since all the ice was removed by the primary freeze drying method. After primary freeze-drying ill ice has sublimed, bound moisture is still present in the product; residual moisture content may be as 7-8 % we had done the process of isothermal desorption

8.The we had done the Lyophilisation of the sodium alginate, chitosan/alginate and gelatin/alginate scaffolds with the help of lyophilizer.

9. Freeze dried scaffold was washed thoroughly with water.

1o. Lyophilisation is done again with the help of lyophilizer and scaffolds were kept in airtight container.

4.2.2Characterization of scaffold

4.2.2.1. X-Ray diffraction analysis

The XRD patterns of Chitosan/Sodium Alginate and Gelatin/Sodium Alginate scaffolds were obtained at room temperature using an analytical powder diffractometer. We had placed scaffolds in liquid nitrogen at −197 ◦C for 60 minutes and then made fine powder of it by using

mortar and pestle. The powdered biocomposite scaffolds were analyzed in the 2θ angle of 5–70 at a speed of 2° min−1.

4.2.2.2. SEM analysis

SEM analysis of the scaffolds was performed to study the surface structure of the scaffold which is very important for the cell seeding rough surface provide good anchorage. It is important to perform electron microscopy of the scaffold to observe the pores formed and interconnectivity of pores etc. Thus, we can say that for understanding the morphology of the scaffolds, electron microscopy is vital. S-3700N model of Hitachi SEM was used for performing the analysis. 15 kV of constant voltage was maintained while performing the microscopy.

4.2.2.3Porosity measurement

Liquid displacement method was used for calculating porosity of the scaffolds. In this method the scaffolds are completely immersed in the known volume of liquid (V_1) present in a measuring cylinder. The scaffolds remain drowned in the liquid till they are not saturated. The total volume of scaffold and liquid is V_2. Thus, volume of the scaffold skeleton is (V_2-V_1). Once the saturated scaffold is removed from the liquid then the volume of remaining liquid is V_3.

Therefore, volume of liquid present in the scaffold will be (V_1-V_3) and the total volume of scaffold will be (V_2-V_1) + (V_1-V_3) = V_2-V_3

Porosity (Π) is given by (V_1-V_3)/ (V_2-V_3).[3o]

4.2.2.4. Shape retention

Swelling behavior and structural stability of scaffolds are critical for their practical use in tissue engineering. Most natural polymers swell readily in biological fluids. Studies showed that early swelling is required and the resultant increase in pore size helps in cell attachment and growth in a three-dimensional fashion. However, continuous swelling would lead to loss of mechanical integrity and production of compressive stress to surrounding tissue. Swelling behavior of scaffold that we have fabricated is to be tested at different pH [31].

4.2.2.5. In vitro biodegradation behaviour

The rate of degradation of the biocomposite scaffolds was studied by the following procedure: The scaffolds were equally weighed and initial weight was noted as (*wi*), then scaffolds were fully submerged in 1× PBS which was having lysozyme similar to that amount which we have in

our body (10,000 U/L). They were incubated at 37 ◦C for different time intervals (24 h, 48 h and 72 h). After completion of each period of incubation, the ions were removed by washing the scaffolds with deionised water and then scaffolds were dried with the help of blotting paper. The dry weights of the scaffolds were noted as (*wt*). The experiment was performed in triplicates. The degradation percentage was calculated by using the following formula:

$$\text{Degradation \%} = \frac{wi - wt}{wi} \times 100$$

4.2.2.6. Hemolysis

Hemolysis studies were carried out on scaffold powder form. Red blood cell were freshly collected from a healthy human and washed three times with 1× D-phosphate buffer solution (pH 7.4) by centrifugation at 850g for 15 minutes until the supernatant became clear. The red blood cells adjusted to a density at 1×PBS (pH 7.4) and an aliquot (250 µl) of the suspension was added to each appendoff tube. Each testing material in PBS (50 µl) was added to each corresponding tube. Triton X-100 (0.2%) was used to generate 100% cell lyses as a positive control. The cells were incubated with the testing material at 37°C for one hour on a three dimensional rotation platform. After incubation, 150 µl of supernatant was transferred to a new appendoff tube after centrifuge at 850 g for 15 minutes. The hemoglobin released from the ruptured red blood cells was measured by colorimeter at 540 nm and absorbance was recorded. Hemolysis % was calculated by following formula:

$$\text{Hemolysis\%} = \frac{A\ test\ samle\ -\ A(-ve)control}{A(+ve)control\ -A(-ve)control}$$

Where A = Absorbance

5.0. RESULT & DISSCUSSION

5.1. Characterization of scaffold

5.1.1.SEM analysis

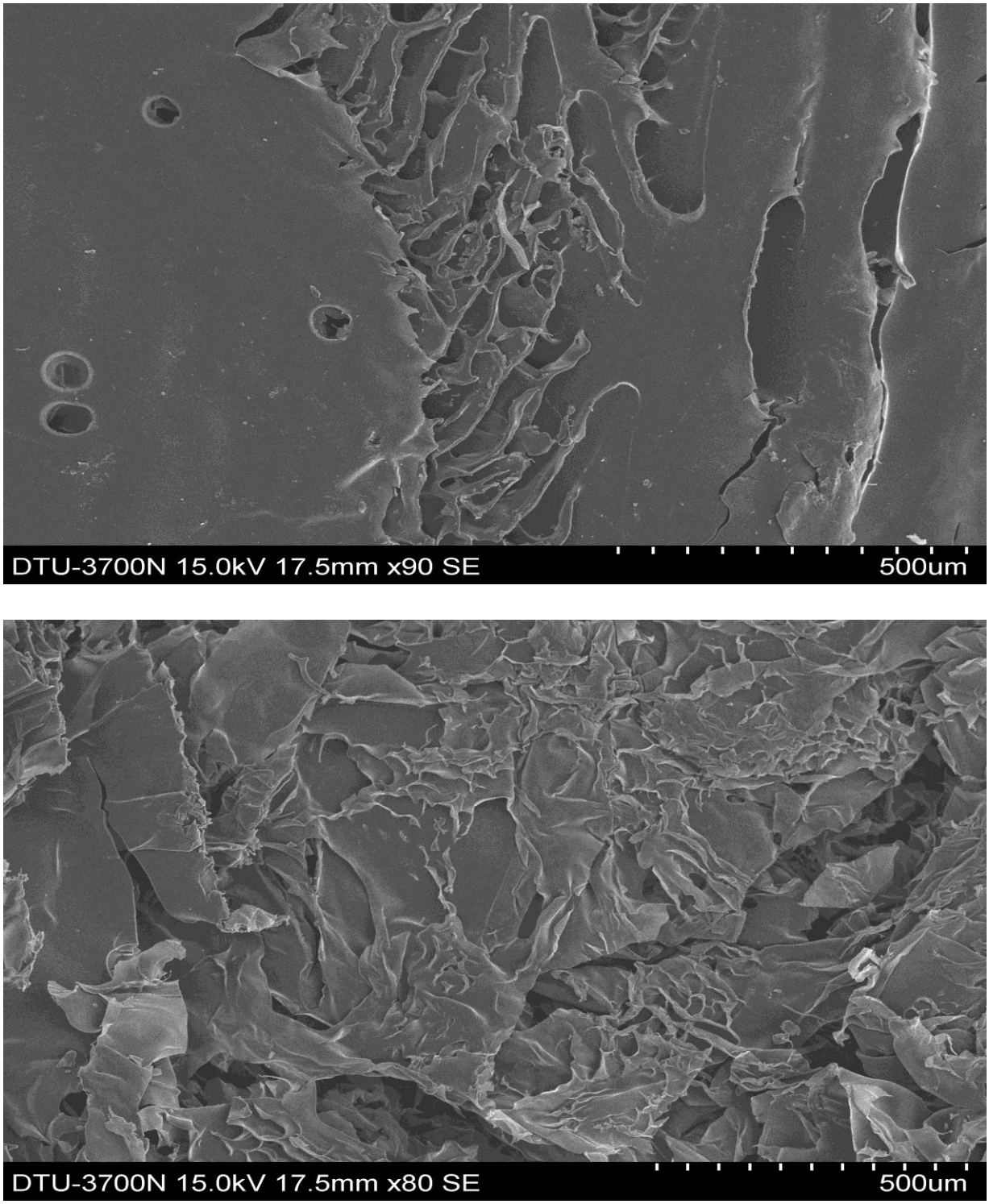

Fig 1.o SEM images of (a) sodium alginate at -20º and (b) sodium alginate at -80°

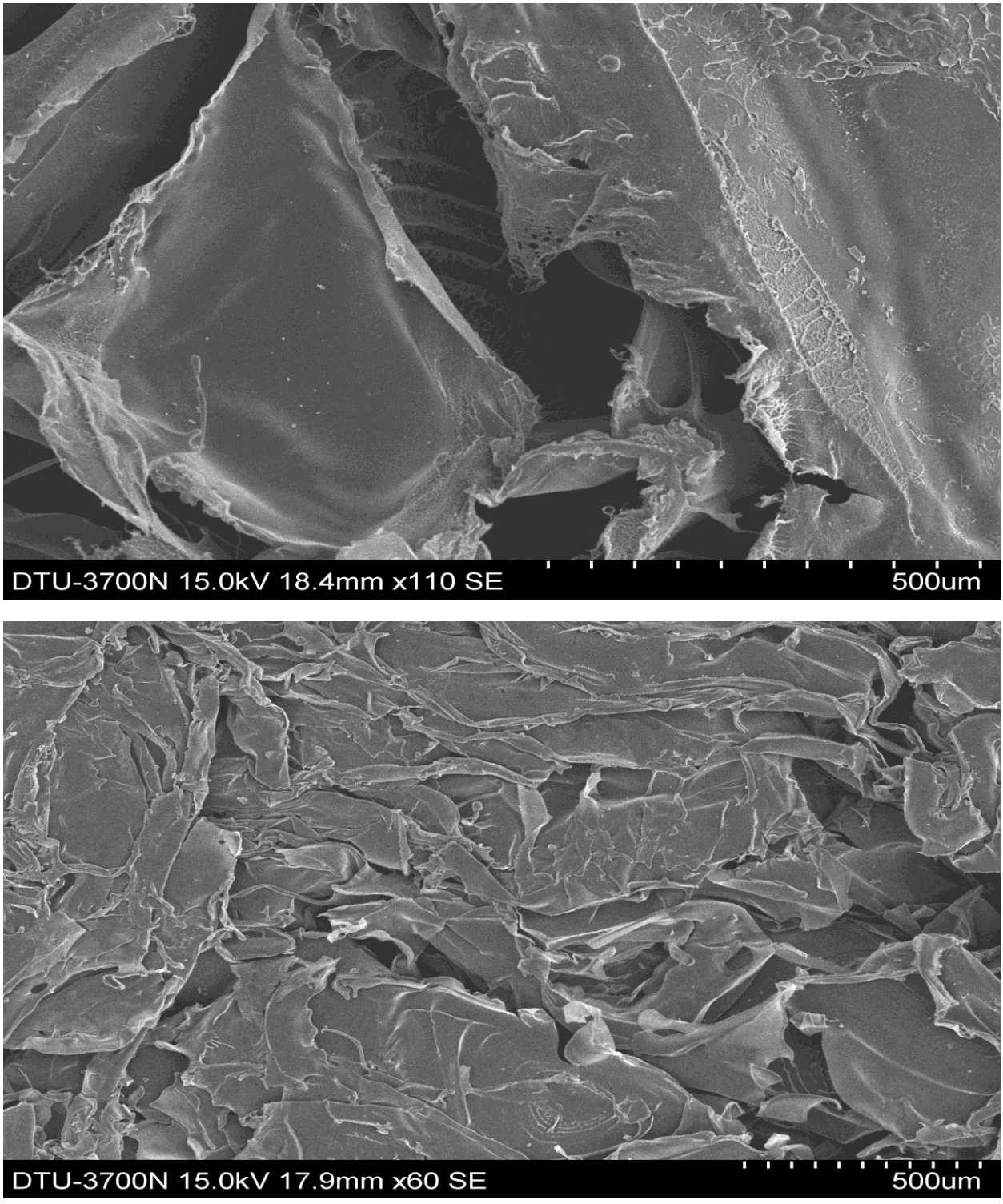

Fig 2.0 SEM images of (a)sodium alginate/chitosan(-20°) (b) SA/chitosan (-80°)

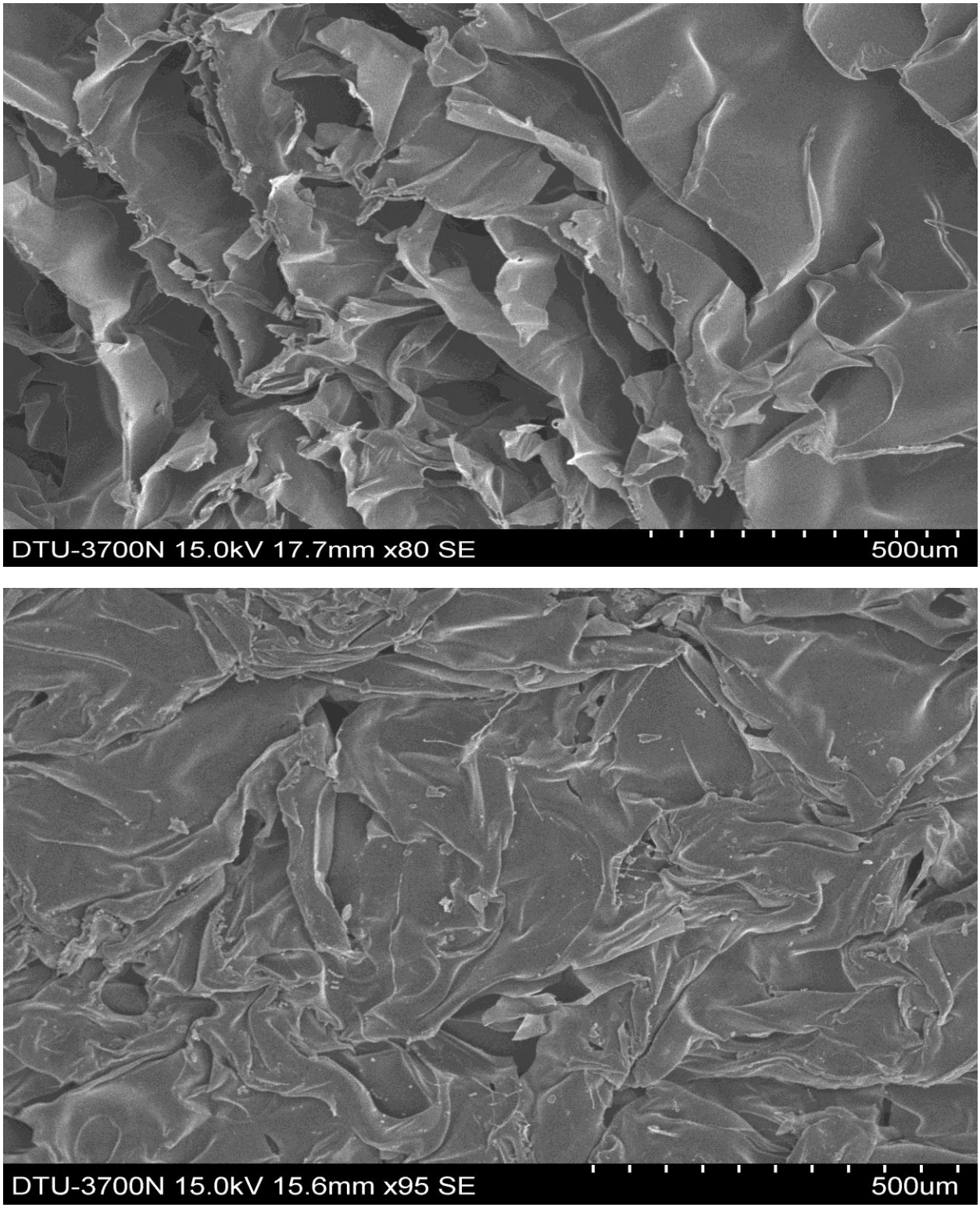

Fig 3.0 SEM images of (a) SA + gelatin (-20°) (b) SA + gelatin (-80°)

5.1.1.2. X-ray diffraction analysis

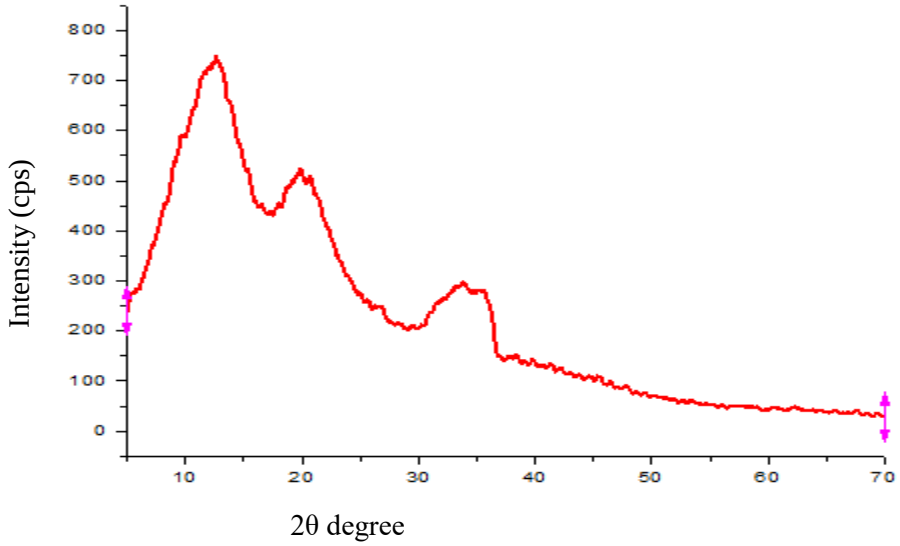

Fig 4.0 XRD pattern of sodium alginate/chitosan scaffold

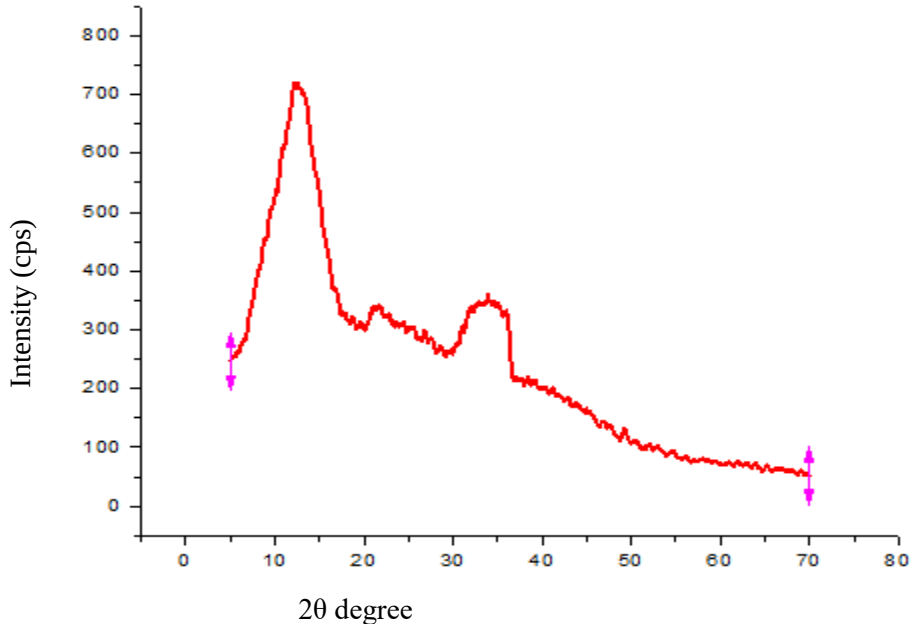

Fig 5.0 XRD pattern of sodium alginate/gelatin scaffold

Morphology of the scaffolds is characterized by the SEM analysis. The rough structure is very helpful for cell seeding as well as it should also contain pores so that blood vessel can enter inside the scaffold for the supply of the blood. As depicted in the SEM image surface structure of alginate is quite rough and also porous in nature as compared to the SA/ge. SA/ch is almost have the same structure but SA/ge is more rough and porous than the SA/ge

XRD pattern shows good blending of sodium alginate, chitosan and gelatin.

5.1.1.3 Porosity

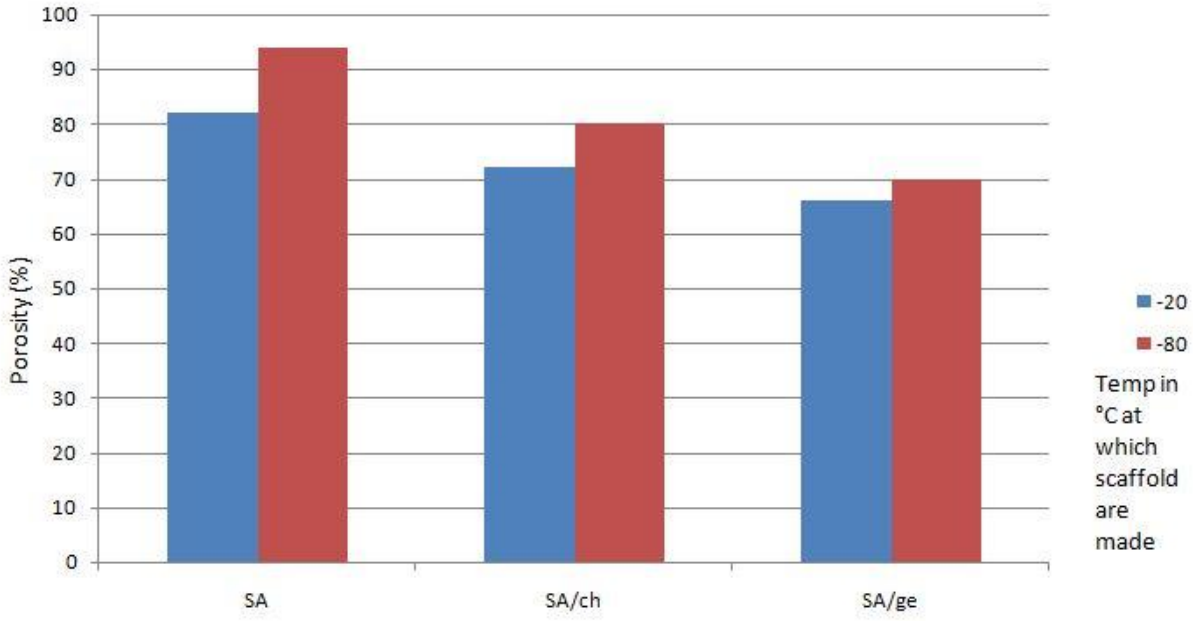

Fig 6.0 Graph showing porosity of sodium alginate (SA), sodium alginate/chitosan(SA/ch) and sodium alginate/gelatin(SA/ge).

Scaffold used in tissue engineering should have a high porosity structure to provide the sites cell attachment, proliferation and differentiation porosity of alginate is highest about 80% at -20°c and above 90% at -80°c. Chitosan also showing porosity of 80% from this graph it is evident that porosity is more when the scaffold is made at -80°c. Temperature plays important role in the morphology of the scaffold.

5.1.1.4 Swelling behavior

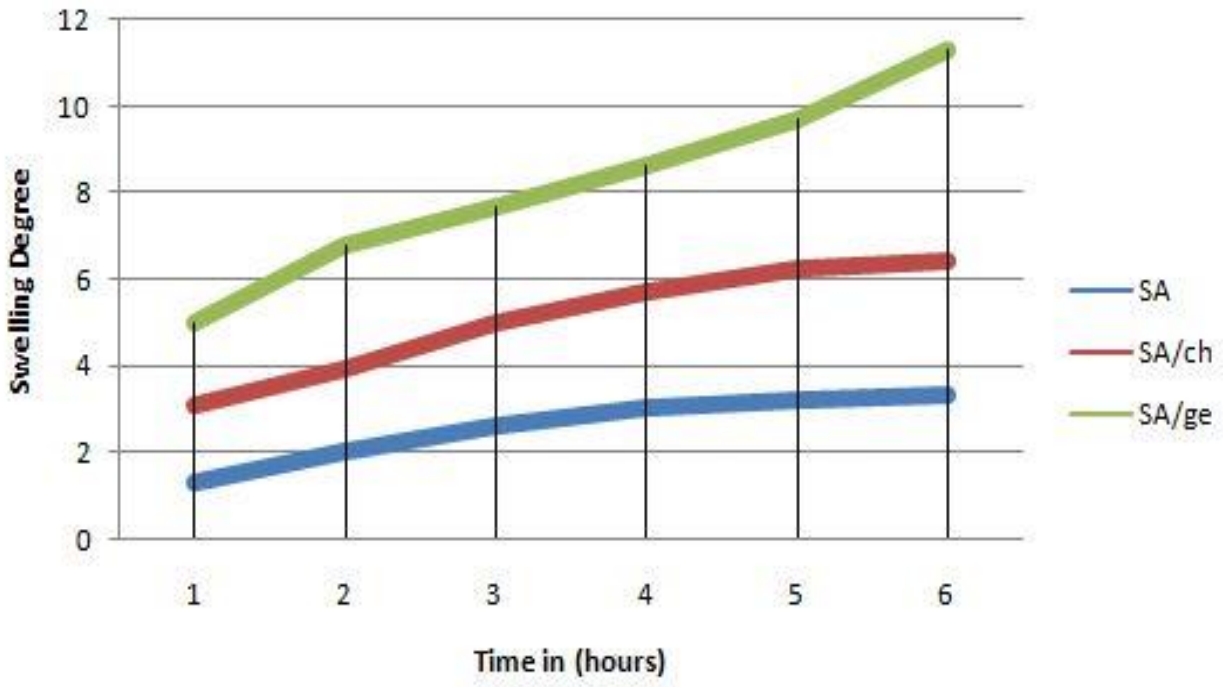

Fig 7.0 Swelling behavior of sodium alginate(SA), sodium alginate/chitosan(SA/ch) and sodium alginate/gelatin(SA/ge) at pH 3.5.

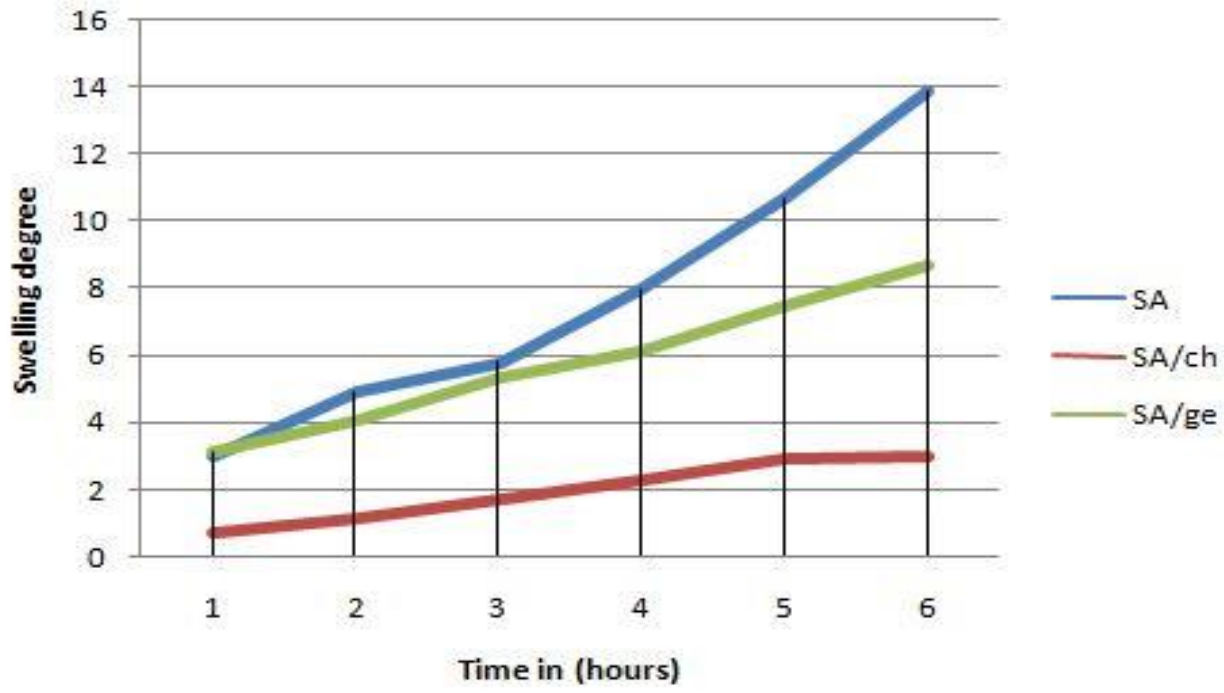

Fig 7.0 Swelling behavior of sodium alginate (SA), sodium alginate/chitosan(SA/ch) and sodium alginate/gelatin(SA/ge) at pH 7.4.

Swelling refers to the ability of fluid uptake by the scaffold material under physiological conditions. Swelling behavior and structural stability of scaffolds are critical for their practical use in tissue engineering. In vitro culture studies showed that early swelling is required and the resultant increase in pore size helps in cell attachment and growth in a three-dimensional fashion. However, continuous swelling would lead to loss of mechanical integrity. It is shown here that sodium alginate absorbs water very quickly and is capable of absorbing water in good quantity which is shown in the graph while sodium alginate at lower pH (3.5) shows less water absorbing capacity. At 3.5 pH SA/ge has the maximum water absorption but at physiological pH (7.4) SA/ge shows moderate water absorption. SA/ch at physiological pH absorbs very less water and after some time becomes constant , almost no swelling is seen after six hours.

5.1.1.5 Enzymatic degradation

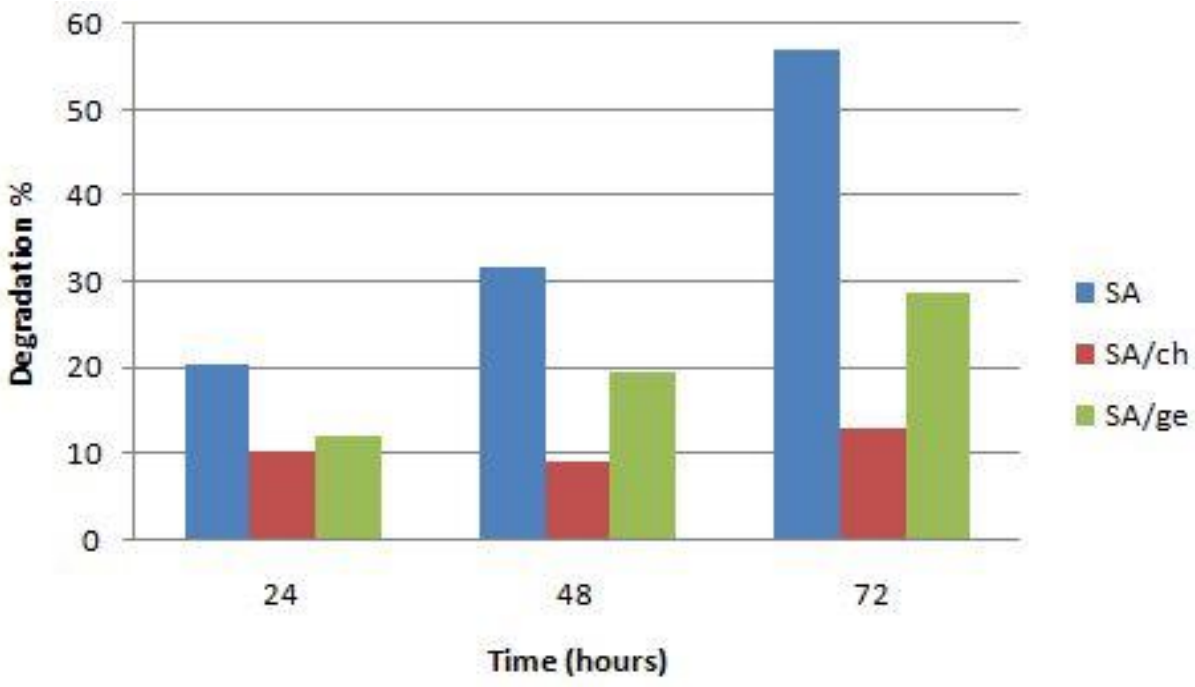

Fig 8.0 *In vitro* biodegradation of sodium alginate(SA), sodium alginate/chitosan(SA/ch) and sodium alginate/gelatin (SA/ge) in PBS containing lysozyme at 37°C.

The crucial parameter to be considered in tissue engineering is the degradation rate of scaffold as it provides space for tissue in growth and matrix deposition, which is essential for quality and quantity tissue regeneration, lysozyme, is the primary enzyme responsible for *in vivo* degradation and it acts on the (NAG) groups of the chitosan chain. A significant degradation was observed in

the sodium alginate tissue scaffold with increase in time but degradation in uncontrolled and not constant. In the case of sodium alginate/chitosan the degradation is very less. In case of sodium alginate/gelatin the degradation is substantial and is also constant. Constant rate of degradation gives the as much space as regenerating tissue needs, if more scaffold is degraded and tissue regeneration is constant than there will be a void created and the load bearing capacity can be affected and set up can collapse. That's why constant degradation with time is needed.

5.1.1.6 Haemolysis

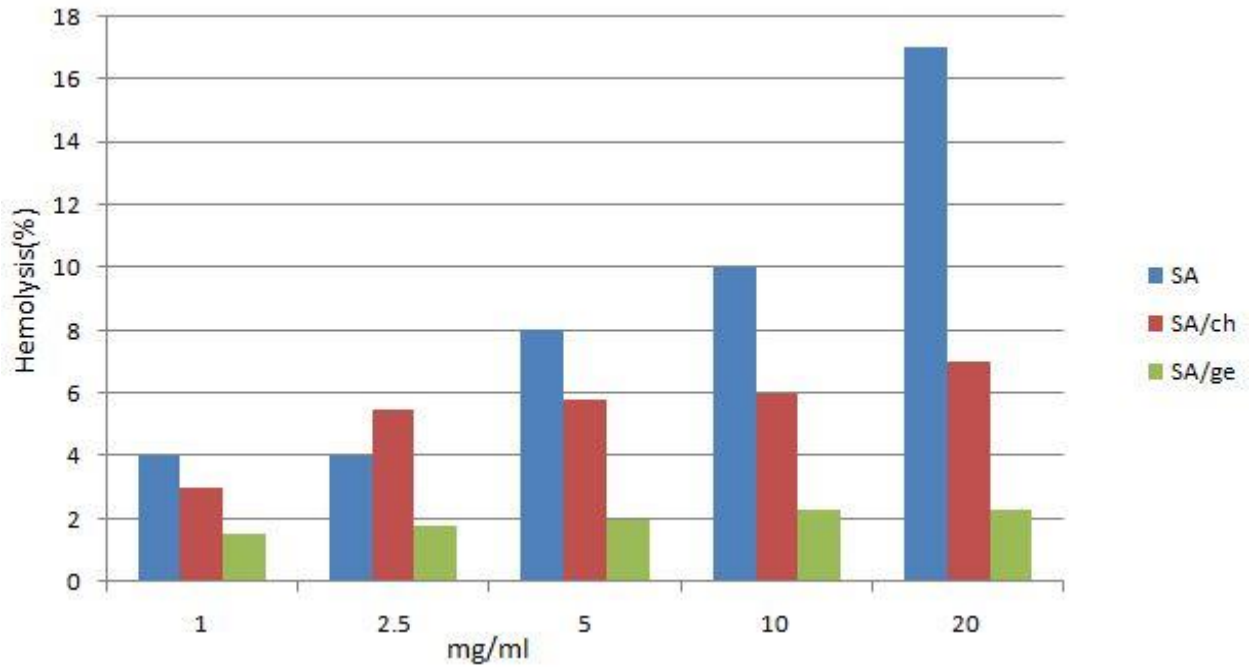

Fig 9.0 Graph showing the Hemolysis of sodium alginate (SA), sodium alginate/chitosan (SA/ch) and sodium alginate/gelatin (SA/ge) at different concentration.

Hemolysis studies were performed on scaffolds of different biopolymer composite and the result are demonstrated by the help of graph. The results show that alginate is causing maximum haemolysis and it is increasing with the increase in concentration while in the case sodium alginate/chitosan it is increasing but not as much as in the case of alginate and in sodium alginate/gelatin the haemolysis is lowest and also becomes constant with increasing concentration.

6.0. CONCLUSION

Here we made composite scaffold of sodium alginate, sodium alginate/chitosan and sodium alginate/gelatin by freeze drying method and characterization was done with the help of different technique and experiments. SEM analysis showed that sodium alginate/chitosan has most suitable morphology while sodium alginate/gelatin has almost same morphology with a little less rough structure while sodium alginate has more plane structure. Effect of temperature is clearly visible as scaffold made at lower temperature are more porous more pores and rough surface structure for the anchorage of the cells. Porosity of sodium alginate is highest; sodium alginate/chitosan is having moderate porosity while sodium alginate/gelatin is very less almost 70% porosity is required by the scaffold for the tissue engineering. Swelling degree of alginate is highest at physiological pH (7.4); most suitable swelling is shown by sodium alginate/gelatin. Enzyme degradation of sodium alginate /gelatin is constantly increasing with time in a controlled manner which is suitable as tissue will also regenerate constantly with time. Degradation of sodium alginate/chitosan is very slow which not good for the tissue, while sodium alginate has uncontrolled degradation. Sodium alginate/gelatin has minimum Hemolysis, it has minimum toxicity, and overall sodium alginate is most suitable among the three scaffolds for tissue engineering

7.0. REFERENCES

1. Persidis A. Tissue engineering. Nat Biotechnol 1999; 17:508–10.

2. Ma PX. Biomimetic materials for tissue engineering. Adv Drug Deliv Rev 2008; 60:1842198.

3.Malda J, Woodfield TB, van der Vloodt F, Wilson C, Martens DE, Tramper J, van Blitterswijk CA, Riesle J. The effect of PEGT/PBT scaffold architecture on the composition of tissue engineered cartilage. Biomaterials 2005;26:63–72.

4.Yang S, Leong K-F, Du Z, Chua C-K. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. Tissue Eng2001;7:6792689.

5.Niklason LE. Engineering of bone grafts. Nat Biotechnol 2000;18:929–30.

6.Service RF. Tissue engineers build new bone. Science 2000;289:1498–500.

7.Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18:959–63.

8.Motamedian SR, Hosseinpour S, Ahsaie MG, Khojasteh A. Smart scaffolds in bone tissue engineering: A systematic review of literature. World J Stem Cells 2015;7:657.

9.Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials 2005;26:5474–5491.

10.Bharatham BH, Bakar A, Zuki M, Perimal EK, Yusof LM, Hamid M. Development and characterization of novel porous 3D alginate cockle shell powder nano bio composite bone scaffold. BioMed Res Int 2014;2014:11 pp.

11.Yang S, Leong K-F, Du Z, Chua C-K. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. Tissue Eng2001;7:6792689.

12.Guo G, Lu L, Ji H, Ma Y, Dong R, Tu J, Guo X, Qiu Y, Wu J, Zhang D. Low intensity pulse ultrasound stimulate chondrocytes growth in a 3-D alginate scaffold through improved porosity and permeability. Ultrasonics 2015;58:43–52.

13.Zhang, J., Wu, L., Jing, D., and Ding, J. A comparative study of porous scaffolds with cubic and spherical macropores. Polymer 46, 4979, 2oo5.

14. Porter BD, oldham JB, He SL, Zobitz ME, Payne RG, An KN, et al. Mechanical properties of a biodegradable bone regeneration scaffold. J Biomech Eng 2ooo;122:286–8.

15.Thomson RC, Yaszemski MJ, Powers JM, Mikos AG. Fabrication of biodegradable polymer scaffolds to engineer trabecular bone. J Biomater Sci Polym Ed 1995;7:23–38.

16.Van Tienen TG, Heijkants RG, Buma P, de Groot JH, Pennings AJ, Veth RP. Tissue ingrowth and degradation of two biodegradable porous polymers with different porosities and pore sizes. Biomaterials2002;23:173121738.

17.Becker TA, Kipke DR, Brandon T. Calcium alginate gel: a biocompatible and mechanically stable polymer for endovascular embolization. J Biomed Mater Res 2oo1;54:76–86.

18.Cortizo AM, Ruderman G, Correa G, Mogilner IG, Tolosa EJ. Effect of surface topography of collagen scaffolds on cytotoxicity and osteoblast differentiation. J Biomater Tissue Eng 2012;2:125–132.

19.Mashhadikhan M, Soleimani M, Parivar K, Yaghmaei P. ADSCs on PLLA/PCL hybrid nanoscaffold and gelatin modification: Cytocompatibility and mechanical properties. Avicenna J Med Biotechnol 2015;7:32.

20.Lim J, Chong MSK, Teo EY, Chen GQ, Chan JK, Teoh SH. Biocompatibility studies and characterization of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate)/polycaprolactone blends. J BiomedMater Res Part B: Appl Biomater 2013;101:7522761.

21. Sachlos E. & Czernuszka J.T. (2oo3). Making tissue engineering scaffolds work Review on the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. European Cells and Materials, Vol. 5, pp. 29-40, ISSN: 1473-2262.

22. Manzanedo D. (2005). Biorubber (PGS): evaluation of a novel biodegradable elastomers. Thesis. Massachusetts Institute of Technology, Department of Materials Science and Engineering.

23.Willerth S. & Sakayama-Elbert Sh. (2oo7). Approaches to neural tissue engineering usingscaffolds for drug delivery, Advanced Drug Delivery Reviews, Vol. 59, No. 4-5, pp.325–338.

24. Kricheldorf H. R. (2oo1). syntheses and application of polylactides. Chemosphere, Vol. 43, No.1, pp. 49-54.

25.Alves NM, Shi J, Oramas E, Santos JL, Tom_as H, Mano JF. Bio-inspired super hydrophobic poly (L-lactic acid) surfaces control bone marrow derived cells adhesion and proliferation. J Biomed Mater Res Part A 2009;91:480–488.

26.Chim H, Ong JL, Schantz JT, Hutmacher DW, Agrawal C. Efficacy of glow discharge gas plasma treatment as a surface modification process for three-dimensional poly (D, L-lactide) scaffolds. J Biomed Mater Res Part A 2003;65:327–335.

27. Reed A.M. & Gilding D. k.; (1981). Biodegradable Polymers For Use in Surgery Poly (lactic acid)/Ploy(glycolic)Homo and Copolymers:2.In vitro Degradation, Polymer, Vol. 22, pp. 494_498.

28. Miller DC, Thapa A, Haberstroh KM, Webster TJ. Endothelial and vascular smooth muscle cell function on poly (lactic-co-glycolic acid) with nano-structured surface features. Biomaterials 2004;25:53–61.

29. Mosahebi. Mohammadi., 2oo1. Genetic Labelling and Transplantation of Schwann cells to Enhance Pheripheral Nerve Regeneration, Royal Free & University College Medical School, University of London.

30. Ljungberg C.; Johansson-Ruden G.; Bostrom K.J.; Novikov L. & Wiberg, M. (1999). NeuronalSurvival Using a Resorbable Synthetic conduit as an Alternative to Primary Nerve Repair, Microsurgery, Vol. 19, No. 6, pp. 259_264.

31. Venugopal, J.; Zhang Y.Z. & Ramakrishna S. (2oo5a). Fabrication of modified and functionalized polycaprolactone nanofibre scaffolds for vascular tissue engineering.Nanotechnology, Vol. 16, No. 1o, pp. 2138-2142

32. Schnell E.; Klinkhammer K.; Balzer S.; Brook G.; Klee D. & Dalton P. (2oo7). Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-ecaprolactone and a collagen/poly-e-caprolactone blend, Biomaterials, Vol. 28, No. 19, pp. 3o12-3o25.

33.Tang Z, Black R, Curran J, Hunt J, Rhodes N, Williams D. Surface properties and biocompatibility of solvent-cast poly [e-caprolactone] films. Biomaterials 2004;25:4741–4748.

34. Sundback C.A.; Shyn J.Y.; Wang Y.; Faquin W.C.; Langer R.S.; Vacanti, J.P. & Hadlock T.S. (2oo5). Biocompability analysis of a poly(glycerol sebacate) as a nerve guide material. Biomaterials, Vol. 26, No. 27, pp. 5454-5464.

35. Flynn L.; Dalton P.D. & Shoichet M.S. (2oo3). Fiber templating of poly(2-hydroxyethyl methacrylate) for neural tissue engineering. Biomaterials, Vol. 24, No. 23, pp. 4265-4272.

36. Elmstedt N. (2oo6). Development of biosynthetic conduit for spinal cord and peripheral nerve injury repair, invitro study. thesis. Stockholm, Sweden.

37. Chunlin Y.; Hillas P.J.; Buez J.A.; Nokelainen M.; Balan J.; Tang, J.; Spiro R. & Polarek J.W. (2oo4). The application of recombinant human collagen in tissue engineering, BioDrugs, Vol. 18, No. 2, pp. 1o3_119.

38.Zhang Q, Lu H, Kawazoe N, Chen G. Pore size effect of collagen scaffolds on cartilage regeneration. Acta Biomater 2014;10:2005– 2013.

39.O'Brien FJ, Harley B, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen-GAG scaffolds. Biomaterials 2005;26:433–441.

40. Ber S, Torun K€ose G, Hasırcı V. Bone tissue engineering on patterned collagen films: An in vitro study. Biomaterials 2005;26: 1977–1986.

41. Matin S. (2oo4). Spinal cord regeneration via collagen entubulation. Thesis. Department of Aeronautics and Astronautics, John Hopkins University, Massachuset Institute of Technology.

42. Khor E. & Lim L.Y. (2oo3). Implantable applications of chitin and chitosan, Biomaterials, Vol.24, No. 13, pp. 2339_2349.

43. Madihally S.V. & Matthew H.W.T. (1999). Porous chitosan scaffolds for tissue engineering, Biomaterials, Vol. 2o, No. 12, pp. 1133_1142.

44.Lien S-M, Ko L-Y, Huang T-J. Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering. Acta Biomater 2009;5:670–679.

45. Cuy J. (2oo4). Biomaterials Tutorial: Natural Polymers. University of Washington Engineered Biomaterials

46. Ebner R.; Lackner J.M.; Waldhauser W.; Major R.; Czarnowska E.; Kustosz R.; Lacki P. & Major B. (2oo6). Biocompatible TiN-based novel nanocrystalline films, Bulletin of the polish academy of science, Vol. 54, No. 2, pp. 167-173.

47. Martins A.; Araújo J.V.; Reis R.L. & Neves N.M. (2oo7). Electrospun nanostructured scaffolds for tissue engineering applications. Nanomedicine, Vol. 2, No. 6, pp. 929-42.

48. Yang L.; Fitie´ C.; van der Werf K.; Bennink M.; Dijkstra P. & Feijen J. (2oo8). Mechanical properties of single electrospun collagen type I fibers. Biomaterials, Vol. 29, No. 8, pp. 955-962.

49. Fang D.; Xong X.; Chen W.; Cruz S.; Hsiao B. & Chu B. (2oo1). Nanostructured electrospun poly-D,L-lactideco-glycolide membranes for anti-adhesion applications. Apparel Technol, Vol. 1, Special issue: The Fiber Society, Spring 2oo1 Conference, Raleigh NC.

50. Buchko C.J.; Chen L.C.; Shen Y. & Martin D.C. (1999). Processing and microstructural characterization of porous biocompatible protein thin films. Polymer, Vol. 4o, No. 26, pp. 7397-74o7.