Development of PVA-Carrageenan based scaffolds and evaluating its efficacy on osteosarcoma upon cryopreservation

Department of Biotechnology,
Thapar University, Patiala
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CERTIFICATE

This is to certify that the thesis entitled "***Development of PVA-Carrageenan based scaffolds and evaluation of its efficacy on osteosarcoma upon cryopreservation***" for the partial fulfilment of the Master degree in Biotechnology submitted to Thapar University, Patiala, Punjab, is a record of bonafied research work carried out by **Mr. Pankaj Chopra**, Roll No. 301301008 under my supervision and guidance at Department of Life Science, National Institute of Technology, Rourkela. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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CERTIFICATE

I hereby certify that the thesis entitled "***Development of PVA-Carrageenan based scaffolds and evaluating its efficacy on osteosarcoma upon cryopreservation***", which is submitted by Mr. Pankaj Chopra, in fulfillment of the requirement for the award of Masters in Biotechnology in Department of Biotechnology, Thapar University, Patiala, Punjab, India is a record of candidate's own independent and original research work carried out by him under the supervision and guidance of Dr. BismitaNayak, Assistant professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha, India and Dr. VikasHanda, Assistant Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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DECLARATION

I hereby declare the thesis entitled "***Development of PVA-Carrageenan based scaffolds and evaluating its efficacy on osteosarcoma upon cryopreservation***", submitted to the Department of Biotechnology, Thapar University, Patiala, Punjab for the partial fulfilment of the Master Degree in Biotechnology is a faithful record of bonafide research work carried out by me under the guidance and supervision of Dr. Bismita Nayak, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

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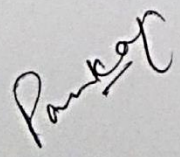

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Chapter 1

Introduction

1.1 Tissue Engineering

Tissue loss or organ failure, due to accidents of diseases, represents a life threatening situation, hence tissue repair by tissue transplantation or autologous cell transplantation; where the donor and recipient are the same, is proved to be one of the most promising technique for tissue repair and regeneration. Organ replacement is been the subject of debate since a long period of time. This field of engineering, where tissue is engineered *in vitro* and damaged tissue is repaired *in vivo*, has originated about two decades ago only [1, 2]. In fact, Tissue engineering has originated from reconstructive surgery in which tissue from an allogenic donor is transplanted in order to repair functionality of a damaged tissue. Tissue transplantation comes with a number of difficulties such as graft rejection, insufficient donor organs, cause immune response and pathogen transmission [3-5]. These difficulties leaves patients waiting for the donor for long period of time and when they receive one, they have to take immunosuppressive medicines for rest of their life and still they are in a risk of needing another replacement organ within some years or sometimes within days after the surgery. Using an autogenic tissue transplant (patient's own cells) counteracts most of the limitations associated with the use of allogenic tissue for transplantation, it also avoids graft rejection and pathogen transmission. Additionally, patient do not have to depend on donors. Tissue engineering uses assembly of cells, materials and engineering methods, and suitable physicochemical and biochemical factors for replacing or to improve biological functions. Tissue engineering aspects are used nowadays as the support to regenerate or repair damaged or diseased organs [1]. Therefore, tissue engineering is a multidisciplinary field comprising of using life science and engineering application to gain understanding on structural and functional relationship between tissues, so that their biological substitutes can be designed that are able to maintain, restore and improve function of a tissue. So, the main objective of tissue engineering is thus to develop responsive living tissues with properties similar to those of the injured tissues that need to be replaced and the scaffold would be able to replace almost every tissue throughout the body such

as cartilage, blood vessels, bone, etc. [1]. For developing a functional biological substitute of tissues, the natural environment or the circumstances such as function, structure or environment of the specific tissue has to be understood primarily. Biological tissues are basically composed of signaling systems, cells and ECM (Extra Cellular Matrix). Cells are the core of tissue but can't work alone, they require both, ECM and signaling system for proper cell growth. Signaling system consist of transcriptional products of differentially active genes, which then urges cues for repair, regeneration and differentiation [4]. The ECM lies within extracellular space which supports cell attachment and proliferation. Hence, the biological substitute must mimic extracellular matrix in order to allow cell attachment and proliferation [6, 7].

. Tissue engineering involves three strategies:

(1) *Cell based therapies*; where cells that fulfils the required function are isolated from a donor and used for transplantation.
(2) *Induction of tissue formation by introducing soluble signaling factors*; involves delivering cell growth and proliferation inducing substances such as growth factors or differentiation factors.
(3) *Artificial ECM (scaffold) as biocompatible support;* involves growing cells on 3D (Three-Dimensional) matrix i.e. scaffolds, on which cell seeding is done *in vitro* or can be recruited from host tissues *in vivo.* Scaffold guided tissue engineering involves seeding cells onto a porous 3D scaffold that supports in vitro tissue formation and maturation. The resulting tissue is envisioned to be implanted in a patient where it further grows, going through self-repair remodeling.

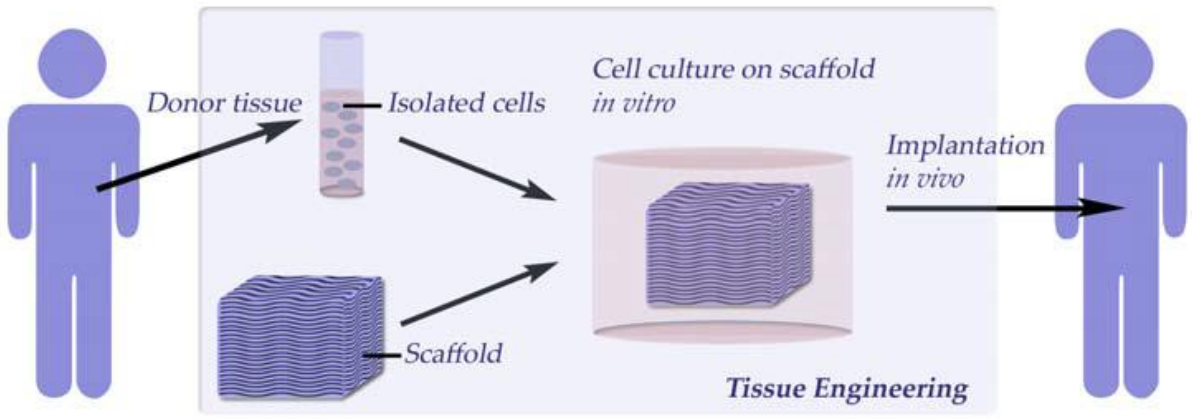


Figure 1: Schematic representation of tissue engineering principle.

Fig. 1 shows the basic principle of tissue engineering based on introduction of a biocompatible scaffold. Cells are isolated either from the patient (autogenic) or from donor (allogenic). After isolation of cells, these cells are cultured *in vitro* and then subsequently introduced to a scaffold. Then finally, these cell cultured scaffold or cell scaffold construct is implanted into the patient. Upon implantation scaffold gets degraded and dissolved slowly to promote the particular tissue regeneration. Isolation and *in vitro* culturing of cells require optimal environmental and processing conditions such as temperature, pH, and medium composition. Each tissue type require a distinct and different conditions, hence tissue engineering requires proper understanding of specific natural biological conditions and environment *in vivo* to allow optimization of cell culturing *in vitro.* Supplementing the culture with tissue inducing factors can be combined within scaffold based approach for guiding cell behavior by triggering specific reactions via pathway activation [8, 9, 10]. Another approach for guiding cell behavior lies within architectural design of scaffold. A scaffold should provide good and proper support for cell adhesion, differentiation and proliferate. Also, it must provide proper allowance for supply of nutrients to cells and elimination of waste from the cells [11, 12]. Design of a proper functioning tissue engineered construct requires optimizing these aspects for specific application. Therefore, current research in this area is driven towards the fabrication and characterization of scaffolds for tissue engineering applications.

1.2 Scaffolds

Scaffolds are the major components in tissue engineering field, in which scaffold function as the templates to allow cell to adhere and regenerate which ultimately promotes new tissue growth and provide temporary structural support. These scaffolds can be biocompatible which could mimic the ECM and upon implantation they would dissolved slowly to promote the specific tissue regeneration [13]. Followed by implantation the scaffold should be bio stable so that the tissue that is needed to be fabricated allows long term implantation [14]. The biodegradation of these scaffolds is carried out by the cell's own ECM proteins that favors remodeling and regeneration of the corresponding tissue without affecting the structural integrity of tissue. Scaffolds must have biocompatibility, optimum porosity [57, 58, 59], with appropriate mechanical strength, provide cell adhesion, large surface area to volume ratio, and when needed, releases growth factors [1, 2, 5]. These 3D scaffolds can be fabricated from synthetic and natural polymers. The selection of the polymer depends upon its application. Natural materials such as Chitosan [15], hyaluronic acid (HA) [16], and collagen [17] are advantageous in being non-toxic and provide appropriate cell adhesion and proliferation. But these natural materials also have some disadvantages of being less abundant, difficult to process and maintain, poor mechanical properties. Synthetic materials such as PVA (Poly Vinyl Alcohol), PLA (Poly Lactic Acid), PCL (Poly Capro Lactone) etc. are readily available and are found ubiquitously. Synthetic polymers are generally cheaper as compared to natural polymers and can be modified easily. But, these synthetic materials lacks bioactivity. Henceforth porous 3D scaffolds plays an important role in modifying cell function and regulation along with regulation of the new organ or tissue formation [18]. Bone and cartilage tissue regeneration requires a material that is supportive enough with high tensile strength which can support bone regeneration with its load bearing property. Materials such as PVA, PCL, PLA, HA etc. are used extensively in bone and cartilage tissue engineering.

Briefly an impeccable or ideal scaffold must comprise of following characteristics:

- It should allow cells to attach, proliferate and differentiate.
- It should be bio-compatible.
- It should be biodegradable and can gets easily eliminated out from the body.

- It must have appropriate porosity to provide enough space for cell attachment and homogeneous cell distribution to promote homogeneous formation of cells.
- It should contain appropriate mechanical strength, supportive physicochemical and biological properties.

There are numerous fabrication techniques to fabricate these synthetic or natural materials into a biological substitute. Each fabricating approach have significant impact on physicochemical properties of the scaffolds. Common methods that are used for scaffold fabrication includes solvent casting or porogen leaching, fiber bonding, freeze-drying, gas foaming, microsphere sintering, phase separation.

1.3 Scope and outline of the thesis

The scope of the thesis is to fabricate a novel PVA-Carrageenan biocompatible scaffold of various composition and to find out that which composition is best suited for implantation. The scaffolds were cryopreserved after seeding with cells, for evaluating their cell sustaining ability. The scaffolds was designed using freeze drying technique which includes drying the blends of two constituents through lyophillization. After fabricating the scaffold, its characterization is needed to be done, where its various mechanical and physicochemical properties including biocompatibility and hemocompatibility are evaluated. *In vivo* incubation of these scaffolds with cells shows cell adhesion on scaffold surface. After cell adhesion, these cell-scaffold constructs are cryopreserved for various time intervals and after various time intervals, the cell-scaffolds are thawed and cell viability along with scaffold integrity is evaluated to analyze that whether the cell scaffold construct is sustaining required parameters or not.

If the scaffold is able to maintain its integrity and cell viability, then these cell-scaffold construct can be used directly as a direct alternate implant for damaged tissue repair. It will minimize the time required for designing the scaffold at the time of treatment of a patient as these cryopreserved cell-scaffolds can be taken out and implanted immediately.

Chapter 2

Review of Literature

Tissue engineering (TE) aspects can be applied to improve numerous clinical situations such as those fractures that failed to heal i.e. non-union fractures, joint replacement, fusion of spinal bone, segmental defects occurred due to removal of a tumor etc. [13]. TE requires scaffolds that can act as an artificial extra cellular matrix. These scaffolds are advantageous because of their biocompatibility, bioavailability, mechanical and physicochemical properties, degradation rate and structure [1, 5, 4, 14]. All of these can be controlled precisely by composition and fabrication of the polymeric materials. Cells upon seeding promotes tissue growth, mineralization and remodeling. These scaffolds acts as ECM and along with the course of tissue growth, scaffold gets degraded and been absorbed by the body [14]. The design, development and functioning of the scaffold depends on the porosity of that scaffold that provide suitable mechanical strength and sufficient surface area to cells for attachment, proliferation, migration and differentiation [15]. During tissue regeneration, factors like recombinant signaling molecules, isolated cells and 3D matrices plays important role in healing process [16].

2.1 Tissue engineering approaches for bone regeneration

2.1.1 Bone tissue

Bone being highly mineralized tissue composed of highly mineralized extracellular matrix embedded with blood cells, bone cells and nerves. It is a dynamic tissue that undergoes remodeling with time as it is been constantly rebuilt and reabsorbed, after any injury [17-19]. Despite of self-regenerating ability after severe injury, bone cells in some cases are not able to regenerate and bone is not able to heal properly. These type of scenarios, where bone in not able to heal correctly and is not able to regain its mechanical function, are known as non-unions and hence these cases requires additional treatment for supporting the healing process.

Bone also suffers from some diseases, most of them take place due to an imbalance between formation and breakdown processes of bone resulting in local or systemic bone loss. These

diseases includes bone sarcoma, Paget's disease, hypercalcemia, hyperthyroidism and malignancy. This Paget's disease is a metastatic disease on bone that cause bone loss in rheumatoid arthritis, local deterioration of bone in jaws in periodontal disease, cause immobilization due to absence of any type of mechanical stimuli [22]. For overcoming these diseases, blocking of bone resorption in required, but it is not possible at all times. Bone tissue engineering, in such cases, provides a solution and hopes to the patients suffering from these diseases. Reviewing bone anatomy and revisit to concepts of bone repair and regeneration can help in these tissue engineering strategies for the regeneration of bone [23, 24]. Understanding mechanism involving bone formation, repair and regeneration can be the basis for developing new strategies for regeneration of bone tissues [25].

2.1.1.1 Overview of bone biology

Bone, a natural composite material consisting of 10% water, 30% collagenous matrix, 60% minerals and [26]. Bone provides at least four major functions in a human body that includes:

- Bone provides structural framework to human body, against which various organ maintain their positions in the body and against which muscles can contract in order to move the body [27].
- Bones or the skeleton system of body serve as a mineral reservoir (predominantly phosphate and calcium) [24, 27].
- Bones delivers guard to the internal most vital organs [24, 27].
- Bone provide a milieu or an environment via bone marrow for development of immune system [25].

To carry out these functions, bone gets continuously break down and regeneration [25]. Osteoclasts carry out resorption that dissolves the bone minerals and subsequently digests the bone matrix. Bone resorption, then takes place in osteoclastic cell membrane, in a specialized area called as the "ruffled border".

Bone contains three main bone specific cells i.e.

Osteoblasts: Single nuclei cell that synthesizes bone [26, 27]**.** Osteoblasts are cuboidal, plump cells, organized in layers and works in connection with other cells to make bone and matrix [20], alone it cannot synthesize whole bone rather the bone is made by the unit of cells called osteon. Osteoblasts secretes the bone matrix, which proceeds subsequently to mineralize extracellularly [20, 24, 26]. They are terminally differentiated products of mesenchymal stem cells which are secreted in response to an inducing effect. This differentiation is a stepwise process involving temporal expression of genes for osteoblast phenotype markers and the process is divided into three distinct periods:

1. Growth period or proliferation period
2. Matrix development period, and
3. Mineralization period

During these process, cells starts secreting characteristic products of osteoblast cells, that includes all constituents of ECM, such as Type 1 collagen (constitutes 90% of matrix) and non-collagenous proteins such as osteonectin, osteopontin, biglycan, bone sialoprotein, matrix gla proteins, osteocalcin and decorin [20, 27]. Osteoblasts are rich in Alkaline phosphatase enzyme that participates in mineralization process. Osteoblast cells deposits around 0.5 μm of matrix each day. Some osteoblast cells gets buried within matrix where they are known as osteocytes, other cells gets flattened on the surface of bone and are known as lining cells [28, 29].

Osteoblasts secrete very dense and rich cross linked collagen-ECM that enables mineralization. This ECM contains smaller quantities of osteocalcin and osteopontin.

Osteocytes: Found in mature bone, derived from osteoprogenitors and some of which gets differentiated into osteoblasts. Osteocytes resides in lacunae and their processes take place in canaliculi. They are responsible for signal transduction over long distances and hence communicates with other osteocytes, mechanical stress signals are sensed by them and sends signals for bone tissue growth and remodeling.

Osteoclasts:

Osteoclast is a type of bone tissue that resorbs bone tissue. They help in maintaining and repairing of bone. These are macrophage like cells that resorbs and degrade bone structure through various acidification and protease secretion processes.

Macroscopically, a fully developed mature skeleton consists of two type of bones:

- Compact or cortical bone (80%)
- Trabecular or cancellous bone (20%)

Cortical bone is anisotropic and is different from cancellous bone in its spatial orientation of its organic components and minerals, and by its characteristic position in skeleton. Cortical bone comprises outer tubular shells of long bones and outer surface of flat and small bones. Cortical bone is denser than cancellous bone and consists of osteons (parallel cylindrical units) or Harversian systems. Osteons determines the mechanical property of a bone [30].

Trabecular bone is less dense than compact bone and composed of an array of rodes and plates of bone tissue and forms open cell foam. It is also anisotropic and provides resistance to stress occurring in its particular location [31].

2.1.1.2 Formation and calcification of bone

Bone formation takes place through three processes:

- Endochondrial process,
- Intra-membranous process, and
- Appositional process

In intra-membranous, formation of bone takes place when progenitor of mesenchymal cell condenses to form clusters of cells and this cluster finally differentiate directly into osteoblasts, while in endochondrial process, the mesenchymal progenitor cells first differentiates to form cartilage which is later replaced by the bone [22, 27, 32]. Intramembranous ossification corresponds to the development of flat bones from skull and this process is also responsible for adding bone to the periosteal surfaces of the long bones. Endochondrial ossification is responsible

for forming vertebrae, long bones and repairing of fracture [22, 32]. Besides these processes, distinct embryonic lineages are also involved formation of different parts of skeleton.

Appositional formation occurs during bone remodeling and bone enlargement. In this process, osteoblast cells attaches to the existing bone and then secretes matrix, generally in layers. All the three type of formation occurs spontaneously and constantly and a particular type of bone can be synthesized through any combination of these various developmental schemes [33].

Independently of the process of bone formation, the collagen matrix secreted by osteoblast cells undergoes mineralization [33, 34]. Osteoblasts are entrapped in spaces known as lacunae and are separated by calcifying matrix. These entrapped osteoblast then loses its ability to produce matrix. These osteocytes communicate with other osteocytes via canaliculi (a long process), which gets organized before getting calcified. There is a time interval between matrix formation and its mineralization i.e. both process do not take place spontaneously, which is believed to occur due to separation between calcified bone (bone after calcification process) and the overlaying cells. Mineralization if matrix is a two-step process which corresponds to growth and nucleation of calcium phosphate crystals. The process of nucleation can be either heterogeneous or homogeneous [24, 33]. In homogeneous nucleation, supersaturation of local environment with appropriate ions results in the formation of crystal. The heterogeneous nucleation takes place only at the surface only, where interaction between ions and the surface lowers interfacial energy requirement which allows nucleation to proceed at concentrations much lower than that of supersaturation. After this process, amorphous calcium phosphate precipitates first, then converted to octacalcium phosphate and finally into hydroxyappetite [33]. As the woven bone is now formed and calcified, it gets remodeled to form mature lamellar bone [24]. Both woven as well as lamellar bone can be found, on a larger scale, either in cortical bone or in trabecular bone. Rate of generation of lamellar bone is much slower than that of woven bone and also the lamellar bone is less mineralized than woven bone [22, 24].

2.1.1.3 Importance of Mechanical environment on bone formation and regeneration

Bone has an enormous capacity to grow, regenerate and remodel itself. One of the major factor involved in these processes is the mechanical environment. Mechanical loading of physiologically relevant magnitudes have proved to directly initiate bone formation and modeling in animal

models [35, 36]. Although mechanical loading is not the only factor contributing these changes [37]. In contrast, lack of load on bones have shown to promote bone loss and tissue atrophy [35, 38]. In fact, during development and growth, skeleton optimizes its structure by various adaptations to thousands of those repetitive mechanical loads to which it is exposed almost daily. This mechanism of adaptation involves a multistep cellular mechanotransduction process, which includes:

(i) Mechanocoupling: Mechanical forces are converted into mechanical signals, such as shear stress by body fluids, which initiates a response by bone cells.
(ii) Biochemical coupling: A mechanical signal is transduced into biochemical response which involves various pathways within the cytoskeleton and cell membrane.
(iii) Cell-to- cell signaling: It involves signaling from the sensor cells (probably bone lining cells and osteocytes) to effector cells (i.e. osteoclasts and osteoblasts) using nitric oxides and prostaglandins as signaling molecules [39-41].
(iv) Effector response: Response can be either bone resorption or formation to cause appropriate structural or architectural changes. These changes tends to improve the bone structure and its mechanical environment [40].

2.1.1.4 Summary of bone Regeneration [42]:

Stem cell → osteoprogenitor cell → preosteoblast → osteoblast → resting → proliferation → matrix deposition → mineralization

Bone repair and regeneration is a symphony of cellular activity that begins with an acute inflammatory response, then infiltration of granular tissue followed by osteogenic cell recruitment, proliferation and differentiation, mineralization and formation of matrix and then remodeling. This process takes place on virtue of mechanical and biological signals. Biologically, expression of growth factors, cytokines and hormones leads to regulation of repair mechanism by conditioning the wound and directly influencing cell migration to the site of action, proliferation and differentiation [43, 44]. Similarly, mechanical forces transmitted via ECM being assembled, influences the patterning or architectural changes of this cellularly orchestrated activity. On a general prospective, success of a bone repair and regeneration will depend upon the generation of

necessary signaling cascades, viability and availability of progenitor cells, nutrition availability and proper mechanical environment.

2.2 Tissue Engineering strategies:

Conventional approaches for bone repair involves biological grafts such as autografts, allografts and xenografts. Autografts are most commonly used in clinical settings as they do not show immune or graft rejection or disease transfer [45, 46]. However, limited availability of autografts have fueled the continuous growing interest in development of alternate methods to repair bones based on tissue engineering strategies [47, 48].

As stated above, ultimate goal of tissue engineering is to repair, replace or enhance biological function of absent, damaged or dysfunctional elements of an organ or tissue. This goal can be accomplished by using cells which are manipulated through their extracellular environment in order to develop and generate engineered tissues that can serve as biological substitute for lacking or missing tissues [49]. Various strategies can be used for developing engineered tissue. For selecting the best strategy for developing hybrid materials for regenerating a specific tissue defect several factors have to be considered, such as host-graft interaction, technical feasibility and properties required for implant [49].

Basically three different strategies have been adapted for creating a new tissue [1, 50-53] (represented schematically in Figure 2.1). These are:

2.2.1 Cell self-assembly

This approach corresponds to direct *in vivo* implantation of cell substitutes or isolated cells. This approach is based on synthesis of their own matrix by the cells. This approach inhibits complications of surgery and allows replacement of only those cells that permits manipulation of the cells before infusion and supplies the required function. The limitations to this approach include infused cell failure in maintaining its function in recipient and immune rejection.

2.2.2 Acellular scaffold

Basis of this approach is to directly implant biomaterials *in vivo* and cell from the body acquire support with the implanted biomaterial and attaches and proliferates or differentiates. In this

approach, Scaffold or the matrix is loaded with therapeutic agents or growth factors. This approach solves the problem related to cell sourcing but success through this approach depends upon infiltration and recruitment of appropriate cell types from the body to populate the construct and hence a proper tissue repair. In case of bone, this 3D process depends on surface properties of implant, its porosity, its 3D structure and its mechanism and rate of degradation. These properties of materials can enhance cell adhesion, relocation and distribution of the cells that are responsible for response of bone healing. When these constructs are implanted adjacent or into bone, cells from the surrounding tissues moves into the available voids or pores of the matrix. At those voids of scaffolds, bone cells differentiate and proliferate which latter forms bone.

2.2.3 Cell seeded scaffolds

The scaffold in this approach provides an adhesive substrate for implanted cells and provides physical support to organize new tissue formation [54]. Cells to be transplanted adhere to the surface of scaffold, proliferate, differentiate and form its own ECM and hence stimulate new tissue formation. During the process of tissue formation, scaffold gradually degrades and eventually gets eliminated from the body.

This is considered as classic tissue engineering approach and is studied most for bone regeneration because, as most of the bone healing processes requires a sufficient amount of osteoblastic cells which is limited in most of the clinical settings such as sites with large bone defects. There are also some systemic conditions that limit the function or number of progenitor cells. In these cases, the implantation of isolated cells or osteoconductive material either alone or loaded with osteoinductive growth factors would not be enough to develop an optimal and reliable bone healing response [55].

Therefore, the key to successfully repair or regenerate bone is to provide the site with ample amount of osteogenic progenitor cells attached to a suitable matrix or scaffold which will ensure osteoblastic differentiation. Interfering tissue must be excluded out, vascular penetrance must be enhanced and appropriate mechanical cues must be engineered for proper working of the scaffold. Once proper information about reactivity and identity of all the triggering factors is gathered, massive bone regeneration can be carried out by combination of scaffolds and those instructional agents [56].

The last approach is the basis of the experimental work done in this thesis, which corresponds to developing a scaffold, characterizing its morphology and physicochemical properties and in vitro culturing of osteoblast cells on these scaffolds to make a cell-scaffold construct.

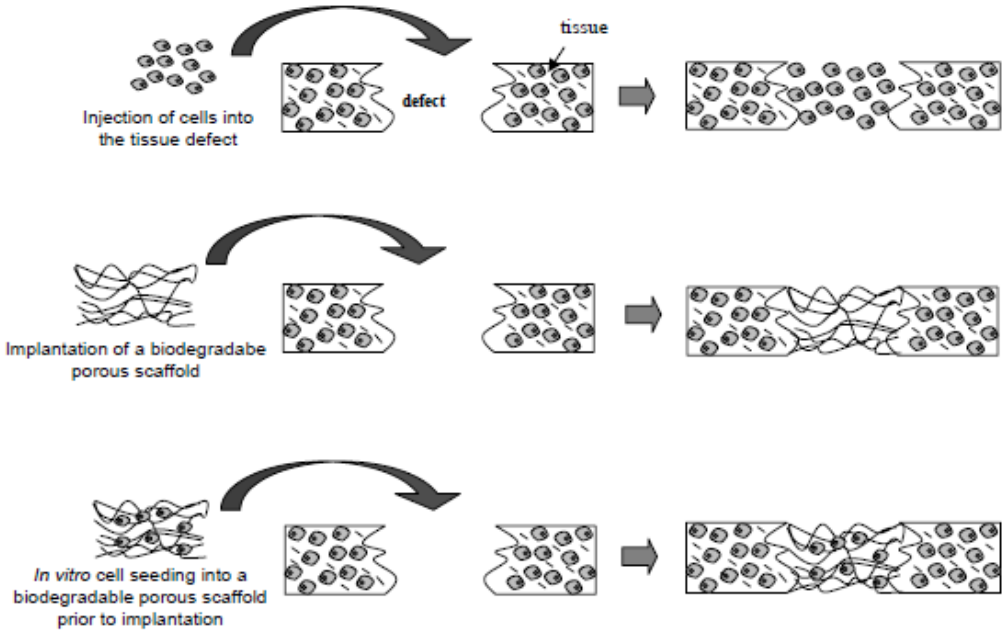


Figure 2.1: Schematic representation of the three tissue engineering approaches a) Cell self-assembly; where the cells are directly implanted into the repair site b) acellular scaffold; where a porous scaffold is implanted into repair site which allows cell to migrate in and occupy voids and then proliferate c) cell-seeded polymeric scaffold; where a scaffold seeded with cells is implanted in the repair site

2.3 Scaffolds for bone tissue engineering: Design and Processing

2.3.1 Requirements of an ideal scaffold

The requirement of a scaffold material to be considered ideal are very complex and depends mainly upon the tissue to be repaired or restored and on the site and size of the damage to be treated.

Scaffolds are the major components in tissue engineering field, in which scaffold function as the templates to allow cell attachment, regeneration which ultimately promotes new tissue growth and provide temporary structural support. These scaffolds are biocompatible and mimic the ECM and upon implantation they gets degraded and dissolved slowly to promote the particular tissue regeneration [17]. Followed by implantation the scaffold should be stable, biologically, so that the tissue that is needed to be fabricated allows long term implantation [18]. The biodegradation of these scaffolds is carried out by the cell's own ECM proteins that favors remodeling and regeneration of the corresponding tissue without affecting the structural integrity of tissue. Scaffolds must have several characteristics like biocompatibility, optimal porosity, appropriate mechanical strength, and large surface to volume ratio, ability to provide cell binding, and when needed, release growth factors.

The key characteristics that a scaffold must possess includes:

(i) *Biocompatibility:* A scaffold should be biocompatible in both implanted as well as degraded form. The products of scaffold must not invoke any adverse effect on the body or surrounding tissue and must not invoke any immune response. It should be non-toxic to the body.

(ii) *Appropriate mechanical properties*: As bone is a hard tissue, it requires a scaffold with an appropriate load bearing properties. It should provide correct and appropriate stress environment for the developing tissue as stress has proved to induce bone formation.

(iii) *Degradation rate*: The scaffold must possess controlled and adjustable degradation rates because each tissue regenerates at different rate and hence the degradation rate of the scaffold must match the regeneration rate of the tissue.

(iv) *Pore size and morphology*: The scaffold must contain appropriate pore size, porosity and pore structure, as these factors directly influence nutrient supply to transplanted cells. Pore size must be greater than the diameter of the cell (10μm typically). Various authors describe

various pore sizes as the optimal pore sizes for maximal tissue growth, some say it has to be from 200 µm to 400 µm [57], some say it should be from 100 µm -150 µm [58], while some others authors say pore size should be from 100 µm to 350 µm [59]. Due to lack of consensus in pore size it is generally accepted that for optimal growth, it is dependent on the type of the tissue to be regenerated. There has to be interconnectivity between the pores as interconnected pores helps in enhancing diffusion rates of nutrition, oxygen and waste removal as compared to isolated pores.

(v) *Surface Chemistry*: The Scaffold must have an appropriate surface chemistry in order to allow cell adhesion, migration, proliferation and differentiation. Cell adhesion to the surface is the must step, as most of the cell types are anchorage dependent and they perform their cellular functions only after adhesion. Hence, surface chemistry of the scaffolds directly plays an important role in cell attachment [60, 61]. It is not desirable that biomaterials with appropriate bulk properties will allow cell adhesion. So, they requires surface modifications for example, coating, roughening, grafting and blending [60, 61].

(vi) *Easily sterilized*: The scaffold must get sterilized easily without using heavy or long protocols. For example, either by ethylene oxide vapor or by exposure to high temperature or by gamma irradiation. The scaffold must remain unaffected by these techniques.

(vii) *Processing into various 3-D shapes*: The scaffold should be flexible enough that it can be processed into various three dimensional shapes with irregular geometry and must maintain its shape even after implantation.

2.3.2 Scaffolding materials or Biomaterials used for fabrication for constructing scaffolds:

In Scaffold based tissue engineering approach, a suitable scaffold is required to act as anchorage for anchorage dependent cells. Hence, the production and design of a suitable scaffold comes in first place, then the appropriate type of cells that has to be seeded and cultured over the scaffold, prior to implantation. Hence, selecting a suitable raw material is of primary consideration. There is a distinct variety of biomaterials that are biocompatible and can be used to fabricate a scaffold. These biomaterials can be polymers, ceramics or polymers. As metals are not biodegradable, they can be excluded out from being used as raw material for scaffold fabrication. Although, in some orthopedic applications some ceramics such as such as sea coral and tri-calcium phosphate have been used successfully. But they have some limitations such as less brittle which makes it hard to process into porous scaffolds. On the other hand, polymers are flexible and can be processed into any shape. There is a large range of biodegradable polymers available that can be used as scaffolding material. These polymers can be natural or can be synthetic based on their origin.

Different types of polymers that can be used in tissue engineering field are described below:

2.3.2.1 Synthetic Polymers

2.3.2.1a. Poly (lactic Acid) (PLA)

PLA is synthesized by cyclic dimer of isomers of lactic acid i.e. D and L Lactic acid. Instead of poly acid according to IUPAC nomenclature, PLA is polyester. Due to its bio-compatibility, biodegradability, high tensile strength and good mechanical properties it has been used widely in various biomedical applications. PLA can also act as a thermoplastic elastomer which can be stretched up to 300%. It is a biodegradable polymer that is derived from renewable resources like corn starch or sugarcane. PLA can be synthesized through ring opening polymerization or by direct condensation of lactide. PLA can be processed by injection molding, extrusion, sheet and film casting.

PLA degrades by bulk hydrolysis and leads to the production of lactic acid. In case of PLLA, degradation results in L (+) lactic acid, a substance that exists in the human body under natural circumstances as well, therefore PLLA is generally preferred over PDLA [62]. The body transports

the produced L (+) lactic acid to the liver, converts it into pyruvic acid and upon entering the tricarboxylic acid cycle, secreting it as water and carbon dioxide [63].

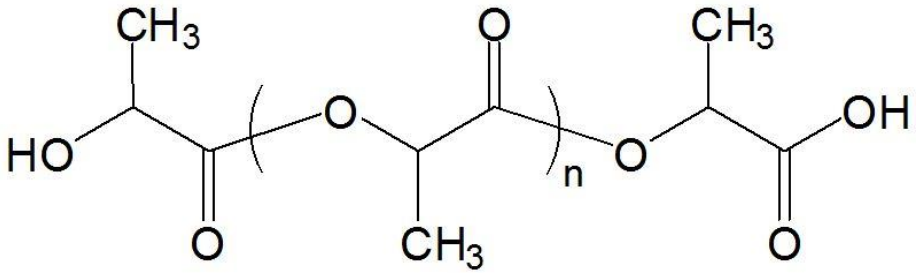


Figure 2.2: Molecular structure of Poly (lactic acid).

Properties:

Crystallinity	37%
Glass transition temperature	60-65^{o}C
Melting temperature	173-178^{o}C
Tensile Strength	2.7-16 Gpa
Degradation time	12-18 weeks
Solvents	Benzene, tetrahydrofuran, dioxane, chloroform, methanol, ethanol, methylene chloride, acetone.

This material is highly tensile, have high modulus hence is more suitable for load bearing applications like orthopedic fixations.

2.3.2.1b. Poly (L-glycolic acid) (PLGA)

Crystallinity	45-55%
Glass transition temperature	35-40^{o}C
Melting temperature	225-230^{o}C
Tensile Strength	7 Gpa
Degradation time	6-12 weeks
Solvents	Hexafluoroisopropanol (HFIP), hexafluoroacetone sesquihydrate,

PGA is an aliphatic, biodegradable, thermoplastic polyester that can be synthesized by ring opening polymerization or polycondensation of glycolic acid. Due to its biocompatibility, hydrolytic instability, good mechanical strength, PGA along with its copolymer poly (lactic-co-glycolic acid), poly (glycolide-co-caprolactone) are used in biomedical applications. PGA is used mostly in making sutures.

Polyglycolide has an ester linkage in its which corresponds to its hydrolytic instability. Degradation process takes place in two steps which polymer is converted to its monomeric form i.e. glycolic acid. First, water get diffused to the amorphous regions of polymer, cleaving ester bonds. Second step starts after the erosion of the amorphous regions which leaves the crystalline portion of polymer available for a hydrolytic attack. Upon collapse of these crystalline regions, polymer chain gets dissolved

Upon exposure to physiological conditions, PGA is degraded by random hydrolysis easily and apparently it can be broken down by enzymes having esterase activity. The degradation product i.e. glycolic acid is proved to be nontoxic, and it can enter TCA or Tri-Carboxylic Acid cycle after which glycolic acid is metabolized and secreted as carbon dioxide and water. A part of glycolic acid gets excreted also through urine [64] .Studies suggested that PGA made sutures have lost half of the material loss and strength after two weeks and gets completely degraded after four weeks.

Poly glycolic acid takes four to six weeks to get completely resorbed [65]. Under in vivo conditions degradation is faster as compared to in vitro conditions, this phenomenon may be due to the cellular enzymatic activity [66].

Techniques such as injection, extrusion and compression molding can be used to process poly glycolic acid. PGA was also investigated for development of bone fixation device (Biofix) [67, 68].

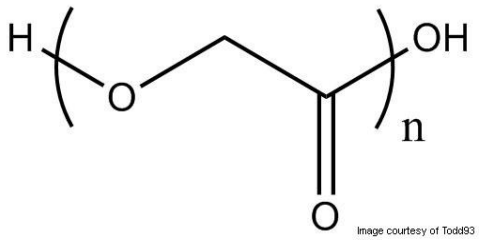


Figure 2.3: Molecular Structure of Poly (glycolic acid)

2.3.2.1c. Poly (Vinyl Alcohol) (PVA)

Poly vinyl alcohol is a synthetic polymer which is soluble in water. It has a great biocompatibility and biodegradability along with appropriate mechanical strength. Poly vinyl alcohol is used as a base material to provide support the fabrication process due to its properties such as biocompatibility, non-toxicity, water solubility and biodegradability [69]. PVA is used in various biomedical and pharmaceutical applications such as drug delivery devices [70], contact lenses [71], orthopedic devices, wound dressing [72] and artificial organs [73]. PVA scaffolds can be used to develop an in vitro model for breast cancer bone metastasis [74].

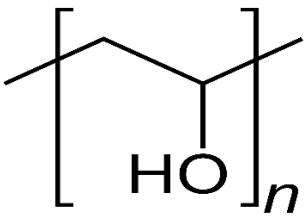


Figure 2.4: Molecular structure of Poly (vinyl alcohol)

2.3.2.1d. Poly (caprolactone)

Glass transition temperature -60^{o}C

Melting temperature 60^{o}C

Degradation time >24 weeks

Solvents Tetrahydrofuran

PCL is a biodegradable polyester and can be synthesized by ring opening polymerization (ROP) of ε-caprolactone using some catalyst such as stannous octoate [75]. PCL can be degraded hydrolysis of esters under physiological conditions and hence can be used in biomedical applications. PCL has been approved for use by the FDA since the 1970s and hence PCL has been used more and more by tissue engineers for making scaffolds for tissue repair therapies.

Bulk hydrolysis breaks the ester linkage, which creates fragmentation and the release of oligomeric species. Low molecular-weight fragments are eventually engrossed by giant cells and macrophages. The byproduct e-hydroxycaproic acid is either metabolized via the tricarboxylic acid (TCA) cycle or removed by direct renal secretion. Poly caprolactone has a very slow rate of degradation which is one of its attractive qualities i.e. it loses 50% of its strength in 8 weeks [76].

PCL has also been blended with higher amounts of starch to increase its degradation rate. With higher starch content, PCL is more susceptible to enzymatic degradation by proteinase K49 [77]. PCL has been blended with several other polymers, including starch, PEO, PLA, and PGA in the past and has been used in many studies relating to biomedical applications.

PCL is used in tissue engineering because of its biocompatibility and desired mechanical qualities which ensures its long term presence and elasticity.

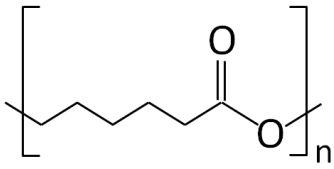


Figure 2.5: Molecular Structure of Poly (ε-caprolactone)

2.3.2.2 Natural polymers

In context of bone and cartilage tissue engineering, the polymer should have an excellent biocompatibility, good tensile strength and good mechanical properties. Polymers of Albumin, hyaluronic acid, chitosan, collagen, agarose, fibrin and alginate derived materials satisfies all the characteristics of an ideal polymer that can be used for bone and cartilage tissue engineering. Natural polymers acts intrinsically as a good template for the attachment of cells. This ligand site representation provides susceptibility to cell-triggered proteolytic degradation and natural remodeling. The inherent bioactivity of these natural polymers has its own downsides. These include a strong immunogenic response associated with most of the polymers, the complexities associated with their purification and the possibility of disease transmission [78].

Much of the interest in these natural polymers comes from their biocompatibility, relative abundance and commercial availability, and ease of processing. The degradation rate of natural polymers varies a lot from patient to patient because concentration of enzymes that degrades those polymers varies from patient to patient.

2.3.2.2a. Collagen

Collagen is one of the main component of Extra Cellular Matrix and is the main structural protein of tissues, mostly connective tissues, in case of animals hence is the most abundant protein in mammals. Collagen is being used widely for various tissue regeneration aspects especially in regeneration of soft tissues. It provides proper cell adhesion and favors cellular recognition for regulation of cell function and attachment. In body, collagen gets enzymatically degraded via body enzymes such as collagenases yielding its corresponding amino acids [79]. The degradation rate of collagen can be modified by altering various treatments. Collagen scaffolds have excellent biocompatibility and porosity which is used for accelerated tissue reproduction.

Collagen is biocompatible, have great mechanical properties, good chemical properties and have good biological properties which makes collagen suitable for application in the field of tissue engineering. Due to its modifiable properties collagen can be processed to form sheets, Nano fibrous powders, tubes, injectable viscous solution. These collagen based scaffolds are used

nowadays in treatment of ulcer and diabetic wounds [80]. The composite of collagen, Tri calcium phosphate (TCP) and Hydroxyapatite is widely used as synthetic graft for bone replacement.

Besides of these advantages collagen also have some disadvantages such as its expensiveness, risk of infection, variable degradation and chemical properties and difficulty in its processing and handling [81].

2.3.2.2b. Fibrin

Fibrin is a 3,62,000 Dalton also known as factor Ia, is a fibrous, non-globular protein which is mainly included in blood clotting, formed by proteolytic degradation of fibrinogen by thrombin, which latter gets polymerized to form clot. Fibrin is extremely bio-compatible and injectable but has a disadvantage of poor mechanical strength which is required mainly for bone and cartilage tissue engineering.

A fibrin scaffold can be used to differentiate and transplant mesenchymal cells for neural cells regeneration that can be used and delivered during injured spinal cord engineering [82, 83].

2.3.2.2c. Albumin

This protein is mostly found in abundant in blood plasma. It is member of globular proteins and are water soluble. Albumin is highly biocompatible, blood compatible and human body is capable of degrading albumin, hence it can be used for drug delivery [84]. Scaffold grafts containing Albumin can be used for promoting neocartilage formation [85].

2.3.2.2d. Hyaluronic acid

Hyaluronic acid is an anionic glycosaminoglycan, linear polysaccharide made of repeating alternate units of glucuronic acid and N-acetyl-D-glucosamine. It is a non-sulfated polysaccharide synthesized in plasma membrane instead of Golgi. Its size can be very large often about millions [86]. HA cushions, nerves, joints, fills the eye and hydrates skin and hair. It can be used to treat osteoarthritis of knee by injecting it in the joints but it also have severe adverse effects [87]. Hyaluronic acid hydrogels are proved to help in controlled release of Bone morphogenic protein-2 (BMP-2) that helps in bone and cartilage tissue regeneration [88]. Hyaluronic acid composite with collagen can be used for vascular tissue engineering [89]. Hyaluronic acid is immunoneutral

and promotes tissue repair by epithelial and mesenchymal cell migration, differentiation and also enhances deposition of collagen and supports angiogenesis. Hyaluronic acid can be used also in case of irregular shape defects and can be implanted with minimum immunogenic response. Besides these properties, it has limited application and constrained mechanical properties.

2.3.2.2e. Chitosan

Chitosan is an anionic linear polysaccharide made up of randomly distributed N-acetylglucosamine and β (1-4) linked D-glucosamine. Chitosan is derived from chitin and is made by treating crustaceans like shrimp with sodium hydroxide. Chitosan is soluble in organic solvents such as acetic acid. Solubility of chitosan is highly dependent on its degree of deacetylation. Higher the deacetylation degree, higher will be the rate of solubilization. In vivo, chitosan is highly biocompatible. Chitosan scaffolds have enough mechanical properties and porosity which makes it a suitable polymeric biomaterial for load bearing applications like bone and cartilage tissue engineering. The porosity of chitosan scaffolds can be modified and controlled which directly affects the mechanical properties of scaffolds [90].

2.3.2.2f. Chondroitin sulfate

Chondroitin sulfate is an anionic glycosaminoglycan, linear polysaccharide made of repeating alternate units of glucuronic acid and N-acetylgalactosamine. It is a nonsulfated polysaccharide. It is one of the major component of cartilage and helps in providing resistance to compression [91]. Composite of CS along with glucosamine is used widely used for treatment of osteoarthritis. Studies have showed that chondroitin sulfate scaffolds along with other biomaterials can be used for repair of cartilage tissues.

2.3.2.2g. Alginic acid or Alginate

Alginate is a natural non branched polymer polysaccharide of non-human origin, it is found in cell walls of brown algae and is capable of absorbing water 200-300 times more than its own weight. It is made up of L-glucuronic acid and D-mannuronic acid. It is also highly biocompatible. It can be used as a cell transplantation vehicle and also for wound dressings. Beside of its slow degradability and non-toxic nature it lacks in sufficient mechanical integrity, hence can’t be used for long term implantation.

2.3.2.2h. Gelatin

Gelatin is a natural, flavorless food stuff which is derived from the collagen obtained from animal by-products. It gets easily dissolved in hot water and becomes gel upon cooling. Gelatin is highly biocompatible material, with appropriate biodegradability and almost same biological properties as that of collagen [92]. Gelatin also contains integrin binding sites for cell attachment and differentiation [93]. But it has a disadvantage of weak mechanical properties and its rapid degeneration rate.

Hence Gelatin is needed to be used with other biomaterial as a blend or composite which can provide appropriate mechanical strength and can be used for bone tissue regeneration.

2.3.3 Techniques for fabrication of porous polymeric scaffolds:

A scaffold designed by fabricating from polymers with appropriate properties is necessary in order to meet all of the necessary requirements. But, designing a scaffold is not an easy task, one must possess deep knowledge about all the biomaterials. This knowledge helps in determining the knowledge about those features that may likely to be interfering with cell-scaffold interactions. Selection of the most adequate tissue engineering strategy will help in determining the most appropriate scaffold design. This also helps in determining the required properties, which should be able to induce the needed tissue response [49, 94]. Three-dimensional porous structure has proved to be the most appropriate design to provide cell attachment ant proliferation. Although, other designs and combinations also play an important role in tissue engineering [95].

As described before, a scaffold, besides of biodegradability and biocompatibility must possess some other properties too. Such as, adequate mechanical properties, suitable porosity, suitable pore size, suitable degradation rate, appropriate surface chemistry and interconnectivity between the pores. A scaffold must provide site for cell attachment, structural guidance and mechanical stability upon implantation. It must provide appropriate interface to respond to biological and physiological changes in order to acclimatize with the surrounding native tissue. So, taking these requisites into consideration, the processing technology is expected to provide maximum control over micro and macro structural properties of scaffold without affecting the properties of scaffold adversely. Another important requirement of the process of fabricating scaffold includes accuracy and reproducibility, i.e. the method or the process used should produce scaffolds with steady properties and physical forms when the same processing parameters are used.

There has been a wide variety of fabricating techniques introduced in the past years that helps in developing matrices which will acts as templates for the cell to attach and proliferate. These methodologies usually involves either solvent casting or melting processing. Solvent casting depends upon the solubility of polymer in various organic solvents. And, melt processing involves heating a polymer above its melting temperature(Tm) or glass transition temperature (Tg). Among these processes, methodologies includes particulate leaching and solvent casting [96], fiber bonding [97], phase separation [68, 98], Solid free-form fabrication, emulsion freeze-drying, gas foaming [99], Electrosppinning and 3D plotting technique [100].

These methodologies developed so far have showed a high level of success. But they also have some of the limitations [101].

(i) Manual intervention: Most of the available techniques are based on manually based protocols and are impossible to transform them in industrial scale up methods. Hence, the processes are time consuming and labor intensive. Additionally, the processes are dependent on the skills of user. Also, reproducibility of result is very challenging to achieve.
(ii) Harmful: Some processes like polymer casting requires usage of toxic solvents that can be harmful as they release and withhold harmful residues.
(iii) Use of porogen: some processes uses porogen, but these porogen can be harmful for the contacting cells and have a cytotoxic effect against them. Furthermore, clusters of porogen may lead to inappropriate pore size and porosity.

Following section will define various fabricating methods used to design and fabricate polymers into scaffolds. Those methods are:

- Solid free-form fabrication, emulsion freeze-drying
- Porogen leaching
- Fiber bonding
- Gas foaming
- Electro spinning, sintering, phase separation
- 3D-plotting technique or a mixture of these techniques

2.3.3a Emulsion Freeze Drying:

This technique is based on immiscibility, like with water and oil, among some solvents, which can be called solvent non-solvent pairs. In short, the polymer is dissolved into a solvent and then a non-solvent is added. The solution is then mixed well to form an emulsion mixture. This mixture is poured into a mold and quenched under low temperature or by using liquid nitrogen. The frozen mixture undergoes a freeze-drying process to remove both the solvent and non-solvent. The advantages of this process are that greater than 90% porosity with pore sizes ranging from 15 — 200μm can be obtained. The pores are highly interconnected which is good for nutrient supply,

metabolic waste clearance, cellular ingrowth, and vascularization. However, this technique is user-, equipment-, and technique sensitive and the processing parameters have to be well controlled.

2.3.3b Porogen leaching:

Also known as solution casting/particulate leaching, is a widely used, facile, convenient, and cost-effective method that can be applied with a wide range of polymers to introduce porosity into tissue engineering scaffolds. This method involves the casting of a polymer/porogen solution followed by solvent evaporation and removal of the incorporated porogen through aqueous washing methods. Various porogen, including sodium chloride (NaCl), paraffin spheres, sugar crystals, have been successfully used to fabricate porous structures. NaCl is perhaps the most widely used porogen, but the wide variations of pore sizes, lack of interconnectivity, irregular pore geometry, and the inability to produce submicron and nanoscale pore sizes have limited the use of this porogen in current tissue engineering applications. Therefore, a convenient, cost-effective pore creation technology to fabricate scaffolds with controlled architectures and that can be applied to a wide range of biomaterials would be a valuable tool for the field of tissue engineering.

2.3.3c Electrospinning:

Electrospinning uses an electrical charge to draw very fine (typically on the micro or nano scale) fibers from a viscous solution with enough surface tension and viscosity to make the Electrospinning machine to draw fibers from the liquid. Electrospinning shares properties and characteristics of both electro spraying and conventional solution dry spinning of fibers. This process does not require the use of coagulation chemistry or high temperatures to produce solid threads from solution. So, it makes the electrospinning particularly suited to the production of fibers using large and complex molecules. When a sufficiently high voltage is applied to a liquid droplet, liquid body becomes charged. Due to this charged body surface tension is counteracted by the electrostatic repulsion which leads to stretching of droplet; at a critical point a stream of liquid erupts from the surface. This point of eruption is known as the Taylor cone. If the molecular cohesion or viscosity of the liquid is sufficiently high, stream breakup does not occur (if it does, droplets are electro sprayed) and a charged liquid jet is formed.

As the jet dries in flight, the mode of current flow changes from ohmic to convective as the charge migrates to the surface of the fiber. The liquid jet from Taylor cone is then elongated by a whipping process caused by electrostatic repulsion initiated at small bends in the fiber, until it is finally deposited on the collector. This bending instability aroused due to elongation and thinning of the fiber, this leads to the formation of uniform fibers with nanometer-scale diameters.

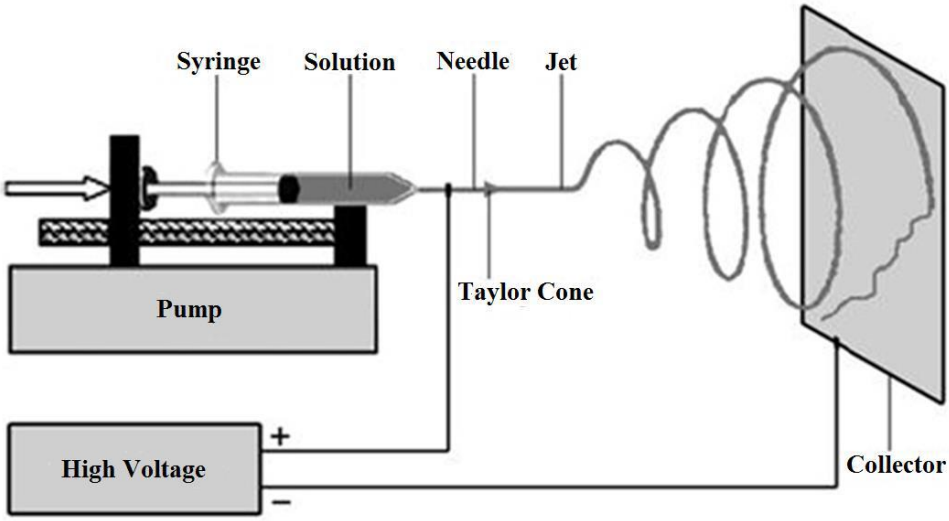


Figure 2.6: Schematic representation of Electrospinning setup

2.3.3d Gas foaming

Generally, this method uses soluble inert gases such as N_2 or CO_2 as a blowing agent to create pores in polymers through pressure quenching [102, 103]. Variation in processing conditions helps in tuning properties of scaffold. Using this method, a composite of biomaterials to fabricate scaffolds for hard tissue engineering. This process in beneficial as it do not require solvent, it eliminates the risk of left over residues and as it is processed at low temperature, it prevents polymer degradation due to high temperatures during processing. The scaffolds produced through foaming have a closed surface and has non percolated pores generally, which is a limitation to this process, limits nutrient transport through scaffold. It may be possible to fabricate scaffolds with open process through foaming, but their pore size are too small to be used in tissue engineering applications. Some post processing steps can be followed to introduce interconnected pores, such as, pulsed ultrasound or pulse treatment to break walls of non-percolated pores [103].

2.3.3e Sintering

Sintering process refers heat treatment of powder to make adhere with each other. This process has applications of fabricating scaffolds for hard tissue engineering. This method mainly uses ceramic powders, but also applicable for other materials like glasses, metals and ceramic particles. This process has advantage of providing controlled and graded porosity but has disadvantage that it creates risk of low interconnectivity between pores [104].

Objectives

1. Fabrication of PVA-Carrageenan based three-dimensional scaffolds via freeze drying technique.
2. Characterization of prepared scaffolds through
    - Microscopy
    - Scanning Electron Microscopy (SEM)
    - X-Ray Powder Diffraction Studies
    - Mechanical Studies
    - Swelling studies
    - Hemocompatibility
3. *In vitro* studies
        - Cell attachment through bright field microscopy
        - Proliferation studies through Florescence Microscopy
        - Cryopreservation of cell seeded scaffolds
        - Cell viability of the cryopreserved scaffolds through MTT assay

Chapter 3

Materials and Methods

3.1 Fabrication of porous scaffolds

3.1.1 Preparation of PVA: Carrageenan Scaffolds

10% w/v Polyvinyl Alcohol (PVA) (Sigma Aldrich) was prepared by dissolving the weighed amount of PVA in distilled water on a magnetic stirrer (REMI, Mumbai) at 100 rpm. And, 2% w/v Carrageenan (Sigma Aldrich) was prepared by dissolving weighed amount of carrageenan in distilled water at 150 rpm at 45ºC. After dissolving, PVA and Carrageenan were mixed at various PVA: Carrageenan ratios (w/w) i.e. 9:1, 8:2, 7:3, 6:4 and 5:5. The mixture was kept on magnetic stirrer at 150 rpm for 25 minutes to make their blends.

3.1.2 Processing of PVA: Carrageenan blends to make composite porous scaffolds

Processing of the blends were done by following the procedure reported by Nair et al [105]. Briefly, the blends were kept for bath sonication for 1 hour. The slurry was poured in molds and freezed in -20ºC for 24 hours. After 24 hours, molds were removed from deep freezer and kept for lyophillization for 24 hours. After lyophillization the samples were soaked in 1% gluteraldehyde for 24 hours and then washed four to five times with double distilled water. The scaffolds are ready for further characterization.

3.2 Characterization

3.2.1 Mechanical testing

Mechanical testing provides information about the suitability of a material for its intended application to help companies design reliable products that will perform as expected. Mechanical testing services measure materials under various temperature, tension, compression and load conditions to determine:

Strength
Hardness
Ductility
Impact resistance
Fracture toughness
Elongation
Stress
Mechanical Test Ranges

Elevated temperature tensile testing of metal parts and specimens in all sizes, from the smallest fasteners to huge tubing and bolts on our 600,000 lb. capacity machines. A 10,000 lb. capacity tensile machine provides plastics testing Elevated temperature tensile testing using a furnace carousel to process up to three samples at once. Samples can be heated to 1800°F Stress rupture and creep testing comply with ASTM standards and can be performed at temperatures up to 2000°F Fracture toughness and fatigue testing with equipment that can generate up to 55,000 lbs. of tensile or compressive force and controls the test temperature between -250°F and +400°FRockwell, Brinell & Superficial hardness testing is available for metals, while a Shore Durometer hardness tester provides plastics testing Charpy impact testing is performed from -452°F to 500°F.

3.2.2 Morphology of scaffold via Scanning Electron Microscope (SEM)

The morphology of the scaffolds were investigated by scanning electron microscopy (Jeol 6480LV JSM Microscope). In preparatory step, samples for SEM analysis were prepared by cutting scaffolds into a thickness of about 1 to 2 mm and then analyzed in Scanning electron microscope after being coated with gold using gold sputter module in a higher vacuum evaporator. Observations were taken under different magnifications at 15Kv.

3.2.3 Chemical composition analysis of the composite scaffolds by Attenuated Total Reflection Fourier Transform Infrared Microscopy (ATR-FTIR)

The Attenuated Total Reflection Fourier Transform Infrared (ATR- FTIR) spectroscopy analysis was conducted to corroborate the possible interaction of PVA and Carrageenan for the fabrication of scaffolds. The ATR- FTIR was performed on a Bruker ALPHA spectrophotometer (Ettlinger,

Germany) with a resolution of 4 cm-1. The samples were scanned in the spectral region between 4000 and 500 cm-1 by taking an average of 25 scans per sample. The prepared samples were cut in small and thick pieces approx. 1mm and kept on the sample holder and the samples were scanned and the result obtained was analyzed through OPUS software.

3.2.4 X-Ray Powder Diffraction studies (XRD)

The X-ray powder diffraction (XRD) patterns of PVA, Carrageenan and PVA: Carrageenan scaffolds were obtained using X-ray diffractometer (PANalytical X'Pert, Almelo, The Netherlands) equipped with Ni filter and Cu Kα (l = 1.54056 Å) radiation source. The diffraction angle was varied in the range of 10-80 degrees while the scanning rate was 5 degree/s.

3.2.5 Swelling Studies

Swelling studies were done to check the water absorbing capacity of the scaffolds. The procedure is as follows:

(i) A thin slice of scaffold weighing 0.5 grams was cut.

(ii) Then, the piece of scaffold was poured into the PBS solution of pH 7.4 and kept on a magnetic stirrer at 30 rpm.

(iii) The weight of samples were analyzed after every 15 minutes and after 2 hours, weight of samples were analyzed every 1 hour.

(iv) The reading of weight of samples were analyzed until the weight becomes constant.

(v) Percent swelling is calculated by the formula:

$$\% \, \boldsymbol{swelling} = \left[\frac{Wt - Wo}{Wo}\right] X \, 100$$

Where, W_t is the weight of the swollen test sample and W_0 is the weight of the dried test sample.

3.2.6 Hemocompatibility Studies

The hemocompatibility test is done to check whether the sample is compatible with RBCs or not. The study was done by following the procedure reported by (Sasidharan et al) [106]. Briefly, the procedure is as follows:

(i) Anti-coagulated peripheral blood was used which is derived from a healthy individual. Blood was collected into a tube containing 3% Sodium citrate and mixed well.

(ii) After collection of blood, a blood suspension was prepared by diluting 8mL blood with 10 mL of Normal saline (0.9% NaCl) which makes a total of 18mL.

(iii) 0.5 gram sample of each scaffold was taken in 15mL centrifuge tubes and 9 mL of saline was added to it along with 0.5 mL of blood suspension.

(iv) Positive control contains 0.5 mL of Blood, 0.5 mL of 0.01N HCL and 9 mL saline.

(v) Negative sample contains 9.5 mL of saline and 0.5 mL of blood suspension only.

(vi) All the samples, positive and negative control was incubated for 2 hours at 37^{o}C.

(vii) After 2 hours, tubes were centrifuged (C24-BL Centrifuge, REMI, India) at 3000 rpm for 7 minutes.

(viii) Pellets was discarded and supernatant was collected and its OD was taken at 545 nm in UV-Vis Spectrophotometer (Lambda 35® (PerkinElmer, Waltham, MS, USA).

(ix) Percent hemolysis was calculated by the formula:

$$\frac{OD\ (Test) - OD\ (Negative\ control)}{OD(Positive\ control) - OD\ (Neative\ control)}\ X\ 100$$

3.3 *In Vitro* Studies

3.3.1 Cell culturing

Cell culturing experiments was performed using Human Osteo-Sarcoma SaOs-2 cells purchased from NCCS, Pune, India, to study cell attachment and proliferation.

SaOs-2 cells were cultured in proliferation medium containing Dulbecco's Modified Eagle's Medium (D-MEM, HiMedia) supplemented with 10 % fetal bovine serum (FBS, HiMedia), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were grown at 37 °C in a humid atmosphere in a CO_2 incubator with 5 % CO_2. Medium was refreshed every two-three days.

3.3.2 Sterilization and conditioning of the scaffolds

The scaffolds were sterilized by fuming them with 37% formaldehyde for 24 hours followed by UV irradiation for 30 minutes. After sterilization, scaffolds were conditioned by incubating them with DMEM media (containing 10% FBS and 1% antibiotic solution) for 24 hours.

3.3.3 Cell Seeding and attachment

After conditioning, the scaffolds were incubated with SaOs-2 cell suspension for 24 hours to provide enough time for cells to attach to scaffold surface. The procedure includes trypsinization of cells. Steps are as follows:

(i) SaOs-2 cells were grown in T-25 culture flasks with the proliferation medium containing Dulbecco's Modified Eagle's Medium (D-MEM, HiMedia) supplemented with 10 % fetal bovine serum (FBS, HiMedia), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were grown at 37 °C in a humid atmosphere in a CO2 incubator with 5 % CO_2. Medium was refreshed every two-three days, until the cell confluency reaches to 90-100 %.

(ii) Medium was discarded from the culture flask and the cells were washed with PBS thoroughly and then PBS was discarded.

(iii) 1 mL of 0.25 % 1X trypsin was added to the culture flask and incubated at 37ºC for 5 minutes in CO_2 incubator.

(iv) After 5 minutes 2mL of DMEM media was added to the culture flask and the surface was washed with medium to detach all loosely attached cells.

(v) Transfer the medium containing cells was transferred into a centrifuge tube and centrifuged for 5 minutes at 1000 rpm.

(vi) Supernatant was discarded and the pellets were thawed and washed with PBS.

(vii) Cells were again centrifuged at 1000 rpm for 5 minutes.

(viii) After 5 minutes, supernatant was discarded and pellets were mixed thoroughly with 2 mL DMEM media. This makes the cell suspension.

(ix) The sterilized and conditioned scaffolds were transferred to the tubes containing cell suspension and incubated for 24 hours at 37^{o}C.

(x) After 24 hours, scaffolds were transferred into 24 well plates with 1mL of DMEM media.

(xi) After 24 hours, scaffolds were analyzed under bright field inverted microscope for cell attachment.

3.3.4 Cell Proliferation

Cell proliferation was determined via fluorescence microscopy and the stain used is Propidium Iodide (PI). Cell scaffold construct was incubated at 37^{o}C and proliferation of cells is observed at 2 and 12 days after the day of cell seeding. Cell density was observed by fluorescence microscopy. The cell scaffold construct was plated in 24-well plate and supplied with DMEM medium containing 10% FBS and 1% antibiotic solution. The medium was replaced with fresh media every 2-3 days. To analyze cell proliferation cell scaffold construct were treated with Propidium Iodide stain that stains living cells. Scaffolds were analyzed at the day after cell seeding and then after 12 days under Epi-fluorescence microscope (Nikon TE 2000E).

The procedure for PI staining is as follows:

(i) Media from the 24 well plate, containing scaffolds, was discarded and scaffolds were washed thoroughly with Phosphate buffer saline (PBS) 2 to 3 times.

(ii) 10µg/mL propidium iodide dye was added to the wells in dark and the plate was incubated for 15 minutes at 37^{o}C in CO_2 incubator.

(iii) After 15 minutes, plates were removed from incubator, dye was removed and the scaffolds were washed thoroughly with PBS to remove the excess stain.

(iv) 1mL of fresh PBS was added to the scaffolds, these scaffolds can be preserved in PBS for 30 minutes.

(v) Scaffolds were then analyzed for cell proliferation under fluorescence microscope.

3.3.5 Cell viability sustainment upon cryopreservation

This test was done to check whether the cells remained viable and attached to the scaffolds or not. The test was performed using two cell lines one is normal epithelial cell line and another is osteosarcoma cell line i.e. HaCat (NCCS, Pune, India) and SaOs-2 (NCCS, Pune, India) cell lines respectively. The cell seeded scaffolds were cryopreserved for 15 days and cell viability was analyzed every third day.

3.3.5a Cryopreservation of cell scaffold constructs:

(i) The scaffolds were seeded with cells and kept in CO_2 incubator for 24 hours.

(ii) After 24 hours, media was removed and the scaffolds were washed with PBS to remove the media.

(iii) After washing, cell seeded scaffolds were transferred to cryovials.

(iv) 1 mL of DMEM cryomedia (containing 10% DMSO and 50% FBS) was added to each of the cryovial containing cell seeded scaffolds.

(v) The cryovials were immediately kept in Mr. Frosty freezing container which provide a cooling rate of -1^{o}C/minute and placed in -80 deep freezer for 24 hours.

(vi) After 24 hours, the cryovials were transferred to liquid N_2 tank.

(vii) At every third day, one vial each of every composition of scaffold were removed from the tank, washed with PBS to remove the DMSO, plated in 24 well plate and incubated with DMEM (containing 10% FBS and 1% antibiotic solution) for 24 hours.

(viii) After 24 hours, cell viability assay of each cell seeded scaffold was performed using MTT Assay.

3.3.5b Cell viability assay; MTT assay

MTT Stands for 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide. This assay is a colorimetric assay for assessing cell metabolic activity. Viable cells contains NADPH dependent oxidoreductase enzyme that converts MTT into purple colored formazon. The MTT assay was done in dark as MTT is a light sensitive dye. The procedure for MTT assay is as follows:

(i) 0.8mg/mL MTT solution was prepared in DMEM Incomplete media (do not contain FBS but contains 1% antibiotic solution).

(ii) The existing medium in the 24 well plates, containing cell seeded scaffolds, was discarded and 500mL of MTT solution was added to each well.

(iii) The plate was incubated at 37^{o}C for 4 hours in a CO_2 incubator.

(iv) After 4 hours, plate was removed from the incubator and MTT solution was removed very slowly, not disturbing the formazon pellets.

(v) 500 mL DMSO was added to the wells to dissolve formazon and incubated again for 15 minutes in incubator.

(vi) After 15 minutes, plates were removed from the incubator and absorbance of the formazan product was read at 562nm in a micro plate reader (PerkinElmer, Waltham, MS, USA).

Chapter 4

Results and Discussion

4.1 Fabrication of PVA: Carrageenan scaffolds

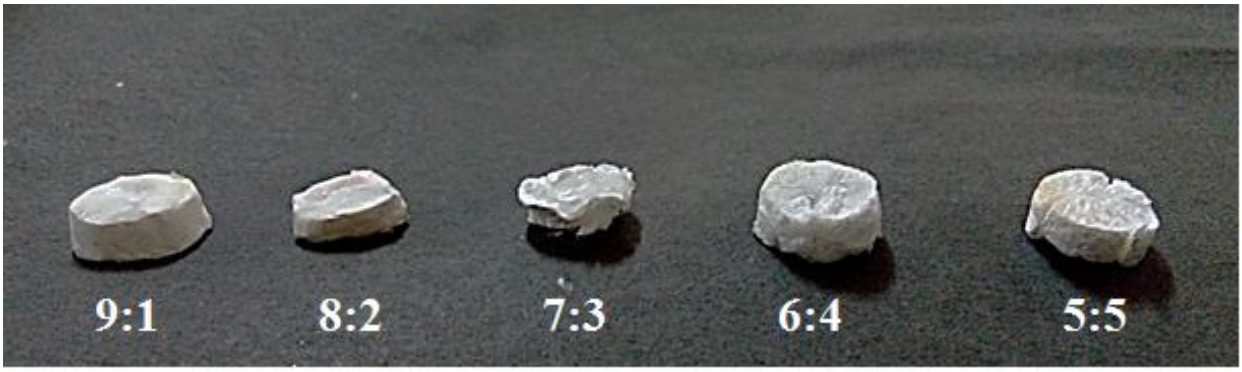


Figure 4.1: Photograph of the designed PVA: Carrageenan scaffolds.

Figure 4.1 shows the photographs of the fabricated scaffolds of PVA and carrageenan formed after blending polyvinyl alcohol (PVA) and Carrageenan at various ratios. Pictures clearly shows the tough and rigid structure of the scaffolds.

4.2 Scaffold morphology by Scanning Electron Microscope (SEM)

4.2.1 Scaffold with PVA: Carrageenan ratio 5:5

Figure 4.2a shows the scanning electron microscopy (SEM) images of prepared PVA: Carrageenan scaffolds of ratio 5:5, showing their surface morphology. Whereas, figure 4.2b shows the pore morphology upon 500X magnification. The average pore size of the pores of scaffold was around 12 µm and porosity was found to be 98.12%.

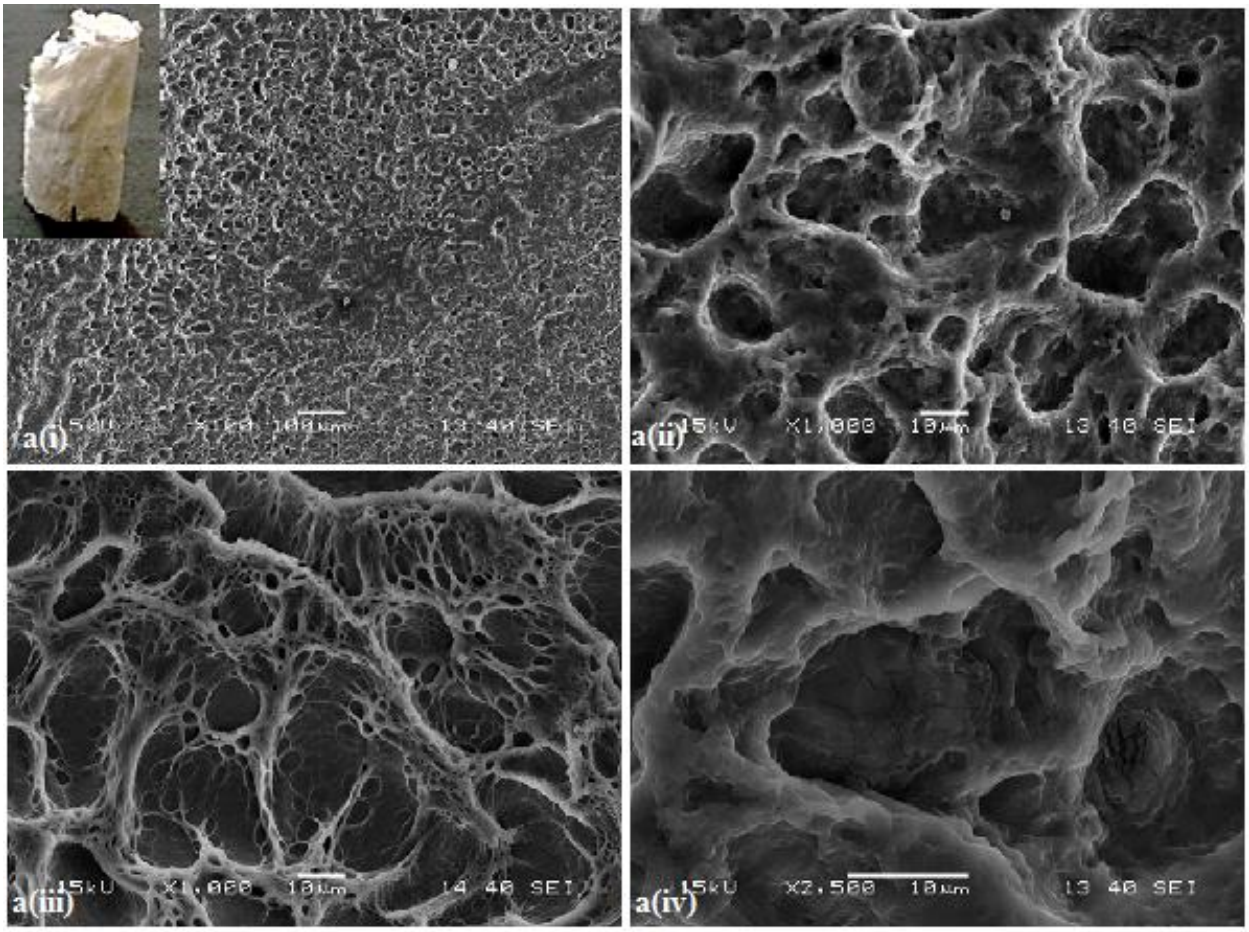


Figure 4.2a: Scanning Electron Microscopy (SEM) images of scaffolds with PVA: Carrageenan ratio 5:5. a(i) 100x, a(ii) 1000x, a(iii) 1000x, a(iv) 2,500x

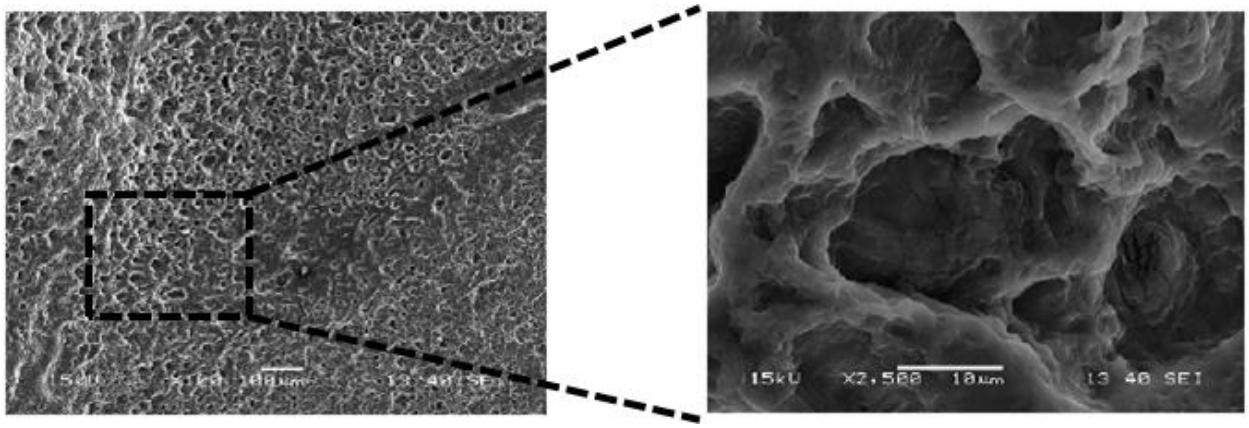


Figure 4.2b: Pore morphology upon 500x magnification

4.2.1 Scaffold with PVA: Carrageenan ratio 6:4

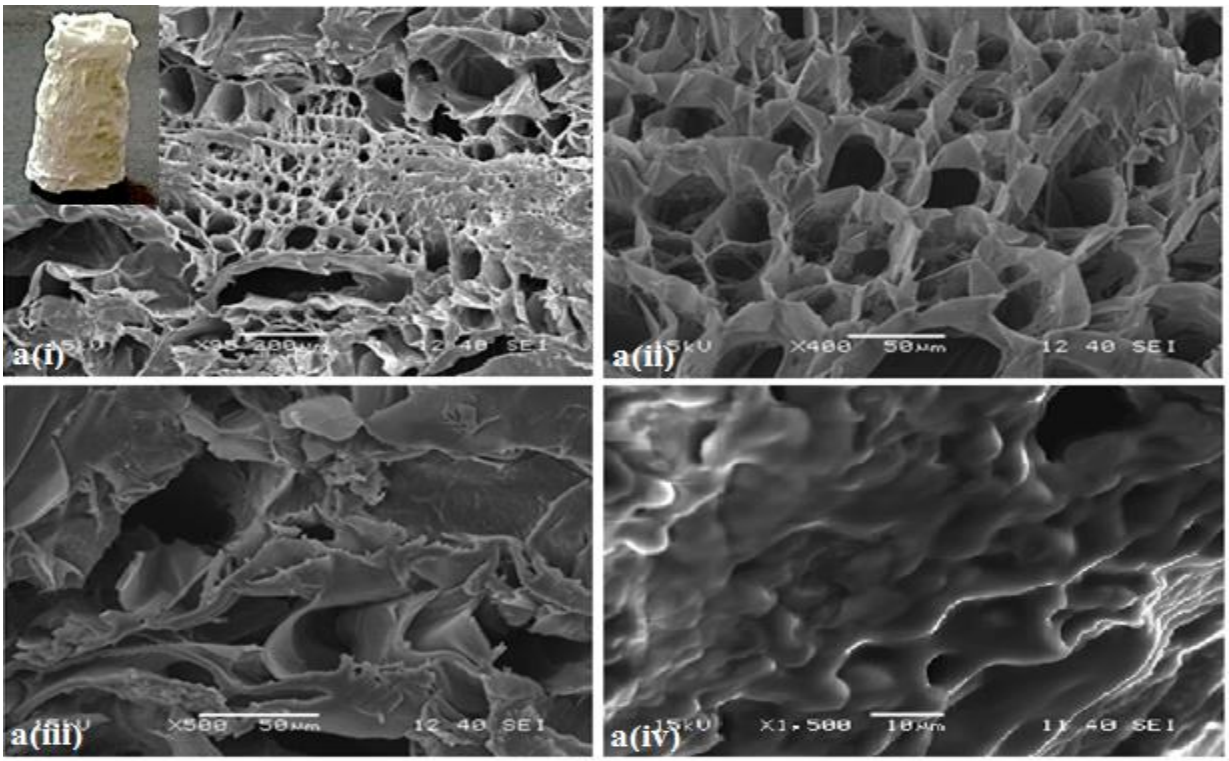


Figure 4.3a: Scanning Electron Microscopy (SEM) images of scaffolds with PVA: Carrageenan ratio 6:4. a(i) 95x, b(ii) 400x, b(iii) 500x, b(iv) 1,500x

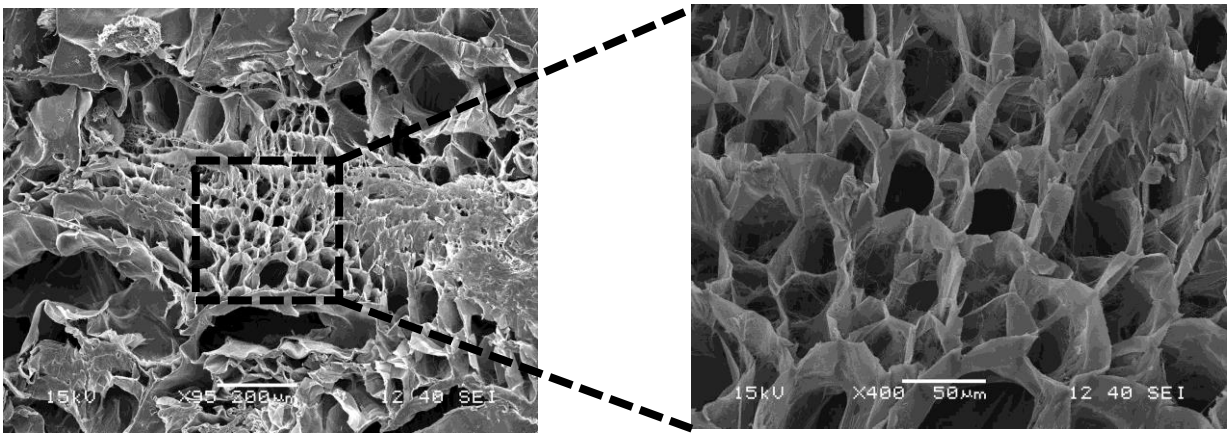


Figure 4.3b: Pore morphology upon 400x magnification.

Figure 4.3 shows the scanning electron microscopy (SEM) images of prepared PVA: Carrageenan scaffolds of ratio 6:4, showing their surface morphology. The average pore size of the pores of scaffold was around 40µm and porosity was found to be around 96.33%.

4.2.2 Scaffold with PVA: Carrageenan ratio 7:3

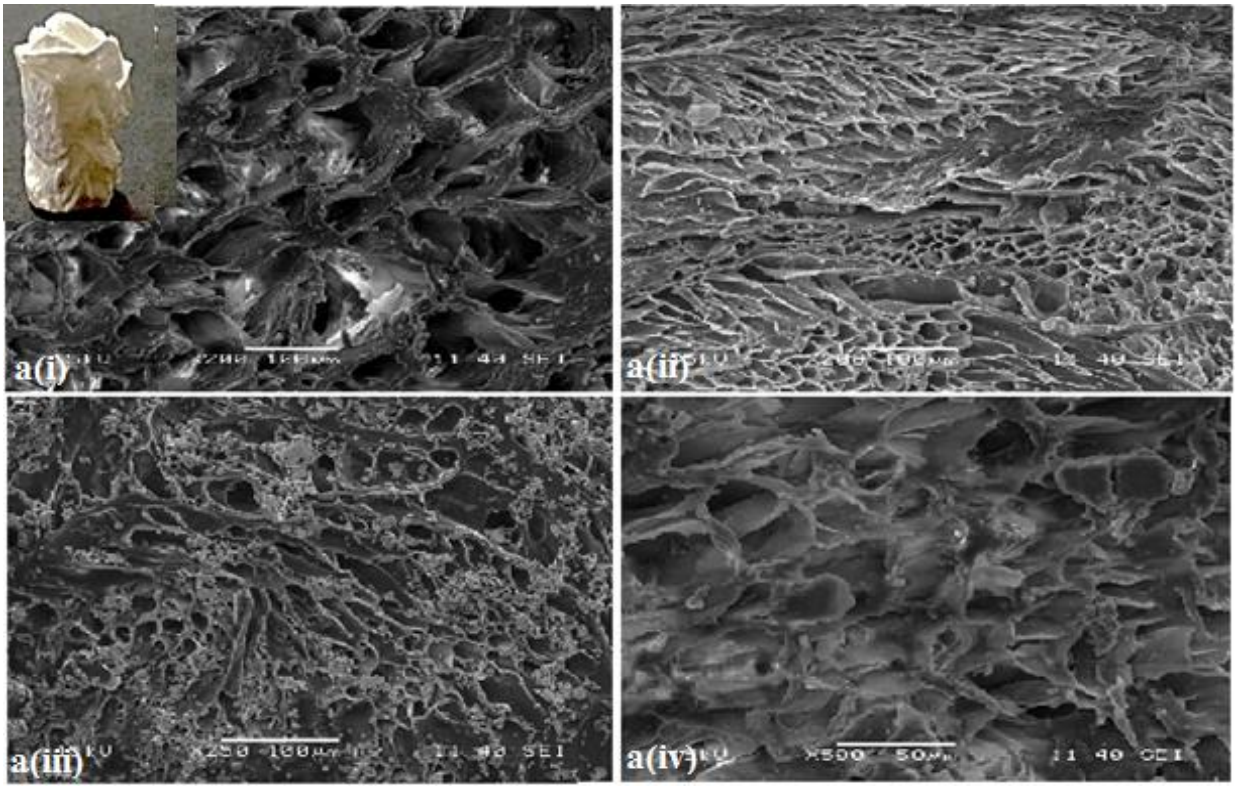


Figure 4.4a: Scanning Electron Microscopy (SEM) images of scaffolds with PVA: Carrageenan ratio 7:3. a(i) and a(ii) 200x, a(iii) 250x, a(iv) 500x

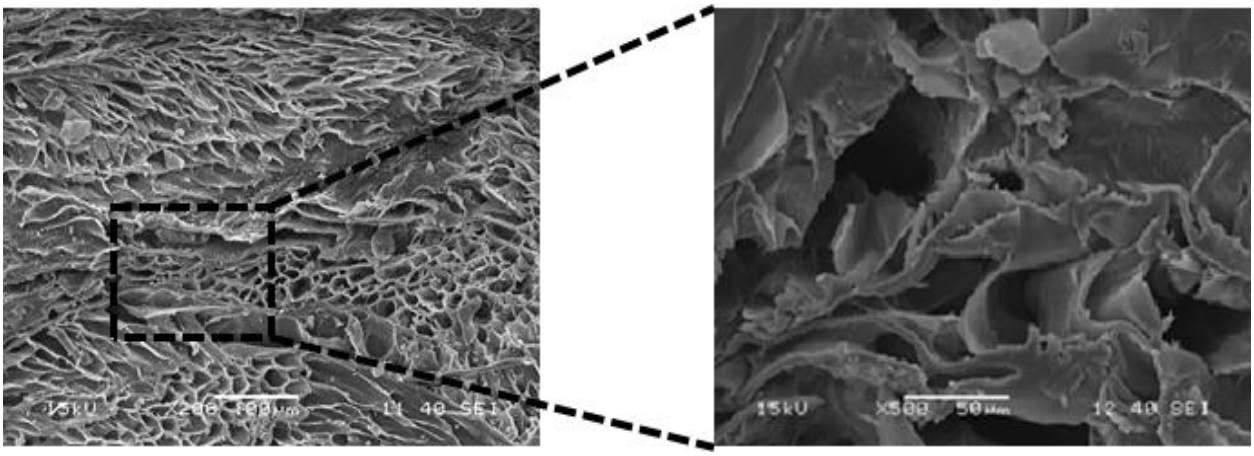


Figure 4.4b: Pore morphology upon 500x magnification.

Figure 4.4 shows the scanning electron microscopy (SEM) images of prepared PVA: Carrageenan scaffolds of ratio 7:3, showing their surface morphology. The average pore size of the pores of scaffold was around 90µm and porosity was found to be around 98.71%.

4.2.3 Scaffold with PVA: Carrageenan ratio 8:2

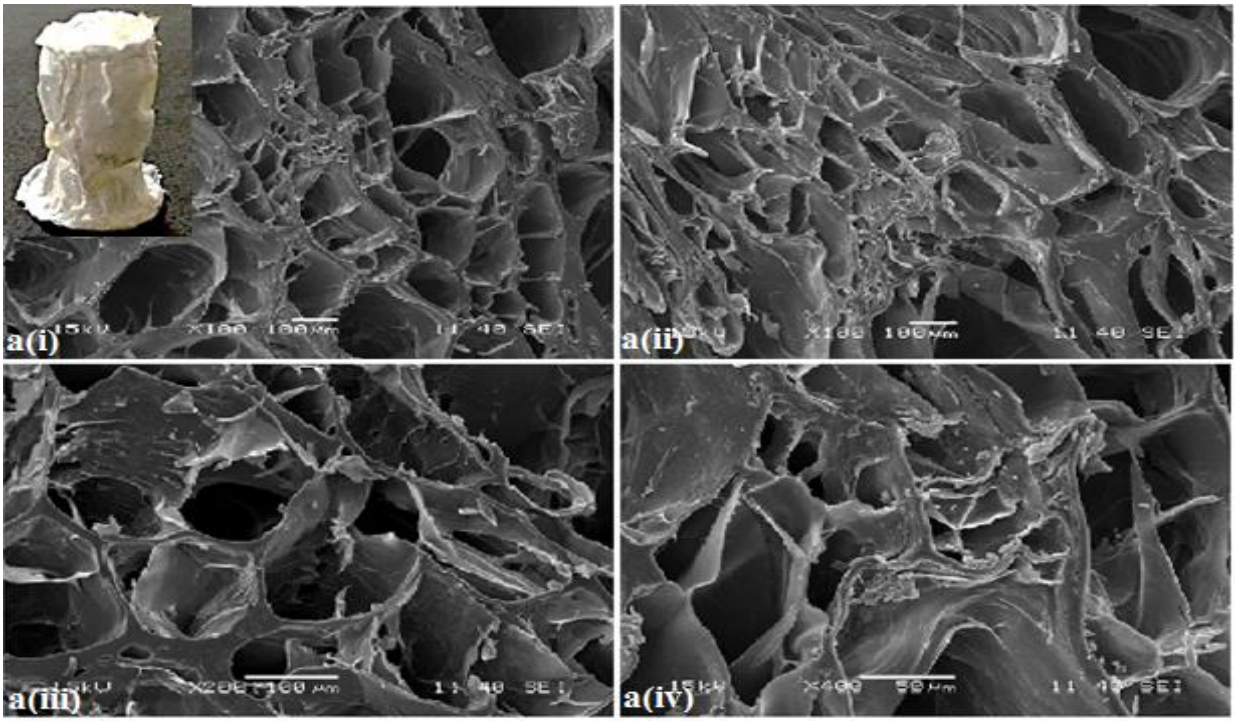


Figure 4.5a: Scanning Electron Microscopy (SEM) images of scaffolds with PVA: Carrageenan ratio 8:2. a(i) and a(ii) 100x, a(iii) 200x, a(iv) 400x

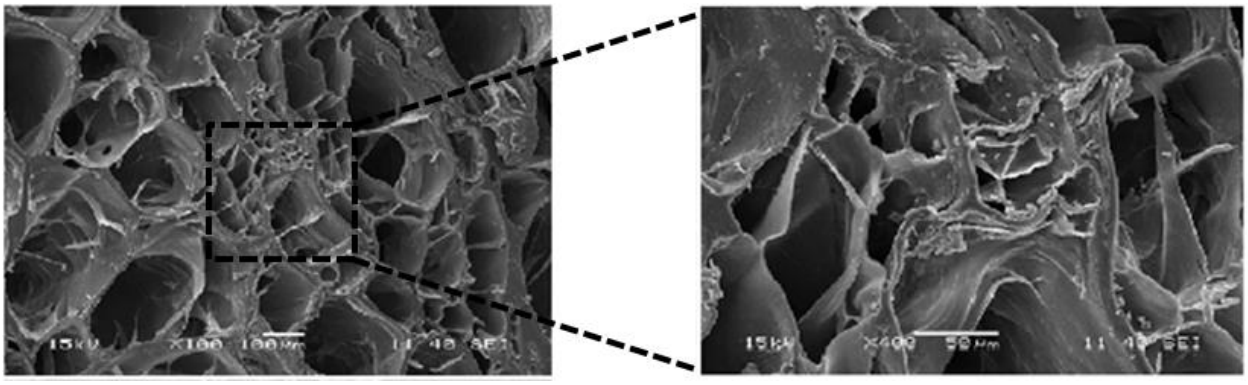


Figure 4.5b: Pore morphology upon 400x magnification.

Figure 4.5 shows the scanning electron microscopy (SEM) images of prepared PVA: Carrageenan scaffolds of ratio 8:2, showing their surface morphology. The average pore size of the pores of scaffold was around 105µm and porosity was found to be around 95.51%.

4.2.4 Scaffold with PVA: Carrageenan ratio 9:1

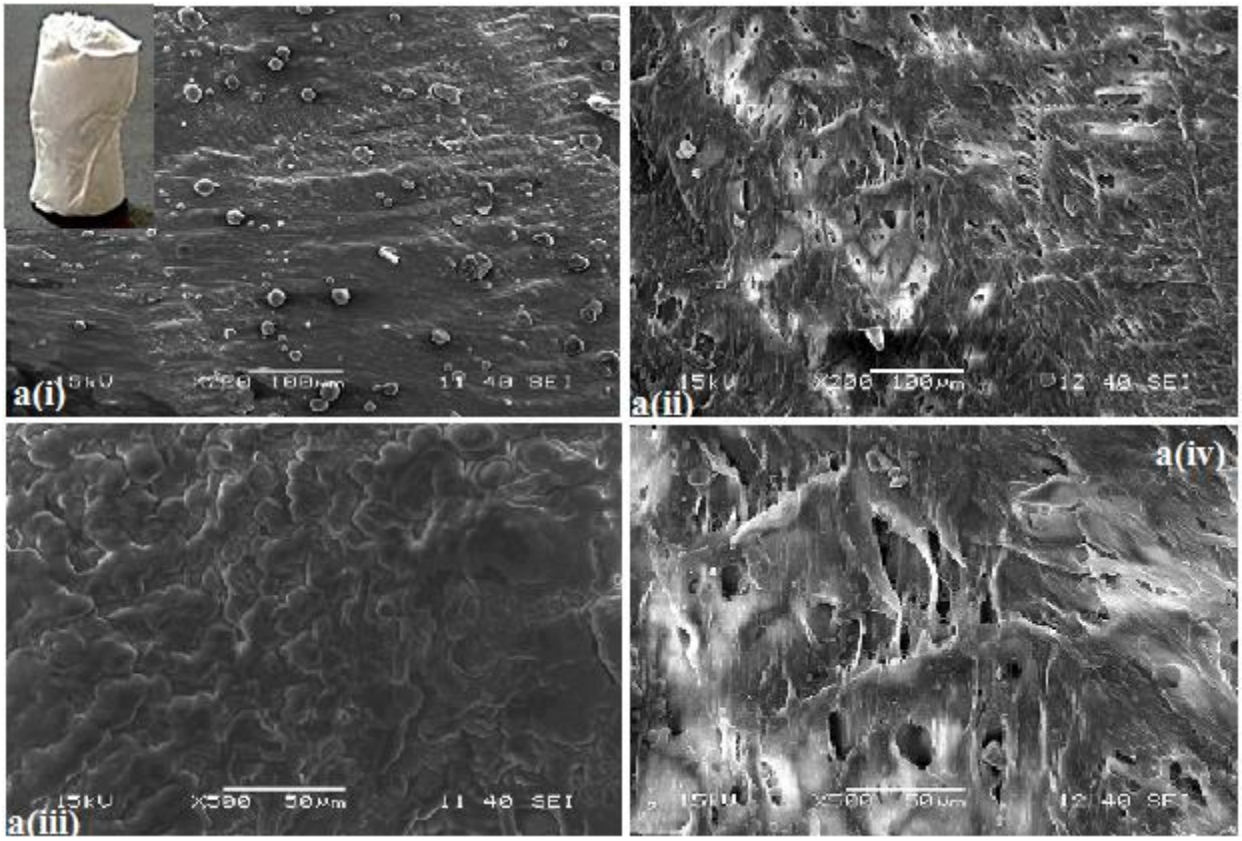


Figure 4.6a: Scanning Electron Microscopy (SEM) images of scaffolds with PVA: Carrageenan ratio 9:1. a(i) and a(ii) 200x, a(iii) and (iv) 500x

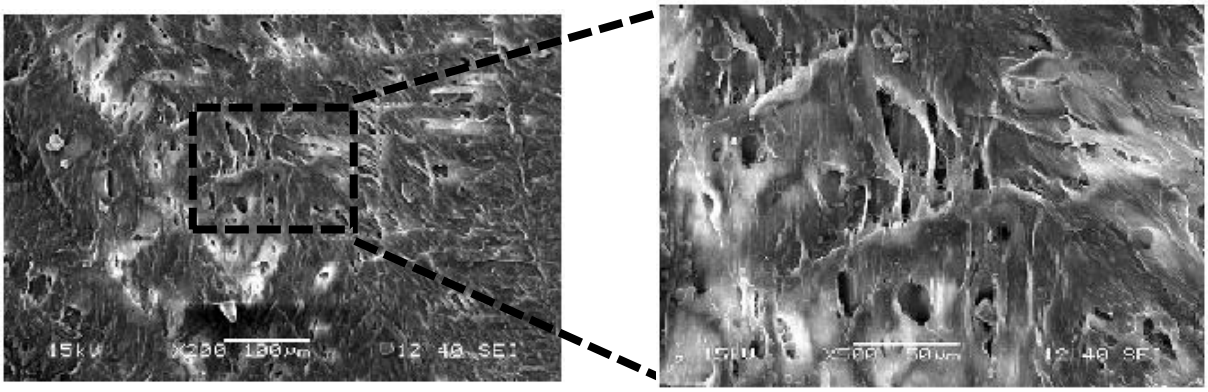


Figure 4.6b: Pore morphology upon 500x magnification.

Figure 4.6 shows the scanning electron microscopy (SEM) images of prepared PVA: Carrageenan scaffolds of ratio 8:2, showing their surface morphology. The average pore size of the pores of scaffold was around 25µm and porosity was found to be around 40%.

4.3 Chemical composition analysis of the composite scaffolds by Attenuated Total Reflection Fourier Transform Infrared Microscopy (ATR-FTIR).

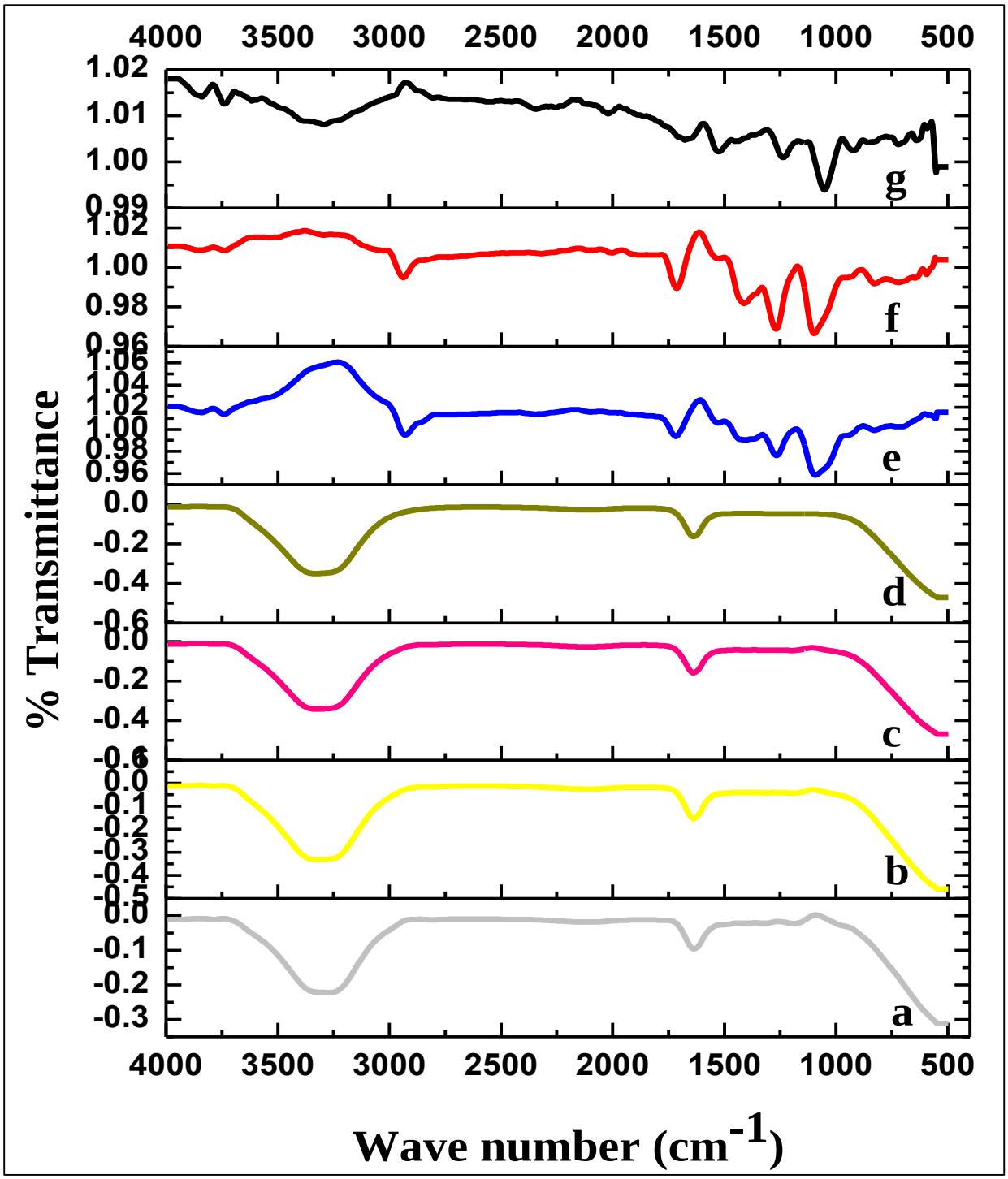


Figure 4.7 : ATR-FTIR spectrum of the PVA-Car scaffolds in different ratios (a) 5:5, (b) 6:4, (c)7:3, (d)8:2 , (e) 9:1, (f) PVA, (g)Carrageenan

The ATR-FTIR measurement of the PVA-Car scaffold samples were carried out in order to identify formation or transformation (shifting) of any chemical bonds formed during the blending of PVA and Carrageenan. Strong bonds associated with amines, alkynes, amides, alcohol, carboxylic acids were observed in the prepared scaffolds when they were scanned in the range of 4000 – 400 cm^{-1}. In 9:1 ratio scaffolds ATR-FTIR bands were observed at 3230.1, 2929.388, 1606.942 and 1264.345 cm^{-1} which were associated with O-H stretching, C-H stretching, N-H bending and C-N stretching respectively. These FTIR bands showed very much similarity with the chemicals present in the starting raw materials i.e., PVA and carrageenan. Similarly in 8:2, 7:3, 6:4 and 5:5 ratio scaffolds spectral bands were observed to be shifting right wards 3334.163 cm^{-1} →3324.006 cm^{-1} →3275.063 cm^{-1} which may be associated with the stretching of primary and secondary amines and the shifting may be due to the increasing concentration of Carrageenan. Another constant spectral band present in all the scaffold ratios at 1635.492 cm^{-1} were observed which corresponds to the primary amines in poly vinyl alcohol Another prominent positive peak was observed at 1093.047 cm^{-1} in 5:5 ratio scaffold which is associated with C-N stretching of aliphatic amines. Figure 4.7 clearly shows the entire scaffolds with different ratios of PVA: Carrageenan confirming their complete blending with each other and all having some of their specific spectral fingerprint.

4.4 X-Ray Powder Diffraction studies (XRD)

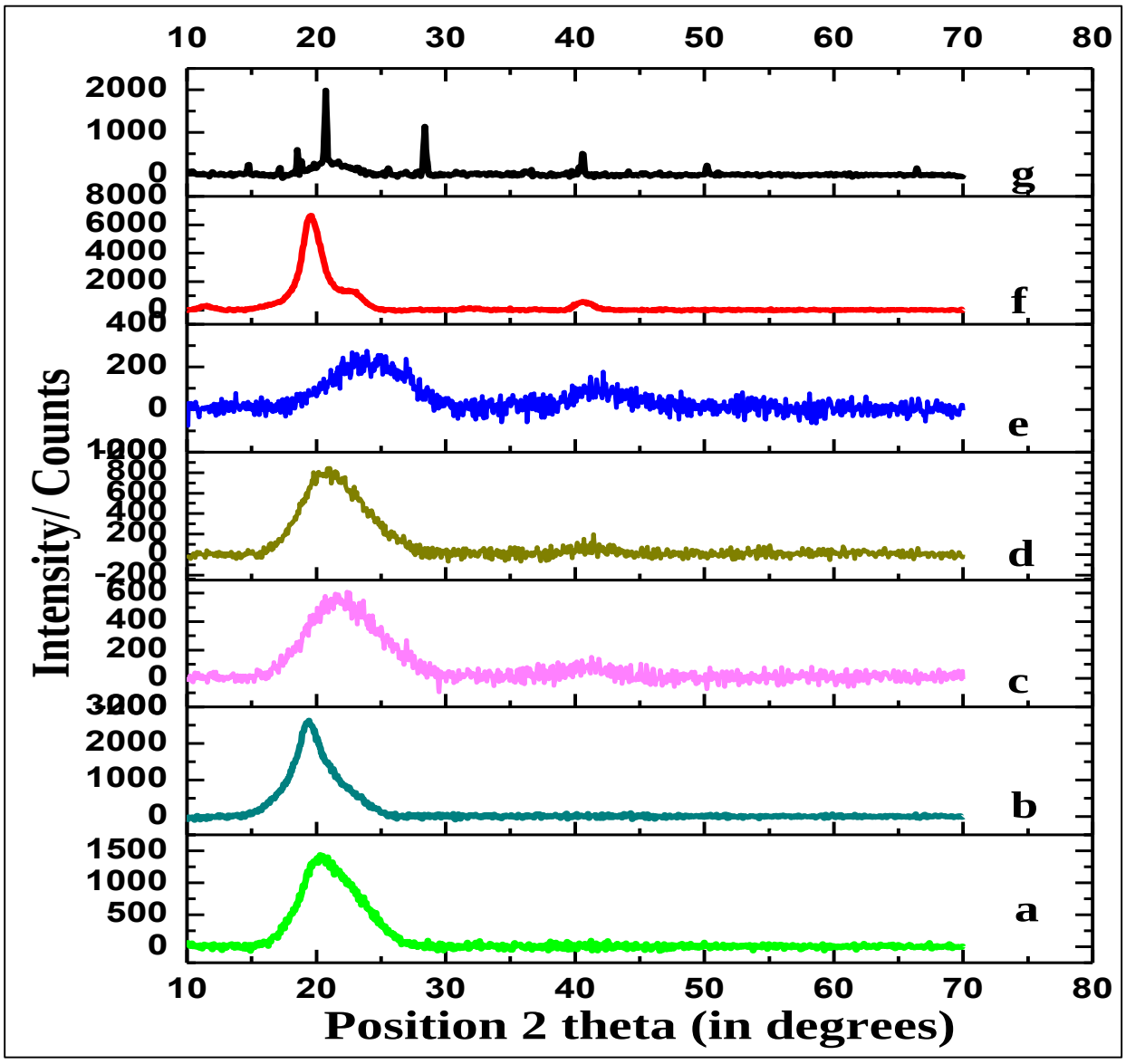


Figure 4.8: X-Ray Diffraction diffractogram of the PVA-Carrageenan scaffolds in different ratios (a) 5:5, (b) 6:4, (c)7:3, (d)8:2 , (e) 9:1, (f) PVA, (g)Carrageenan

Figure 4.8 shows the typical XRD diffractogram of PVA-Carrageenan scaffolds scanned within 20-80° at position 2θ. The starting raw material Carrageenan (g) and PVA (f) showed their typical sharp and intense XRD peaks that means their atoms are periodically arranged which is associated with its crystalline nature. From the figure 4.8 it becomes very clear that the scaffolds formed were properly blended due to the presence of only one prominent peak present around 20° at position 2θ.

4.5 Swelling Studies

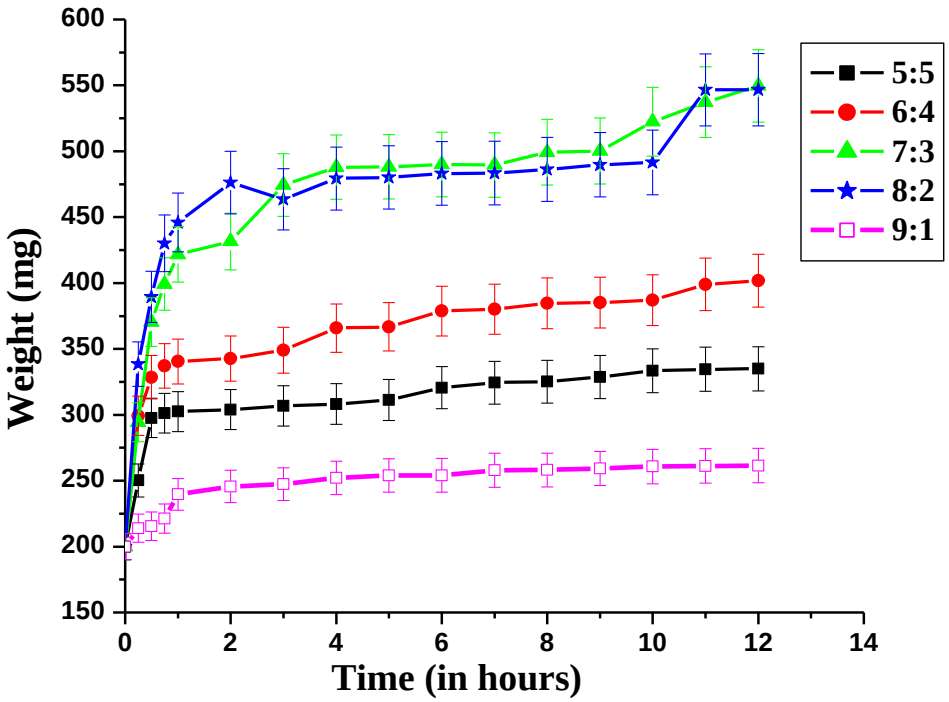


Figure 4.9: Graph showing amount of PBS uptake by the scaffolds.

Figure 4.9 shows a typical graph of swelling behaviors of the scaffolds which defines that how much water it can absorb before getting degraded. A scaffold, upon implantation must be able to absorb enough amount of matrix constituents in order to get stabilized and acclimatized properly. Matrix constituents contains growth factors, proteins or other things that is required for a tissue to repair and regenerate, and if a scaffold is able to absorb those constituents, it would be advantageous. Figure 4.9 indicated below shows swelling behavior of the prepared scaffolds on the basis of amount of PBS absorbed by it in a period of 12 hours. From the graph it can be clearly concluded that:

- Scaffold with composition 7:3 and 8:2 showed similar kind of swelling, both of them showed the maximum swelling then others. Scaffold with composition 7:3 absorbed a little more as compared to scaffold with composition 6:4. Both of the scaffolds showed approximately 2.5 fold increase in weight when compared to the initial weight of them.
- Scaffold with composition 6:4 showed a medium amount of swelling as compared to others. It shows a 2 fold increase in weigh when compared to its initial weight.

- Scaffold with composition 5:5 showed fourth highest swelling, with an increase in weight by 1.75 fold as compared to its initial weight.
- Scaffold with composition 9:1 showed least swelling with only 0.25 fold increase in weight as compared to its initial weight.

Figure 4.10 here shows the percentage swelling of the scaffolds with respective to its initial weight. Scaffold with composition 7:3 and 8:2 showed a similar swelling percentage of 349.5% and 346.7%. Whereas, scaffold with composition 6:4 and 5:5 showed a swelling percentage of 201.7% and 134.9%. Lastly, scaffold with composition 9:1 showed least swelling percentage of 61.4%.

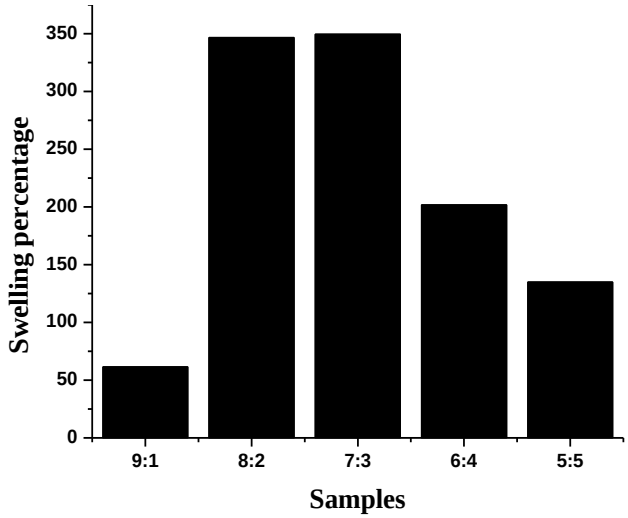


Figure 4.10: Graph showing swelling percentage of the prepared scaffolds.

From the two graphs, it can be concluded that as the PVA concentration in the scaffold increases their swelling capability also increases. Scaffold with ratio 7:3 and 8:2 are found to be best of them all.

4.6 Hemocompatibility Studies

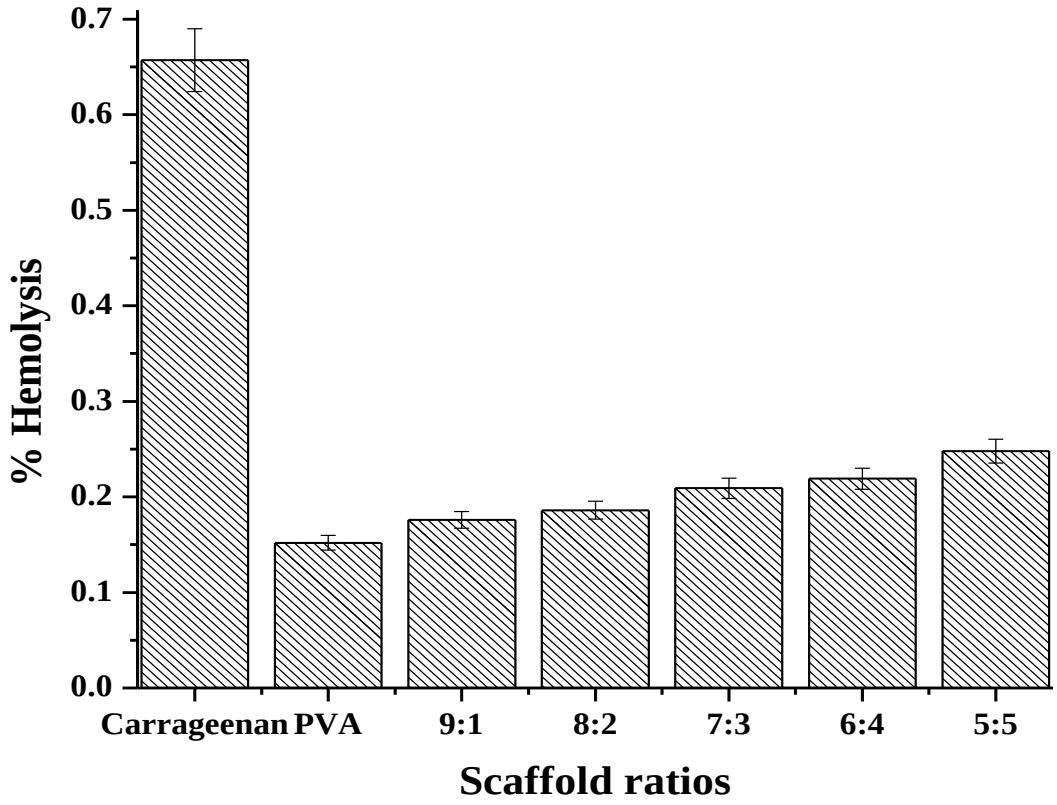
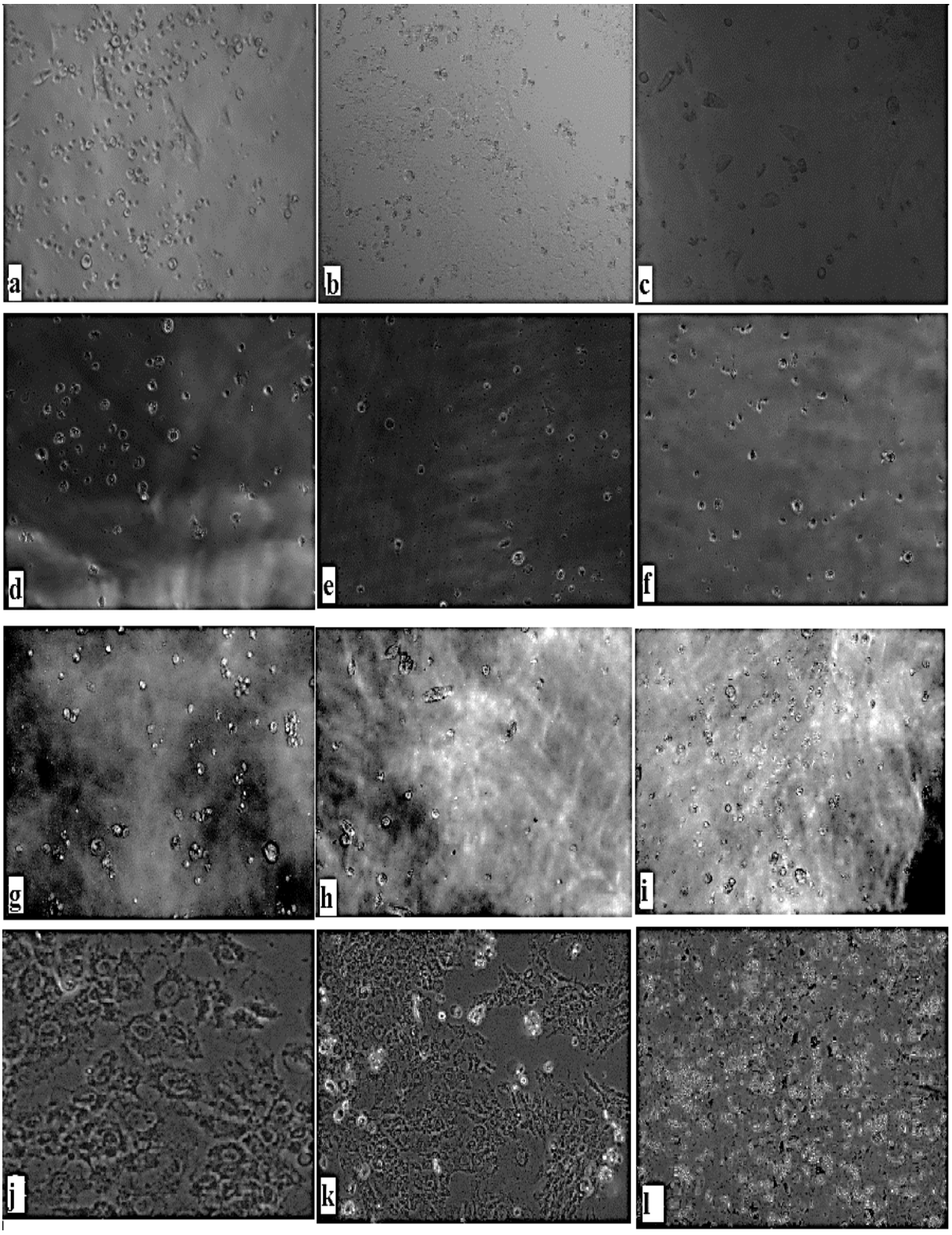


Figure 4.11: Bar diagram showing hemocompatibility of PVA: Carrageenan scaffolds.

Figure 4.11 shows the bar diagram of percent hemolysis of the fabricated PVA: Carrageenan scaffolds. All of the scaffolds show a great degree of hemocompatibility and hence it can be mentioned that these scaffolds are highly hemocompatible. It is clearly observable from the graph that, as the concentration of carrageenan increases, the hemocompatibility decreases. Scaffolds with PVA: Carrageenan ratio 9:1 and 8:2 are found to be most hemocompatible.

4.7 *In Vitro* Studies

4.8 *Cell Seeding and attachment*



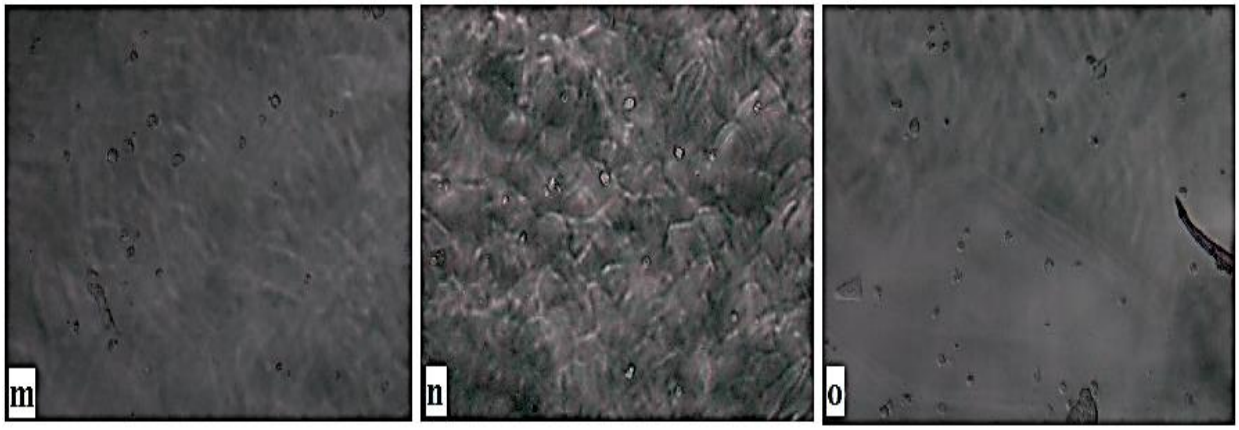
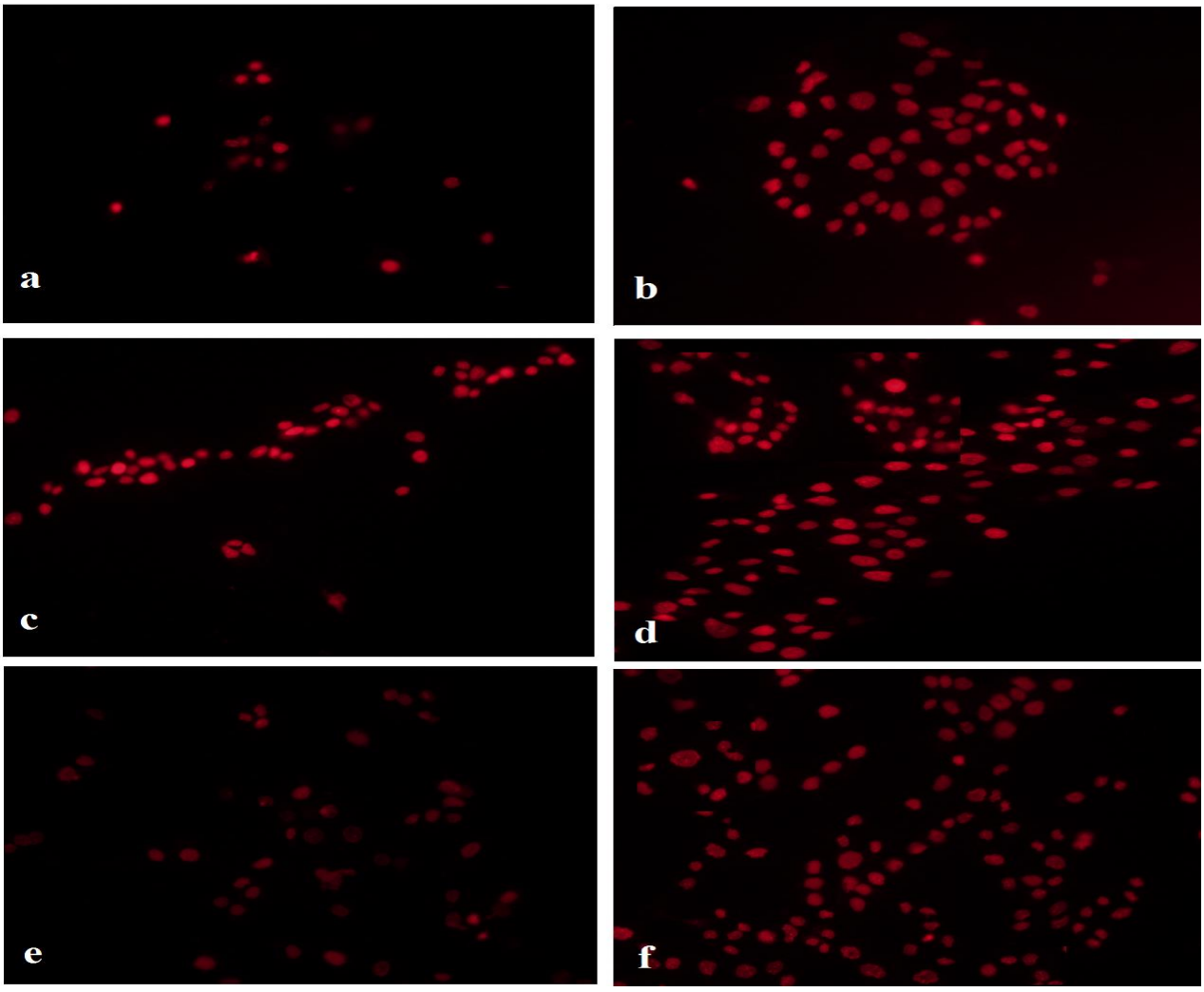


Figure 4.12: Bright field microscopy images of scaffolds seeded with osteosarcoma SaOs-2 cells. **(a, b, c)** 5:5**; (d, e, f)** 6:4**; (g, h, i)** 7:3**; (j, k, l)** 8:2**; (m, n, o)** 9:1**.**

Figure 4.12 shows the bright field microscopy images of the cell seeded scaffolds at 40x magnification. All the scaffolds upon incubation with cell suspension of Osteo sarcoma SaOs-2 cells, showed cell attachment at enough density to provide cells enough space to proliferate and acquire the void spaces. As compared to others, scaffold with compositions 8:2, 7:3 and 5:5 showed better cell attachment with more cell density then others. Hence it can be concluded that, the scaffolds of these three compositions would be useful as a graft for tissue repair. However, further confirmation for the most appropriate composition can be obtained by studying or analyzing the cell proliferation studies that which scaffold provide better cell proliferation then others.

4.9 *Cell Proliferation*

For investigating the cell proliferation, scaffolds were seeded with SaOs-2 cells purchased from NCCS, Pune, India and incubated at 37^{o}C in a CO_2 incubator. The scaffolds were retrieved after 2 and 10 days of incubation *in vitro.* Fig. 4.13 shows representative cross-sectional images of prepared scaffolds of various compositions seeded with cells retrieved after 2 and 10 days and observed under fluorescence microscope. Propidium iodide (PI) stained nuclei are shown in red color. All the scaffolds showed cell proliferation. The row to the left containing images a, c, e, g and i corresponds to the cell seeded scaffold retrieved after 2 days of incubation. And, the row to the right containing images b, d, f, h and j corresponds to the cell seeded scaffolds retrieved after 10 days of incubation. From the two images, it is easy to predict that the designed scaffolds acted suitably and are able to provide surface for cell attachment and proliferation.



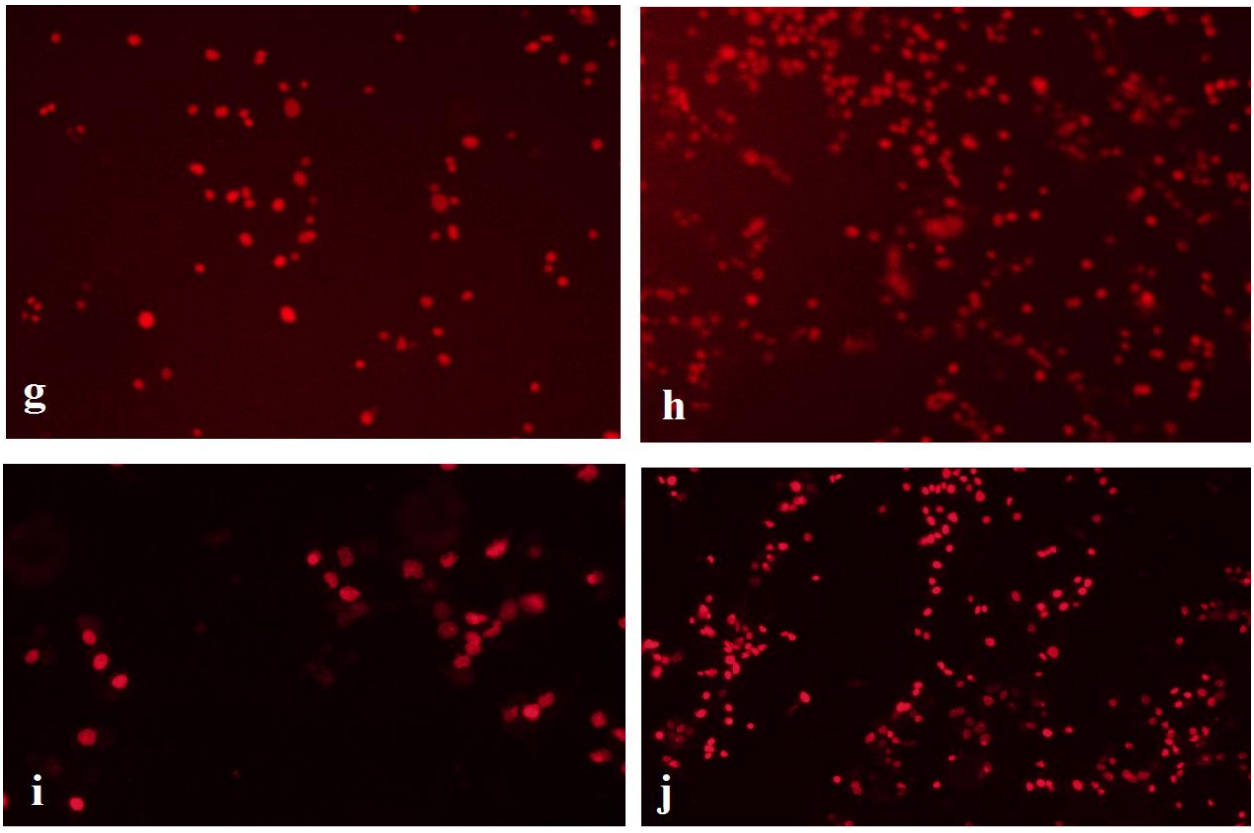
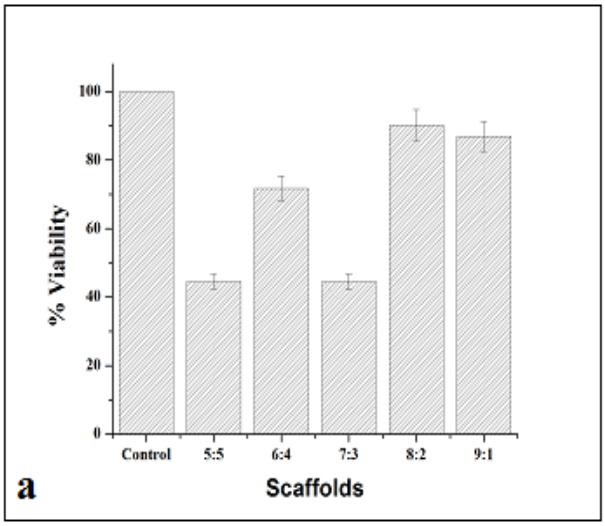
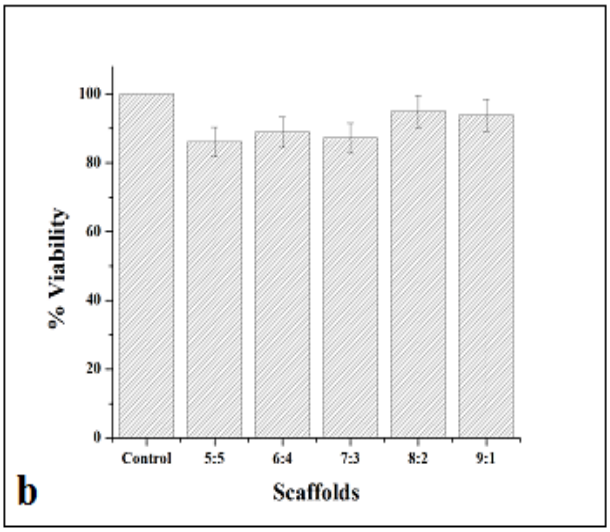
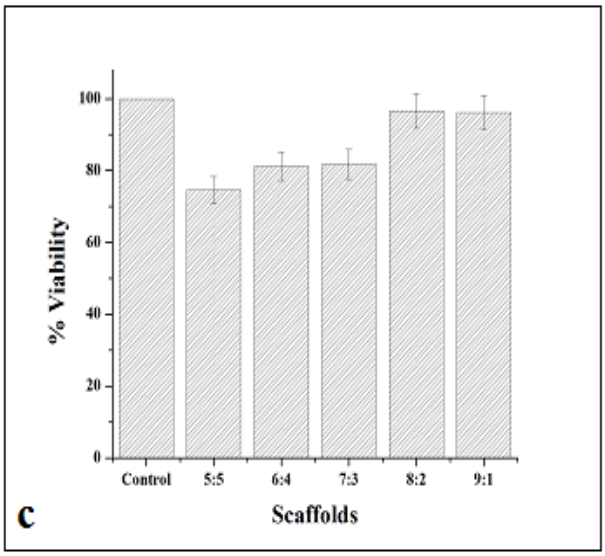


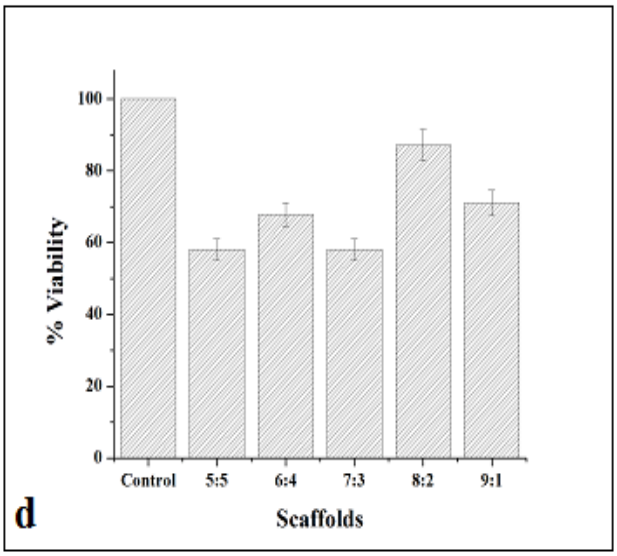
Figure 4.13: Fluorescence Microscopy images of Propidium iodide (PI) stained cell seeded scaffolds retrieved after 2 and 10 days. Where, **a, c, e, g and i** corresponds to cell seeded scaffolds with composition **5:5, 6:4, 7:3, 8:2 and 9:1**, retrieved after 2 days and stained by PI. Whereas, on the other side, images **b, d, f, h and j** corresponds to cell seeded scaffolds with composition 5:5, 6:4, 7:3, 8:2 and 9:1, retrieved after 10 days and nuclei stained with propidium iodide. All images have the same magnification.

4.10 *Cell viability sustainment upon cryopreservation (MTT Assay)*









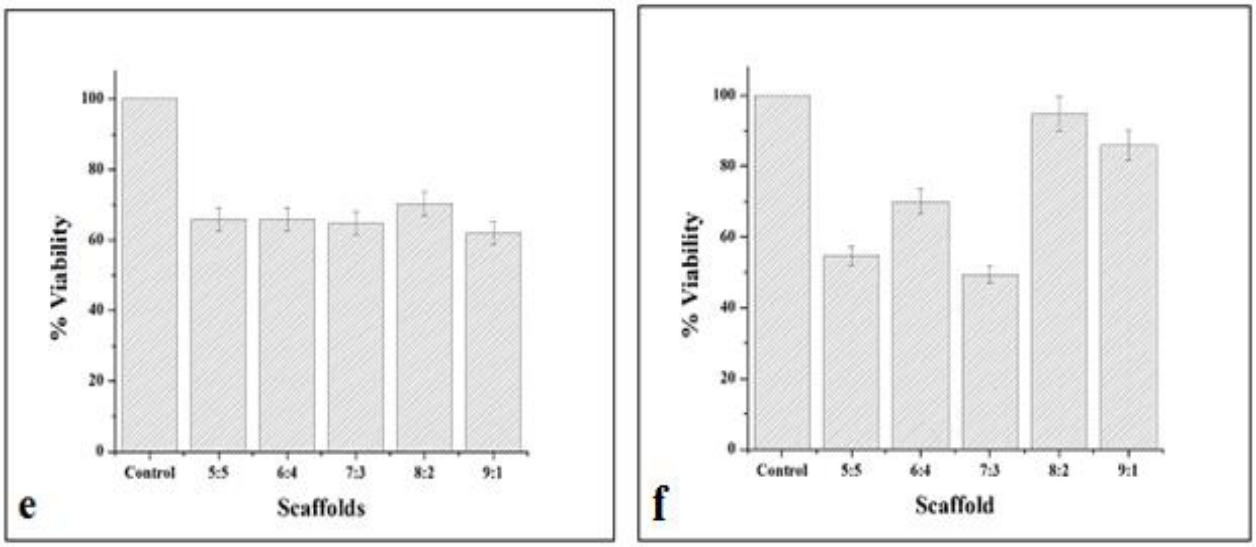
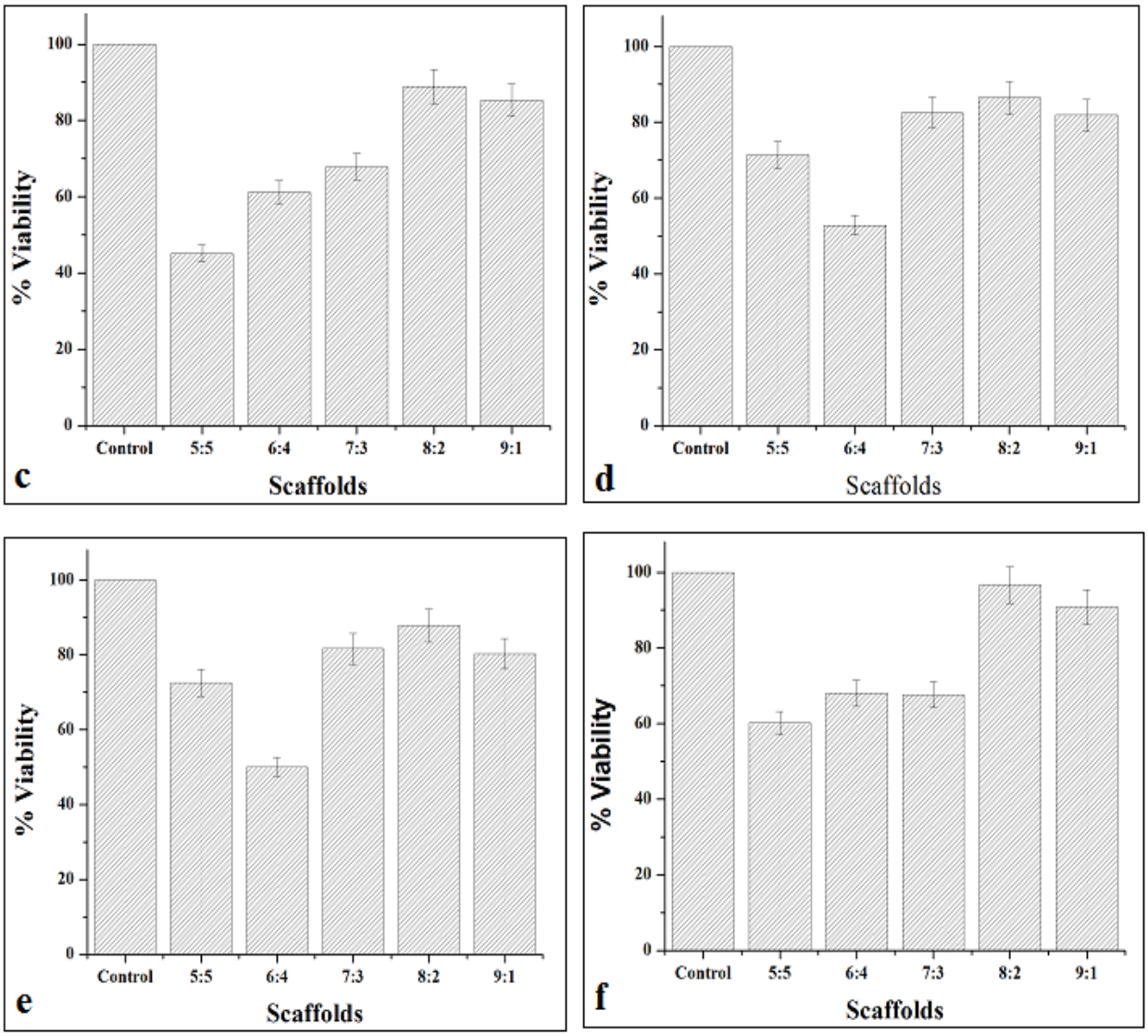


Figure 4.14: Graphs showing percent viability of Osteo sarcoma SaOs-2 cells seeded on scaffolds and cryopreserved for 15 days. **Graph (a)** shows cell viability on day zero i.e. just after cell seeding and attachment. **Graph b, c, d, e and f** shows percent cell viability of cells on day 3, 6, 9, 12 and 15 respectively after seeding and cryopreservation. Control contains only cells i.e. they are not seeded on scaffolds.

Figure 4.14 shows the MTT assay graph of SaOs-2 cells seeded scaffolds predicting their cell viability upon cryopreservation for a period of 15 days. The cell viability was calculated every third day after cryopreservation. From the calculations it can be clearly deciphered that the scaffold with PVA: Carrageenan ratio 8:2 showed maximum cell viability followed by the scaffold with ratio 9:1. Other scaffolds also showed sustainment but not as much as these two.

When the scaffolds were seeded with normal Keratinocyte continuous HaCat cell line, the viability results showed same kind of pattern i.e. scaffolds with PVA: Carrageenan ratio 8:2 and 9:1 showed maximum viability when seeded and cryopreserved for 15 days. Figure 4.15 shows percent cell viability of cells after cryopreservation.



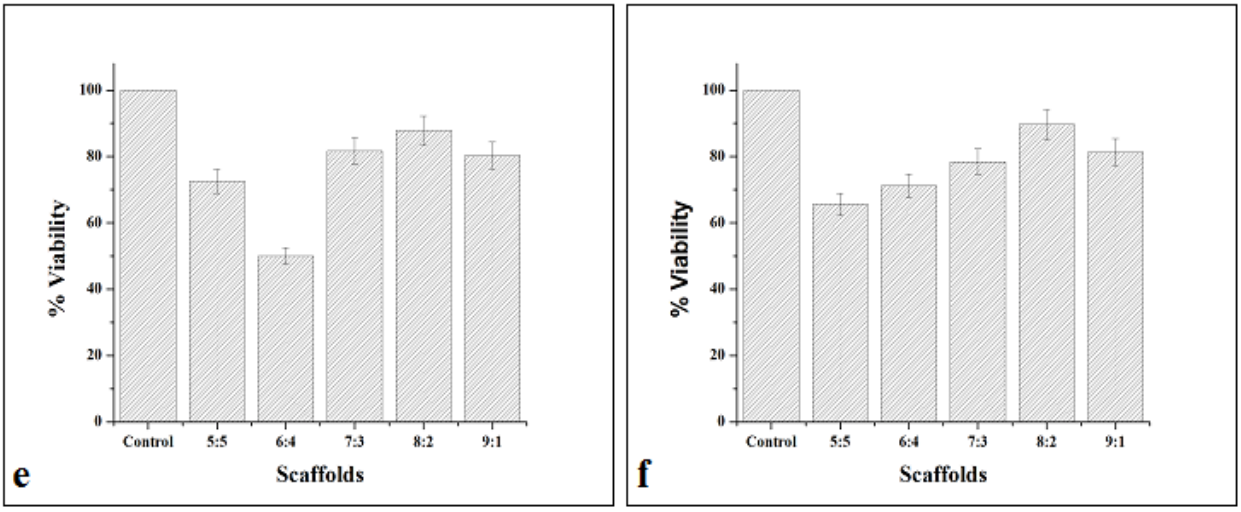


Figure 4.15: Graphs showing percent viability of normal keratinocyte HaCat cells seeded on scaffolds and cryopreserved for 15 days. **Graph (a)** shows cell viability on day zero i.e. just after cell seeding and attachment. **Graph b, c, d, e and f** shows percent cell viability of cells on day 3, 6, 9, 12 and 15 respectively after seeding and cryopreservation. Control contains only cells i.e. they are not seeded on scaffolds.

Chapter 5

Conclusion

The present study demonstrated the fabrication of PVA- Carrageenan based scaffolds in different ratios of PVA and Carrageenan. Among the different formulation, ratio 8:2 was found to be the best scaffolding materials based upon its properties like optimum porosity, optimum pore size, biocompatibility, highest swelling property, provides proper cell attachment, cell proliferation and cell viability upon cryopreservation. From the SEM micropragps the honey comb like structures were clearly visible that compares the porous nature of our prepared formulation. From the ATR-FTIR spectrum, the molecular shifting and resuffling of chemicals groups associated with PVA and Carrageenan were clearly observed with the increase and decrease of the concentration PVA and Carrageenan. Further characterization of the scaffolds through XRD diffraction patterns reveals the atomic arrangement of the prepared scaffolds whose diffraction patterns were completely changed with the mixture of the two base raw materials. The stability of a prepared scaffold is one of the most important parameter which shows its water retention rate along with its degradation rate with increase in time duration. Among the five prepared scaffold ratios, the scaffold with ratio 7:3 was found to be highly stable followed by 8:2 > 6:4 > 5:5 > 9:1. Another important criteria for an ideal scaffold is its hemocompatibility which shows its biocompatibility nature within our body. Scaffold with PVA: Carrageenan ratio 8:2 was found to be highly hemocompatible followed by 9:1 > 6:4 > 7:3 > 5:5. In vitro studies with osteosarcoma SaOs-2 and normal keratinocyte cell line HaCat were done to observe the best scaffold materials upon different days of cryopreservation. Among them scaffold with ratio 8:2 was found to be highly capable of re-growth which was around 93.24% followed by scaffold 9:1 with cell viability percentage 86.87%. Scaffolds of composition 7:3, 6:4 and 5:5 showed 68.51%, 78.50% and 68.23% cell viability respectively. To confirm the results obtained through MTT assay, the cryopreserved cell seeded scaffolds were observed for the cell attachment and proliferation through fluorescence microscopy using Propidium Iodide intercalating dye which binds to nucleic acids and gives red

fluorescence at 617nm and thus we can differentiate between live or dead cells. Finally we conclude that scaffold with PVA: Carrageenan ratio 8:2 is the best among the 5 formulated scaffold which can be utilized for bone regeneration purpose, but again further detail work has to be done before it can be used in human trials.

Further prospective:

- *In vivo* implantation of these scaffolds and study in different mice models.
- Differentiation of MSCs (Mesenchymal Stem Cells) using the PVA-Car based scaffolds.

Chapter 6

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