SYNTHESIS AND CHARACTERIZATION OF CHITOSAN-ALGINATE SCAFFOLDS AND SEEDING MESENCHYMAL STEM CELLS FOR BIOMEDICAL APPLICATIONS

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May 2017

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I hereby declare that the thesis entitled, "**SYNTHESIS AND CHARACTERIZATION OF CHITOSAN-ALGINATE SCAFFOLDS AND SEEDING MESENCHYMAL STEM CELLS FOR BIOMEDICAL APPLICATIONS"**, completed and written by me has not previously formed the basis for the Degree or Diploma or other similar title of this or any other University or examining body.

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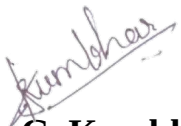

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CERTIFICATE

This is to certify that the thesis entitled, "**SYNTHESIS AND CHARACTERIZATION OF CHITOSAN-ALGINATE SCAFFOLDS AND SEEDING MESENCHYMAL STEM CELLS FOR BIOMEDICAL APPLICATIONS**" which is submitted herewith for the Degree of Doctor of Philosophy in **Biotechnology** of D.Y. Patil University, Kolhapur (Declared u/s 3 of the UGC Act 1956) is the result of original work completed by **Ms. Sneha Gurudas Kumbhar** under our supervision and guidance and to the best of our knowledge and belief the work embodied in this thesis has not formed earlier the basis for the award of any Degree or similar title of this or any University or examining body.

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What you get by achieving your goals is not as important as what you become by achieving your goals.

Zig Ziglar

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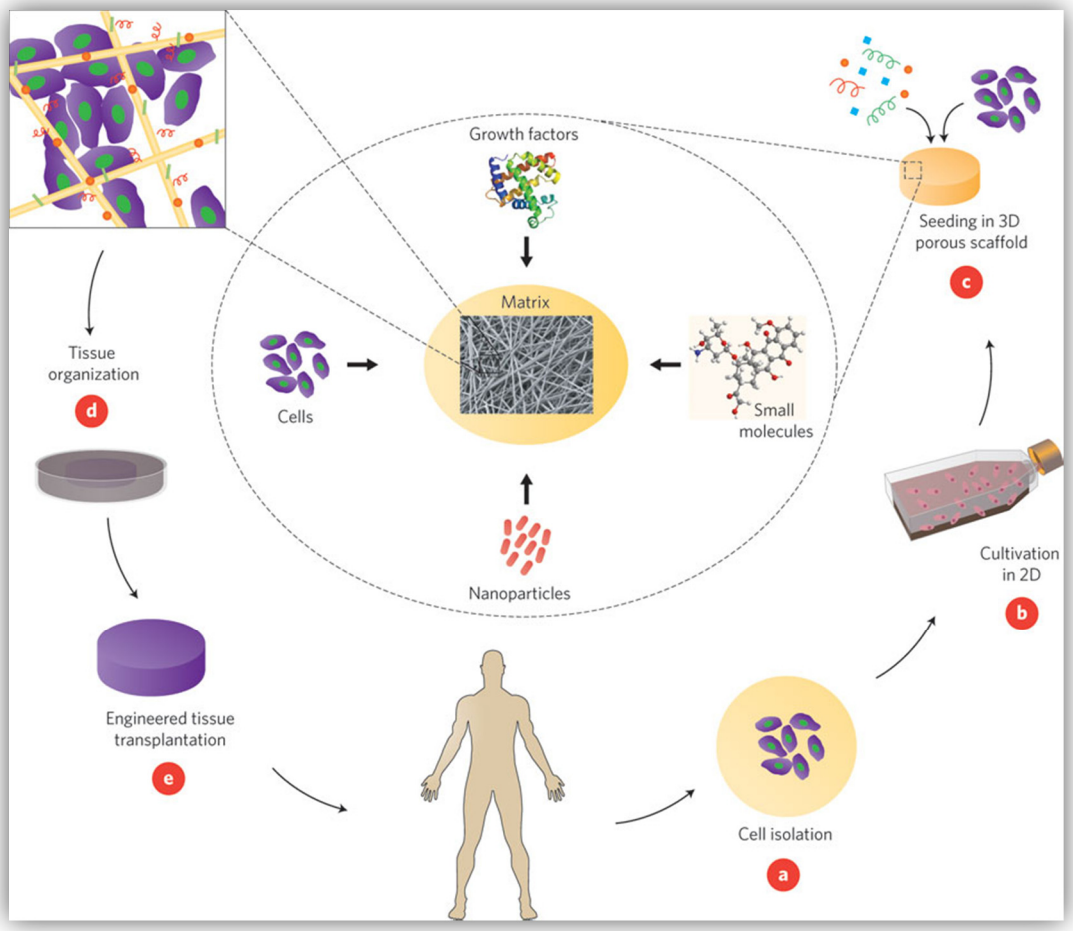
Introduction



1.1 Introduction

A scaffold is an artificial 3-D structure that serves as a mimic of extracellular matrix for cellular adhesion, migration, proliferation, and tissue regeneration in three dimensions. Also, one can define; Scaffolds as material that have been engineered to cause desirable cellular interactions to contribute the formation of new functional tissues for medical purposes. A scaffold in tissue engineering serves as an impermanent skeleton to accommodate and stimulate new tissue growth. It is typically made of polymeric biomaterials, provide the structural support for cell attachment and subsequent tissue development. However, researchers often encounter an enormous variety of choices when selecting scaffolds for tissue engineering [1].

An ideal scaffold should possess the following characteristics:

1. It is highly biocompatible and does not elicit an immunological or clinically detectable foreign body reaction.
2. It is three-dimensional and capable of regenerating tissue and organs in their normal physiological shape.
3. It is highly porous with an interconnected pore network available for cell growth, nutrient and metabolic waste transport.
4. It has a suitable surface chemistry allowing for cell attachment, migration, proliferation, and differentiation.
5. It has controllable degradation and resorption rates that match the rate of tissue growth *ex vivo*, and *in vivo* for biodegradable or resorbable materials.
6. It possesses the appropriate mechanical properties which match with the normal tissue and organs.
7. It has a bioactive surface to encourage faster regeneration of the tissue.

1.2 scaffolds for biomedical applications

Initially, scaffolds were derived from surgical materials. The tendency to adapt materials in current use for other applications offers advantages from the perspective of regulatory agencies such as the Food and Drug Administration, but does not necessarily promote development of optimal materials with regard to performance characteristics needed for different tissues [1-3]. It is desirable that the scaffolding biomaterial can be degraded as cells go through the process of forming their own supportive extracellular matrix (ECM). The permanent presence of implants can be expected to elicit a foreign-body response. Degradation is influenced by material composition, surface chemistry and topology. Bioactive scaffolding materials can be engineered to deliver growth factors/signals, to deliver cells, or to direct the three-dimensional orientation of cells. For example, certain biomaterials can aid hepatocytes to retain epithelial polarization [4].

The creations of a scaffold structure have the appropriate physical, chemical and mechanical properties to enable cell penetration and tissue formation in three dimensions [5]. Scaffold materials are 3-D tissue structures that guide, growth and differentiation of cells. Scaffolds must be biocompatible and designed to meet both nutritional and biological needs for the specific cell population [6]. Scaffold architectures such as high surface area to polymer mass ratio and volume ratio allow for tissue in growth, uniform cell delivery and development of high cell density [7].

An ideal biomaterial designed for clinical applications should fulfill a series of requirements. First of all, biocompatibility and biodegradability are essential; allowing scaffold replacement by proteins synthesized and secreted by native or implanted cells [8-10]. Besides, the material must be clinically compliant (Good

Manufacturing Practice) to minimize inflammatory and immunological response avoiding further tissue damage [8]. Moreover, as cell degradation products are toxic to other cells, it would be important that the material allow host macrophages

to infiltrate and remove cellular debris [11]. Finally, material production, purification and processing should be easy and scalable. Hence, use of appropriate scaffold material is of keen interest in the tissue engineering field [12, 13].

1.2.1 Extracellular Matrix (ECM)-mimicking scaffolds

1.2.1.1 ECM composition and functions

ECM is the natural medium in which cells proliferate, differentiate and migrate, and hence is the gold standard for tissue regeneration. Cell-ECM interaction is specific and biunivocal. Cells synthesize assembly and degrade ECM components responding to specific signals and, on the other hand, ECM controls and guides specific cell functions. This continuous cross-talk between cells and ECM is essential for tissue and organ development and repair. In physiological conditions, ECM composition derives from homeostasis, a fine dynamic balance of regeneration, differentiation and programmed cellular death (apoptosis), which continuously remodels ECM through protein breakdown and synthesis [11,12]. Natural ECM is a condensed matrix mainly composed of locally secreted proteins and polysaccharides, arranged as a molecular network formed by an intricate agglomerate of weaves, struts and gels interconnecting cells with matrix proteins. Amounts and organizations of these molecules are variable with tissue site and type, and during tissue development, in terms of resistance to tensile and compressive forces and transport properties. Dynamic properties of ECM are controlled by proteoglycans and a number of signalling molecules, such as growth

factors (GFs), which mediate cell-ECM and cell-cell interactions. All these molecules are embedded in an amorphous, fundamental substance represented by glycosaminoglycan chains, which form the highly hydrated gel structure imbibing the matrix [14].

1.2.2 Importance of 3D architecture

Recent advances in tissue culture have shown that cells respond differently in attachment, morphology, migration and proliferation on a 3D scaffold then in traditional two-dimensional tissue culture [11]. Many cell types such as fibroblasts, mesenchymal stem cells, epithelial cells, and neural crest cells show different adhesions when grown on 3D matrices as opposed to 2D cell culture. In 2D substrata, cultured cells are restricted to spreading and attaching to a flat rigid glass or tissue culture plastic surface coated with different substrates. The influence of biophysical properties of the material may be overwhelmed by the effect of the rigid surface. However, biophysical properties considerably influence cell adhesion, signaling and functions in 3D environment. Further, the 3D architecture could distribute binding sites differently than 2D architecture. 3D focal adhesions appear distinct from 2D focal adhesions on a rigid 2D matrix and are termed as "3D matrix adhesions" to separate them from 2D counterparts. In addition to proteins present in focal adhesions on 2D matrices, cells may have cytoskeletal adaptor proteins on 3D matrix. Such discrepancy in cell adhesion between 2D vs. 3D causes different signal transduction, subsequent altered cell morphology and rearrangement [12-14]. In response to different physical and chemical signals from surrounding 3D matrix, cells can synthesize ECM components and the degradation of matrix can create spatial advantages for cell

expansion and forward migration, unlike 2D architecture. Pore size and void fraction, stiffness, pore interconnectivity and topography can affect cell colonization in synthetic scaffolds. A majority of the cells are unable to completely colonize scaffolds with pore sizes > 300 nm due to difficulty in crossing large bridging distances. An average pore size range for many cell types is 100 – 150 nm. Hence matrices with that pore size range are preferred in many applications [14].

1.2.3 Types of scaffolds

Scaffold is the central component used to deliver cells, drugs, and genes into the body. The definition of the scaffold is categorized into two main categories: (1) a cell delivery scaffold and (2) a drug delivery scaffold. When cells are seeded into an artificial structure capable of supporting three-dimensional (3D) tissue formation, these structures typically are called "cell delivery scaffolds," and when drugs are loaded into a 3D artificial porous structure capable of high drug loading efficiency and sustained release of a drug for longer duration, they typically are called "drug delivery scaffolds."

Different kinds of polymeric scaffolds for cell/drug delivery are available: (1) a typical 3D porous matrix, which is a highly porous and well interconnected open pore structure that allows high cell seeding density and tissue in-growth, as shown in Figure1.1(A); (2) a nanofibrous matrix that is prepared by electrospinning or self-assembly would provide a better resemblance of the physiological environment Figure 1.1(B); (3) a thermosensitive sol-gel transition hydrogel Figure 1.1 (C); and (4) a porous microsphere Figure 1.1 (D).

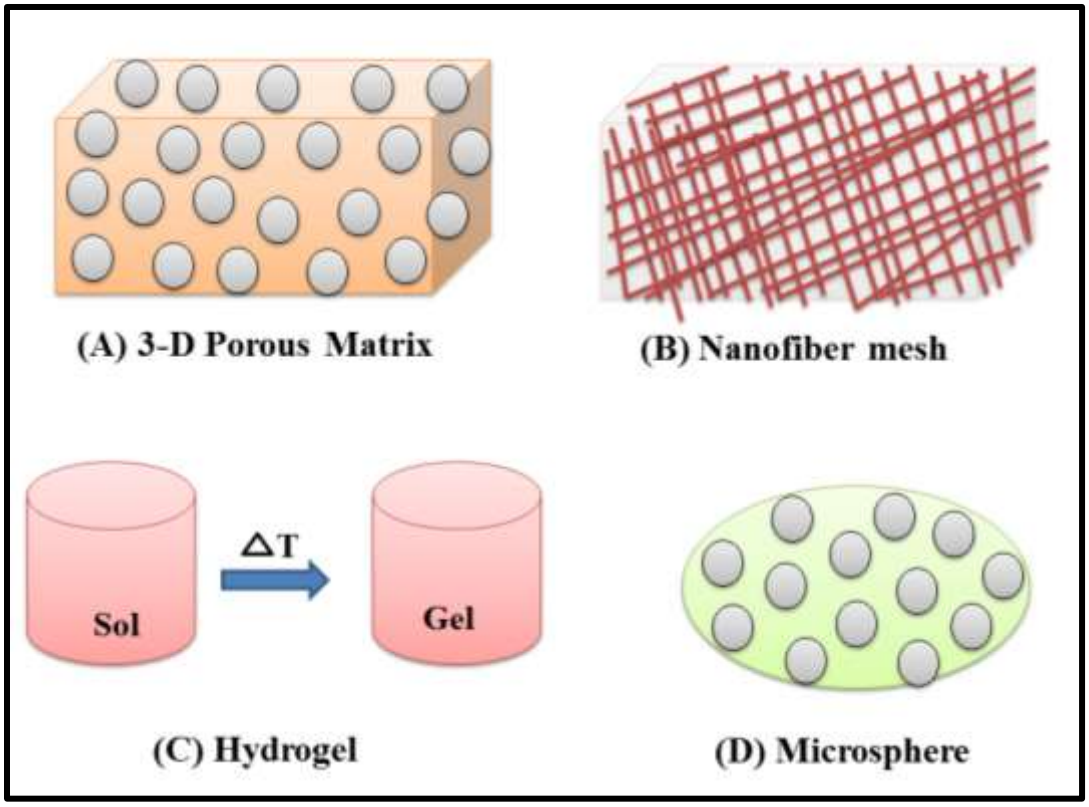


Figure 1.1 Different forms of polymeric scaffolds: (A) 3-D porous matrix; (B) nanofiber mesh; (C) hydrogel; and (D) microsphere used for cell and drug delivery

These are already generally utilized as sustained protein-release formulations and have been applied for the potential use as a cell delivery carrier or supportive matrix. Off the polymeric scaffolds mentioned above, a typical 3D porous matrix and nanofibrous matrix are the implantable forms and a thermo sensitive sol-gel transition hydrogel and porous microsphere are the injectable forms.

1.2.3.1 Porous scaffold

The scaffolds with three dimensional polymeric porous structures and higher porosities having homogeneous interconnected pore network are highly useful for tissue engineering. Their porous network simulates the ECM architecture enables cells to interact effectively with their environment. Sponge or

foam porous scaffold have been used for growth of host tissue, bone regrowth or organ vascularization [50,15]. Though foams and sponges are more mechanically stable compared to mesh structures, their use is still limited due to the open spaces present throughout the scaffold. Porous scaffolds can be developed with specific pore size, porosity, surface area to volume ratio and crystallinity. Porous controlled-release systems contain pores that are large enough to enable diffusion of the drug [15]. Synthetic biodegradable polymers such as Poly L-Lactic Acid (PLLA), Polyglycolicacid (PGA), Polylactic co-glycolic acid (PLGA) [16], Polycaprolactone (PCL) [17], Poly-D L-Lactic acid (PDLLA), Polyethylene oxide (PEO), and Polybutylene terephthalate (PBT) [18] are used as porous scaffolding materials. To control porosity and pore diameter as compared to most fabrication methods, a solvent casting and particulate leaching technique was developed. A recent method for creating porous scaffolds composed of nano and microscale biodegradable fibers by electrospinning is a latest development in this field.

1.2.3.2 Microsphere scaffold

Microsphere-based tissue engineering scaffold designs have significant attention in recent years [19]. Laurencin *et al.* [20] initially used a microsphere-based approach for tissue engineering scaffold. Microsphere scaffolds are increasingly used as drug delivery systems and in advanced tissue engineering applications such as gene therapy, antibiotic treatment of infected bone and so forth [21]. Microsphere scaffolds are generally a polymer matrix used for drug encapsulation for the release of drugs at a relatively slow rate over a prolonged period of time [22]. Polymers with low molecular weight used in developing porous microspheres for the rapid release of the drug, while polymers with high molecular weight for developing microspheres for a slower drug release profile which can be achieved due to its dense nature [23]. Injectable microspheres have also been developed for the controlled delivery of drugs [24]. Microspheres as building blocks gives several benefits, including ease of fabrication, control over

morphology, physicochemical characteristics and its versatility of controlling the release kinetics of encapsulated factors [25]. The methods used to produce microsphere-based scaffolds have utilized heat sintering [26,27], solvent vapor treatment [28,29], solvent/nonsolvent sintering method [30] or nonsolvent sintering technique [19]. Composite microspheres are also used for the production of polymer-ceramic matrices for bone applications. Chitosan microsphere scaffolds have been produced for cartilage and osteochondral tissue engineering [31].

1.2.3.3 Hydrogel scaffold

In the last decade, hydrogels have played an ever increasing role in the revolutionary field of tissue engineering in which they are used as scaffolds to guide the growth of new tissues. The design and application of biodegradable hydrogels has significantly increased the potential impact of hydrogel materials in the biomedical field and enabled the development of exciting advances in controlled drug delivery and tissue engineering applications [32]. Hydrogels consist of naturally derived macromolecules have potential advantages of biocompatibility, cell-controlled degradability and intrinsic cellular interaction. Hydrogels are made either from synthetic or natural polymers, which are cross-linked through either covalent or noncovalent bonds. Hydrogels are often favorable for promoting cell migration, angiogenesis, high water content, and rapid nutrient diffusion [33]. The hydrogel scaffolds have received an intensive study for their use in the engineering of replacement connective tissues, primarily due to their biochemical similarity with the highly hydrated GAG components of connective tissues. Examples of hydrogel forming polymers of natural origin are collagen [34], gelatin [35], fibrin [36], hyaluronic acid (HA) [37], alginate [38] and chitosan [39]. The synthetic polymers are PLA [40], Polypropylene fumarate (PPF) derived Copolymers [41], Poyethylene glycol (PEG-derivatives), and Polyvinyl alcohol (PVA) [42].

1.2.3.4 Fibrous scaffold

The development of nanofibers has improved the scope for fabricating scaffolds that can potentially mimic the architecture of natural human tissue at the nanometer scale. Currently, three techniques available for the synthesis of nanofibers: electrospinning, self-assembly, and phase separation. Of these, electrospinning is the most commonly studied technique and also seems to exhibit the most promising results for tissue engineering applications. Nanofibers synthesized by self-assembly [43] and phase separation [44] had relatively limited studies that explored their application as scaffolds for tissue engineering. The high surface area to volume ratio of the nanofibers with their microporous structure favors cell adhesion, proliferation, migration and differentiation, all of which are highly desired properties for tissue engineering applications [45,46]. Nanofibers used as scaffolds for musculoskeletal tissue engineering including bone, cartilage, ligament, skeletal muscle, skin, vascular, neural tissue engineering, and as vehicle for the controlled delivery of drugs, proteins and DNA [47]. Natural polymers and synthetic polymers explored for the fabrication of nanofibers such as collagen, gelatin, chitosan, HA, silk fibroin, PLA, Polyurethane (PU), PCL, PLGA, Polyethylene vinyl acetate (PEVA), and PLLA are fibrous scaffold in biomedical application.

1.2.3.5 Acellular scaffold

Acellular tissue matrices can be prepared by manufacturing artificial scaffolds or by removing cellular components from tissues by mechanical and chemical manipulation to produce collagen-rich matrices [48-50]. These matrices slowly degrade on implantation and are generally replaced by the ECM proteins secreted by the ingrowing cells. Acellular tissue matrices have proven to support cell ingrowth and regeneration of genitourinary tissues, including urethra and bladder, with no evidence of immunogenic rejection [50]. Ureteral acellular matrices were utilized as a scaffold for the ingrowth of ureteral tissue in rats [51].

Acellular bladder matrix has worked as a scaffold for the ingrowth of host bladder wall components in rats. The obvious advantage of this scaffold is that it is composed of ECM proteins typically found in the body. When derived from a vessel, the three-dimension architecture is very similar to that of the original, thus conferring appropriate mechanical and physical properties, which is essential in identifying and predicting optimal cell environments in order to develop scaffolds for preliminary analysis and implantation. Naturally derived materials and acellular tissue matrices have the potential advantage of biological recognition. Polymer coating of a tissue-derived acellular scaffold can improve the mechanical stability and enhance the hemocompatibility of the protein matrix.

1.3 Biomedical applications of stem cells

The stem cells and progenitor cell research development in recent years has opened new horizons for sources of cells issues for tissue engineering. In vitro tissue engineering requires the harvest of progenitor cells at various stages of differentiation for expansion and maturation on appropriate scaffolds in culture and subsequent implantation [52]. This form of tissue engineering is complicated by progenitor cell availability, difficulty in culturing some progenitor cell types, the need for cellular patterning, topographic control, and the need for providing a microcirculation for the development of lager, biologically meaningful tissues [53]. The harvest of adequate numbers of progenitor cells for in vitro expansion can be problematic. It depends on the cell source as well as cell type. For example, the harvest of osteoblast is time consuming, relatively few cells are available after dissociation of bony tissue and the cellular expansion rate is relatively low. Most differentiated progenitor cells have a limited capacity for self-renewal. Besides, regular culture techniques are not good for the long-term maintenance of certain cells. For example, the chondrocytes lose their normal morphology and function on petri dishes or in culture flasks. Hepatocytes rapidly lose their liver- specific

characteristics when maintained in standard tissue culture [54,55]. Hence, use of stem cells is increasing in order to avoid complications made by progenitor cells.

Stem cells are undifferentiated cells with a high proliferation capacity, the capability of self -renewal, and the potential for multilineage differentiation. There are two main types of stem cells which exists namely adult stem cells and embryonic stem cells. They can be obtained from living human tissue, from human embryos, as well as in the laboratory as shown in Figure 1.2. The cells contain in some organs are known as adult stem cells. They persist throughout the entire life of an individual and have an important role in repairing tissue. However, their capacity is limited and naturally not all organs include stem cells. Alternatively, embryonic stem cells have the ability to divide almost indefinitely, being the most important source of stem cells for research, as well as for therapy. Unfortunately, the only way known to derive embryonic stem cells supposes the destruction of unimplanted blastocyst-stage embryo at the 6th or 8th day of development. Human embryonic stem cells are isolated from human embryos. This is believed to be unmoral by some people. Hence, use of embryonic stem cells is becoming a limiting factor in tissue engineering field [56].

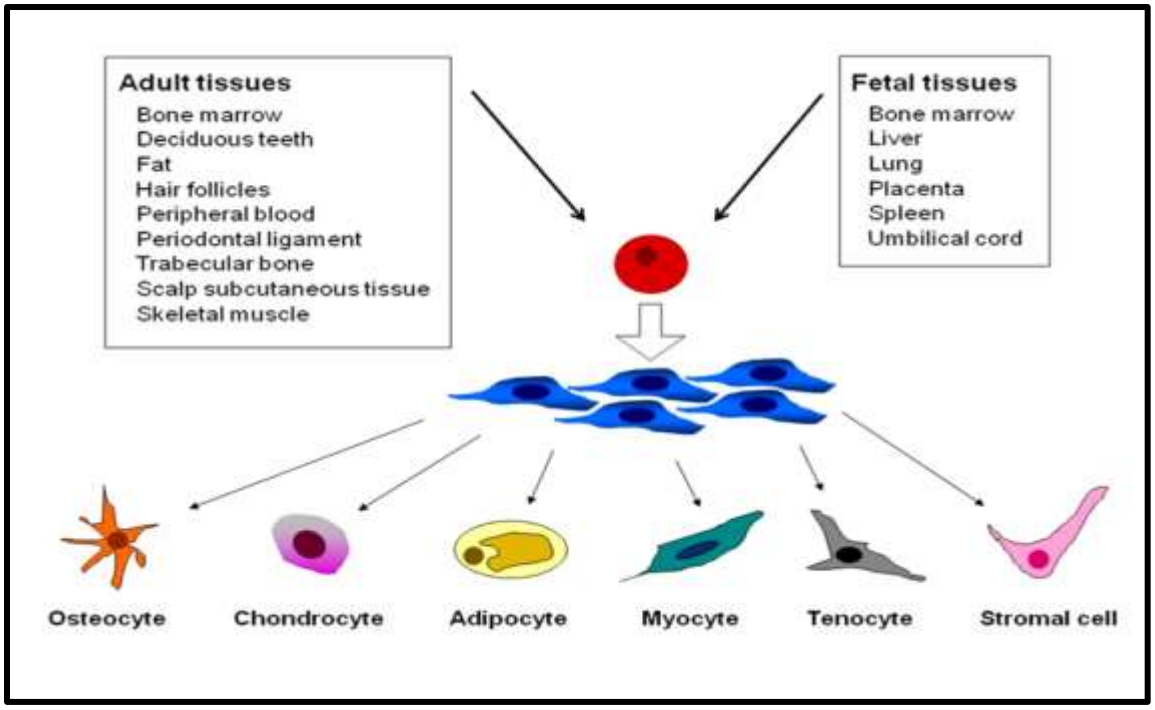


Figure 1.2 Possible sources of stem cells [56]

Totipotent stem cells are derived from the first two divisions of the fertilized oocyte and are able to form the embryo and the trophoblast of the placenta. A few days later, as the blastocyst forms, pluripotent stem cells can be harvested from the inner cell mass and these are known as embryonic stem cells (ESCs) [57]. These cells can differentiated into any tissue arising from three germ layers of the embryo but are not able to give rise to another embryo and its supportive tissue. Finally, multipotent stem cells can be harvested from mature tissues, and these are known as adult stem cells. Figure 1.3 shows schematics of ESCs isolation process and its possible appications. Adult stem cells can are being actively studied for tissue engineering because of the current ethical and sociopolitical debate.

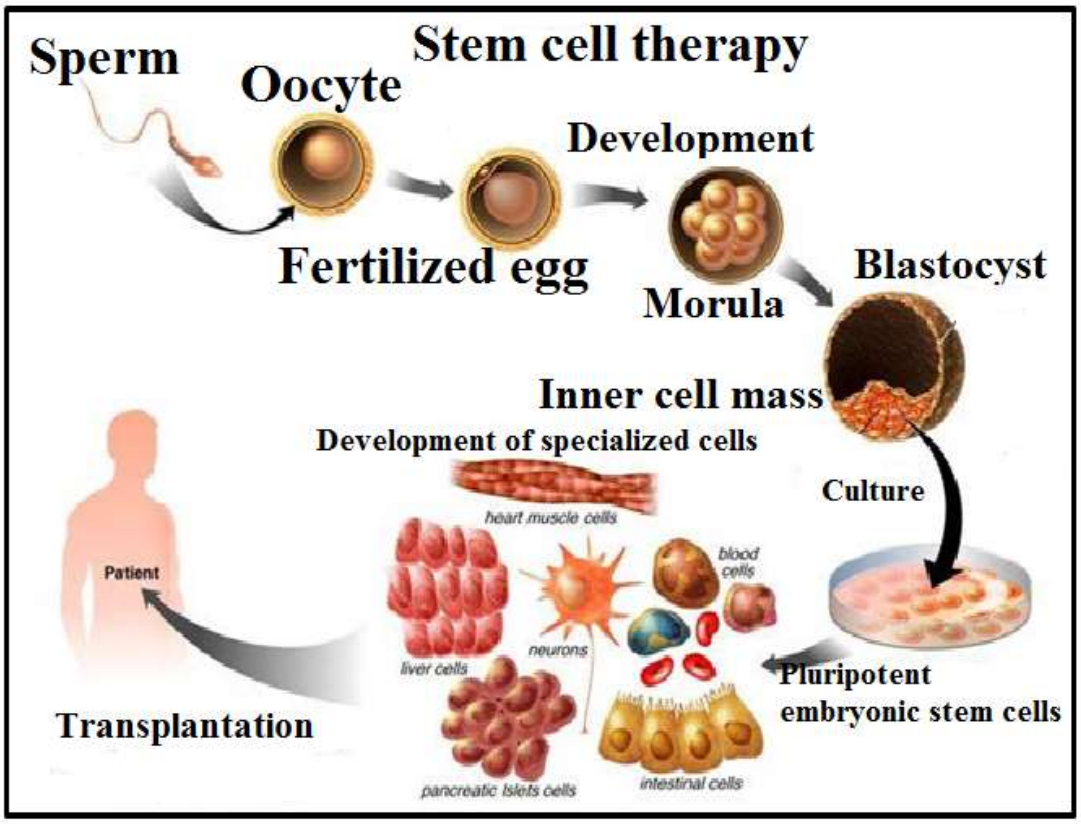


Figure 1.3 Embryonic stem cells and their possible therapeutic applications [57]

Furthermore, adult stem cells have been identified in bone marrow, muscle, periosteum etc. As they were all derived from mesenchyme, they were also called as mesenchymal stem cells (MSCs). MSCs are low in immunogenic nature and they have significant differentiation plasticity [58-60]. For instance, neural stem cells can differentiated into hematopoietic precursors and muscle cells, dermal stem cells can give rise to neurons, glia, muscle and fat cells. Bone marrow stromal cells can be induced to differentiate into osteoblasts, chondrocytes, myocytes, tendon cells, adipocytes, hepatocytes, or neural phenotypes. Limitation with adult stem cell use includes the lack of identifying markers and their scarcity in adults. It is estimated that 1 in 20,000 to 1 in 1,00,000 nucleated bone marrow cells is a stromal cell, representing 0.001%-0.01% of nucleated cells[58-60]. Whereas fat and muscle tissues are less cellular than bone marrow, the adult stem

cell prevalence in these tissues may be as high as 1 per 4000 cells [61]. But adult stem cell numbers and their differentiation capacity decrease with advancing age. The density of MSCs in newborns is approximately 1 in 10,000 as compared with 1 in 2, 00,000 in an 80 year old [62].

1.4 Interaction of scaffolds and stem cells

Since its emergence in the mid-1980s, tissue engineering has continued to evolve as an exciting and multidisciplinary field aiming to develop biological substitutes to restore, replace or regenerate defective tissues. Cells, scaffolds and growth-stimulating signals are generally referred to as the tissue engineering triad, the key components of engineered tissues. Scaffolds typically made of polymeric biomaterials; provide the structural support for cellular attachment and proliferation.

The general strategies adopted can be classified into three groups: (i) Implantation of isolated cells or cell substitutes into the organism, (ii) delivering of tissue inducing substances (such as growth factors), and (iii) placing cells on or within different matrices. The last of these strategies is more frequently associated with the concept of tissue engineering, that is, the use of living cells seeded on a natural or synthetic extracellular substrate to create implantable pieces of the organism. The scaffold is a 3D substrate for cells, and serves as a template for tissue regeneration. Ideal scaffolds should be biocompatible, biodegradable, and promote cellular interactions, tissue development, possess proper mechanical and physical properties.

Scaffold design and fabrication are major areas of biomaterial research, and they are also important subjects for tissue engineering and regenerative medicine research [63]. Scaffold plays a unique role in tissue regeneration and repair. During the past two decades, various kinds of work have been done to develop

potentially applicable scaffold materials for tissue engineering. The developing scaffolds with the optimal characteristics, such as their strength, rate of degradation, porosity, and microstructure, as well as their shapes and sizes are more readily and reproducibly controlled in polymeric scaffolds [64]. The few scaffolds that have displayed biological activity have induced regeneration of tissues and organs that do not regenerate spontaneously. Biological scaffolds are derived from human, animal tissues and synthetic scaffolds from polymers. The first biologically active scaffold was synthesized in 1974; its degradation behavior and exceptionally low antigenicity *in vivo*, as well as its thromboresistant behavior *in vitro*, were described [65]. The initial patent describing these scaffolds was granted in 1977 [66]. Principles for synthesizing a biologically active scaffold, including the critical importance of the degradation rate, were described in detail in 1980 [67]. The first reports of induced regeneration of tissue in an adult (dermis) by a scaffold in animals [68, 69] and humans [70,71], is a peripheral nerve regeneration across a gap of unprecedented length, and regeneration of the conjunctiva.

Table 1.1 Cell sources and biomaterials used for biomedical applications

	Biomaterials used			**Cells**
Tissue Engineering area	Natural	Bioceramics	Synthetic	
Bone	Autologou grafts, Alginate, Chitosan,	Hydroxyapatite	PCL, PEG, PGA, PLA, PLG, PLGA	Adipose Stem Cells, Bone Marrow Stem Cells

	Collagen, Fibrin, Gelatin			
Articular Cartilage	Alginate, Agarose, Chitosan, Collagen, Fibrin, Gelatin, Hyaluronan	----	elastin-based polymers, PCL, PEG, PGA, PLA, PLGA, polyurethane	Chondrocytes, Adipose Stem Cells, Bone Marrow Stem Cells, umbilical cord cells
Heart	Agarose, Alginate, Chitosan, Collagen, Elastin, Fibrin, Gelatin, Hyaluronan, Laminin, M atrigelTM, Platelet gel, Silk, Starch, Vitronectin	----	PCL, PEG, PGA, PLA, PLGA, Polyesters, PPF	Resident Cardiac Stem Cells, Cardiac Myocites, Skeletal Myoblasts, Embryonic Stem Cells, Bone Marrow Stem Cells, Adipose Stem Cells, Endothelial Pluripotent Cells, Umbilical Cord cells
Pancreas	Agarose, Alginate, Collagen, Chitosan,	----	Acrylonitrile copolymers, NIPA, PEG, PLA ,	β-cells, Embryonic stem cells, Stem/Progenitor cells of the Ductal

	Laminin, Matrigel		PLGA,	Epithelium, Bone Marrow Stem Cells, Hepatic Cells
Vasculature	Agarose, Alginate, Chitosan, Collagen, Elastin, Fibrin, Gelatin, Hyaluronan, Matrigel TM	Hydroxyapatite, Silica-based Mocrospheres	PEG PGA, PGLA, PLA, Polycarbonate, Polyethylene oxide, Polyester, Polypropylene oxide, Polyuretane	Adipose-derived stem cells, Endothelial cells, MSCs, Smooth muscle cells
Cancer	Alginate, Collagen, Gelatin, Hyaluronan, Laminin, Matrigel	----	PEG, PLA, PLG, PLGA	Tumoral Cells

Biomaterials play a crucial role in this technology by serving as synthetic frameworks referred as scaffolds, matrices or constructs. The state of the art in biomaterials design has continuously evolved over the past few decades. In recent years, there has been increasing demand of materials that could be used in biomedical areas. Scaffold materials can be synthetic or biologic, degradable or nondegradable, depending on the intended use [72]. The properties of polymers based on the composition, structure and arrangement of their constituent macromolecules. It can be categorized into different types in terms of their

structural, chemical and biological characteristics, for example, ceramics, glasses, polymers and so forth. Naturally occurring polymers, synthetic biodegradable, and synthetic nonbiodegradable polymers are the main types of polymers used as biomaterials.

Natural polymers can be considered as the first biodegradable biomaterials used clinically [73]. Natural materials having the bioactive properties have better interactions with the cells; allow them to enhance the cells performance in biological system. Natural polymers can be classified as proteins (silk, collagen, gelatin, fibrinogen, elastin, keratin, actin, and myosin), polysaccharides (cellulose, amylose, dextran, chitin, and glycosaminoglycans), or polynucleotides (DNA, RNA).

1.5 Chitosan-alginate (Chi-alg) scaffolds

There are varieties of materials that can be used to create a 3D scaffold for cell culture. Among these are chitosan and alginate, two cheap and easily accessible polymers that yield superior qualities when combined to make a scaffold.

Chitosan is a biopolymer that is derived from the shells of crustaceans. It has been shown in previous research to be extremely biocompatible [74,75]. Cells can easily stick to this material and grow. When fabricated into a scaffold, it has a very high porosity and high inter-pore connectivity. When absorbed in solution, chitosan scaffolds absorb large amounts of liquid and swell considerably. It is also very soft and spongy. These characteristics can be remedied by the addition of alginate, a polymer derived from brown algae. The addition of alginate enhances inter-pore connectivity. When the chitosan-alginate scaffolds are in a solution containing calcium ions, these ions interacts and cross-links the alginate. This provides more rigidity for the scaffold, allowing the scaffold to absorb solution without considerable swelling.

When considering a material from which to fabricate an osteochondral tissue-engineered scaffold, chitosan-alginate (chi-alg) was deemed an optimal selection due to its ability to promote both osteogenesis and chondrogenesis, both *in vitro* and *in vivo* [74-76]. Chi-alg scaffolds have been used in cartilage tissue engineering particularly because they are naturally occurring polysaccharides similar in chemical structure to articular cartilage ECM glycosaminoglycans (GAGs) like chondroitin sulfate, keratan sulfate, and hyaluronic acid that provide cartilage with its notable properties such as resistance to compression and swollen state [75]. Further, Chi-Alg scaffolds can be prepared at neutral pH, allowing growth factors and drugs to be uniformly incorporated without denaturation [77].

Several groups have examined the use of chitosan–alginate fibrous scaffolds; these micron-sized chitosan–alginate polyelectrolyte complexed fibers have been examined for gene delivery [78], mesenchymal stem cell encapsulation [79] and annulus fibrosus cell culture [80]. Another group demonstrated the ability to form chitosan–alginate microfibers by spraying a chitosan solution into a stirred alginate solution [81].

1.6 Statement of problem

The dynamic development of tissue engineering and search for new biocompatible substances over the last two decades has changed the proportions of used biomaterials. While at the end of the 1950s, the most popular were metallic materials and their alloys, in the following year's interest in polymers increased. Polymers can be widely used in biomedical and food applications. They are biodegradable, biocompatible, non-toxic, reactive and bioactive and have water binding capacity. Among these, the biodegradable tissue scaffold becomes a new hot spot in the tissue engineering field. They can be gradually degraded in a certain period of time after implantation. Therefore, the biodegradable tissue scaffolds offer a number of distinct advantages over the permanent scaffolds, for

example, avoiding a second surgery to remove the implants [82, 83], reducing rate of rejection, and controlling the release rate of the drug loaded scaffolds [84].

In nature, it is hard to find a perfect polymer, which would possess all of these features. New materials that combine optimal mechanical, physical, chemical and biological features are being looked for currently. The expectations of these properties are quite broad and include mechanical properties as elastic modulus, shear modulus, yield strength, tensile strength and resistance to cracking and fatigue. Important parameters of physical conditions are: density, topography, roughness, shape or electric and magnetic properties. Chemical structure, adhesion, biofunctionality, bioactivity and biodegradability are the advantages of chemical and biological agents. During manufacture or treatment of such material, the essential features are reproducibility, ease of sterilization and reliability. The solution appears to be composites consisting of at least two polymers with different properties.

Hence, in these studies we have used chitosan-alginate biopolymers for fabrication of chitosan-alginate scaffolds which forms macro porous 3D structures, used for tissue engineering applications like cell/stem cell delivery for regeneration and repair of tissue, drug encapsulation for sustained release of drug, and DNA/gene delivery.

A part of this study is devoted to synthesize chitosan-alginate scaffolds by freeze drying method. Such Chi-alg scaffolds can be used for biomedical applications like drug delivery, cell delivery and DNA delivery. In this research, mesenchymal stem cells were isolated from human umbilical cord tissue which can be seeded on to scaffolds for *in vitro* studies. The specific aim of this study, thus, involves:

- To study structural and morphological properties of chi-alg scaffolds synthesized by freeze drying method, due to its overriding advantages such as feasibility, economical and time saving method.

- To study antibacterial property of synthesized chi-Alg scaffolds against *E. coli* and *S. epidermidis*. For this purpose, Agar plate diffusion assay was planned and Antibacterial activity assay carried out using ELISA plate reader.

- To investigate biomedical properties of chi-alg scaffold, like biocompatibility, rheological study and hematocompatibilty assay which proves suitability of chi-alg scaffold for *in vivo* applications. Biocompatibility study of chi-alg scaffolds was planned with the Almar blue assay and MTT assay.

- To isolate mesenchymal stem cells from human umbilical cord tissue and study its growth kinetics in culture. Such isolated cells were seeded onto scaffolds and proliferation study was planned with cell viability and proliferation studies. Histology studies also were planned.

- To investigate effect of chi-alg scaffold on storage conditions of stem cells (cryopreservation of cell-scaffold construct).

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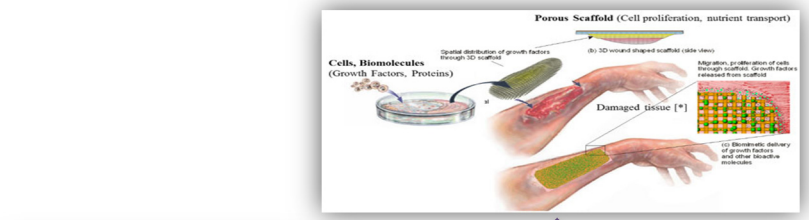
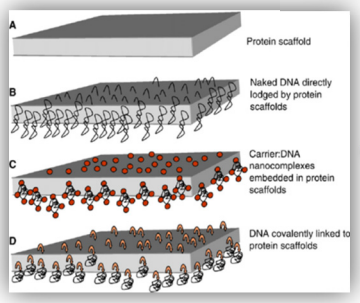
Chapter 2

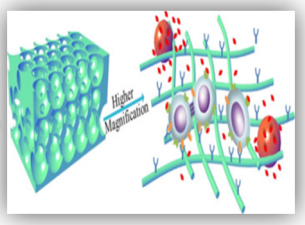
Chitosan-Alginate Scaffolds and Their Biomedical Applications: A Review



Wound Healing

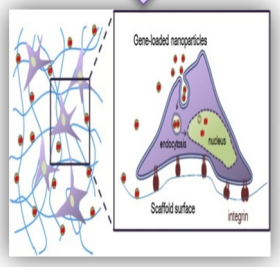


Chitosan-Alginate Scaffolds



Drug Delivery

Gene/DNA



Cell/ Growth Factor Delivery

2.1 Introduction

Tissue Engineering is addressed to create functional three-dimensional (3D) tissues combining scaffolds, cells and/or bioactive molecules [1]. It is an interdisciplinary discipline and involves basically three basic elements: scaffolds, cells and biomolecules [2].

A major goal in Tissue Engineering is the design of scaffolds capable of recreating the *in vivo* microenvironment that is provided mainly by the ECM. Thus, these structures should incorporate the appropriate biophysical, biomechanical and biochemical cues those guide cell proliferation, differentiation, maintenance and function [3]. The appropriate scaffold for tissue engineering will be one that is created with biology in mind. Ideally, the scaffold provides a temporary pathway for regeneration and will degrade while or after healing, thereby obviating the need to remove the material later and eliminating possible side effects allied with leaving materials in the body. Of course, attention must be paid to ensure the degradation products are non-cytotoxic [4-8].

Development of biomaterials also poses significant challenges. Formation of implanted tissue is greatly influenced by the composition, architecture, and Scaffold's three-dimensional environment and biocompatibility of the biomaterial. In addition to being biocompatible, an ideal biomaterial scaffold for tissue regeneration can now be bioactive, biomimetic, biodegradable and bio responsive, providing signalling with spatio-temporal control and response which is selective to defined stimuli. Scaffold material's mechanical strength needs to mimic the mechanical properties of the tissue which is intended to repair or replace. Moreover, material porosity, pore size distribution and continuity greatly influence the attachment of specific cell types and interaction of the biomaterials with the host [9].

Properties of scaffolds

Scaffolds provide cells with a suitable growth environment, optimal oxygen levels, and effective nutrient and waste transportation as well as mechanical integrity. Scaffolds also provided 3D environments to bring cells in close proximity so that they can organize to form tissues. While the scaffold is being degraded, the cells produced their own extracellular matrix (ECM) molecules and eventually formed 3D structures mimicking the native tissue in morphology.

Many factors affect the selection and properties of scaffold. These include but not limit to biocompatibility, porosity, pore size, surface properties and pH, surface charge, biodegradability, mechanical properties and ideally, the ability to recruit variety of cells. Several requirements have been identified as crucial for the production of scaffolds which are represented in Figure 2.1[10, 11].

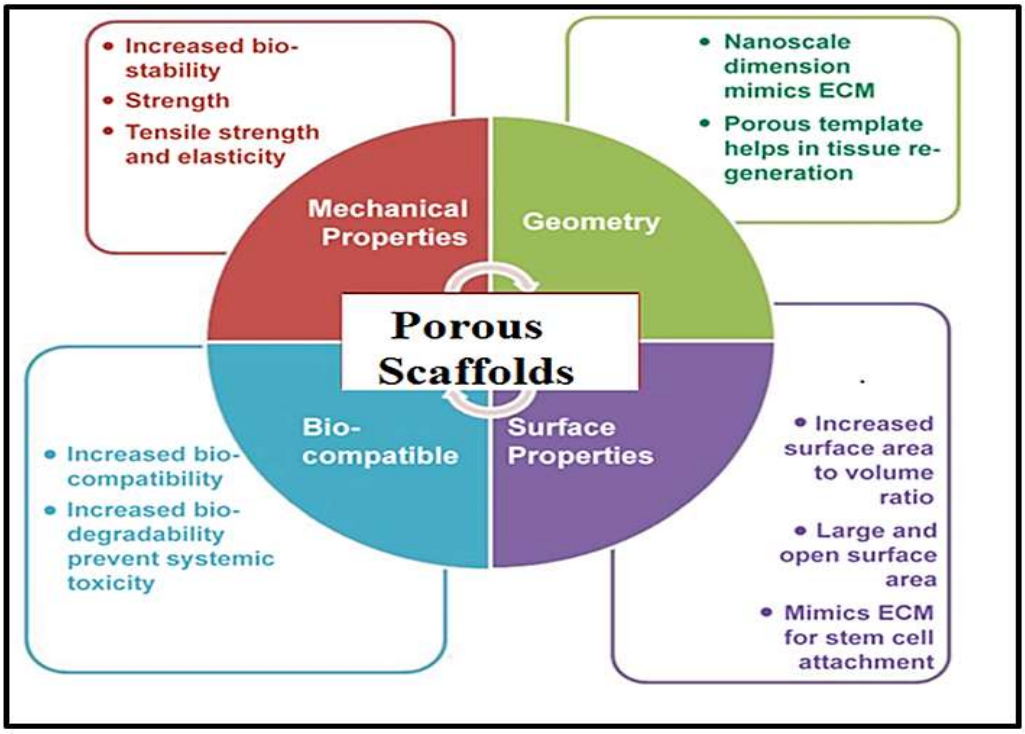


Figure 2.1 Schematic representation of properties of porous scaffold which includes geometry, mechanical competence, bio-compatibility and degradability and surface properties [11]

The scaffold should possess acceptable biocompatibility and toxicity profiles. Biocompatibility is the ability of the scaffold to perform in a specific application without eliciting a harmful immune or inflammatory reaction. If the scaffold is nontoxic and degradable, new tissue will eventually replace it, whereas if it is nontoxic and biologically active, the scaffold will integrate with the surrounding tissue. However, Fibrous capsule may encapsulate the scaffold if it is biologically inactive. In the worst case rejection of the scaffold and localized death of the surrounding tissue can occur when the scaffold is toxic.

The scaffold material should be biodegradable and its degradation products should not be toxic and should be eliminated easily from the implantation site by the body, eliminating the need for further surgery to remove it. The scaffold's degradation rate should be adjusted to match the rate of tissue regeneration so as to get disappeared completely once the tissue is repaired.

2.2 synthesis methods of chitosan-alginate scaffolds

Scaffold design and fabrication are major areas of biomaterial research, and they are also important subjects for tissue engineering and regenerative medicine research [12]. During the past two decades, many works have been done to develop potentially applicable chitosan-alginate scaffold for tissue engineering using various techniques. *Nicola L. Francis et al.* [13] used freeze casting, a directional solidification method to fabricate CA scaffolds with longitudinally aligned pores extending from end to end which produces porous structures through ice templating. They have done slight modification of freeze drying method by introducing a custom-made cylindrical polytetrafluoroethylene (PTFE) mold fitted with a copper bottom plate and secured onto the temperature-controlled copper cold finger of the freeze casting system. Similarly, *Alexander Gamboa et al.* [14] used spray freeze drying instead of casting. Spray freeze-drying using chitosan (SFDC) or alginate (SFDA) was proposed as an alternative to the traditional CA scaffold freeze drying synthesis method. Chitosan and alginate nano-composite

(NP) carriers intended for colonic delivery containing prednisolone and inulin were obtained by two above mentioned processes.

Jing Han et al. [15] prepared alginate-chitosan/hydroxyapatite polyelectrolyte complex porous scaffolds by using a wet chemical coprecipitation approach. Importantly, the PEC formation process provides a simple and effective method to fabricate scaffolds with different surface properties. *Yi-Cheng Ho et al.* [16] used homogenizing interpolyelectrolyte complex method for synthesizing chitosan-alginate PEC gel. Such PEC gel was used for controlled release of growth factor (bFGF). This modified method can be used to produce stably immobilized heparin on the 3-D chitosan-alginate PEC complex. *Shan-hai-Hsu et al.* [17] used hyaluronate in combination with chitosan-alginate scaffolds for cartilage regeneration. They have studied different combinations of such complex like C1A1R, C1A2R, C1A1HR and C1A2HR. Amongst them, they depicted that C1A1HR was a potential scaffold for cartilage regeneration based on the biochemical and histological analysis of the in vivo implants as well as previous in vitro.

Hyeong-Ho Jin et al. [18] synthesized porous hydroxyapatite (HAp)/ chitosan-alginate composite scaffolds through in situ co-precipitation and freeze-drying for bone tissue engineering. They have proven that HAp/chitosan–alginate composite scaffold has been shown to be more effective for new bone generation than chitosan–alginate scaffold. *Hye-Lee Kim et al.* [19] produced chitosan-alginate scaffolds with hydroxyapatite nanoparticles by modified freeze drying method. Furthermore they concluded that the composite scaffolds containing nano-HAp, as compared to the chitosan/alginate composite scaffolds, exhibited improved osteoblastic differentiation for bone regeneration, as determined by ALP activity and the mineralization of alginate/chitosan scaffolds.

Hui Ling Lai et al. [20] synthesized chitosan-alginate sponges instead of scaffolds for controlled drug release studies. They demonstrated that the use of chitosan and alginates together, appears to allow the formulator to manipulate both

the mechanical properties and the drug release properties of the sponges. *Xin Meng et al.* [21] investigated the characteristics and drug release properties of membranes of chitosan and alginate prepared via a casting/solvent evaporation technique. They have produced scaffolds like CA-1, CA-2, CA-3 and CAS-1, CAS-2 respectively. On the basis of the requisite physical properties, the chitosan–alginate PEC membrane can be considered for potential wound dressing or controlled release application.

Dong-Joon Park et al. [22] synthesized chitosan-alginate scaffold through sonication method. They also incorporate mesenchymal stem cells and BMP-2 protein into scaffolds for bone tissue engineering. This study showed that chitosan-alginate gel/MSCs/BMP-2 composites could become clinically useful injectable materials to generate new bone. Similarly, *Dunia M. Garcia Cruz et al.* [23] prepared chitosan scaffolds by combination of freeze gelation and leaching out technique. This study confirmed that GBMSCs seeded in chitosan scaffolds with micro and macro pores are able to attach and gradually proliferate along 4 weeks of culture. Again, *Matthew Leung et al.* [24] prepared chitosan-alginate scaffolds by freeze drying method for studying hepatocellular carcinoma and its drug resistance. This 3D model of HCC, with its ability to more closely mimic the in vitro tumor behavior, may serve as an invaluable model for study and application of novel anticancer therapeutics against HCC. *Sakchai Wittaya-areekul et al.* [25] synthesized chitosan/alginate microparticles by complex coacervation method using sodium alginate as a gel core. The results shows that depending on the preparation method these chitosan coated alginate particles show different mucoadhesiveness whereas their other properties are not statistically significant different.

2.3 Structural behavior of CA Scaffolds

2.3.1 Structure of Chitosan and its structural modifications

Chitosan is a natural, cationic amino polysaccharide (pKa 6.5) copolymer of glucosamine and N-acetylglucosamine gained by the alkaline, partial deacetylation of chitin. The name chitosan is used for a co-polymer with less than 40 percent DA (i.e., more than 60 percent DD) that, in most cases, will be soluble in dilute acid. Biological source and the extraction procedure used influence the Chitosan quality. Chitosan derived from crustacean sources like shrimp and crab shells and squid bone plates have a high molecular weight with low polydispersity. Figure 2.2 represents the structure of chitosan.

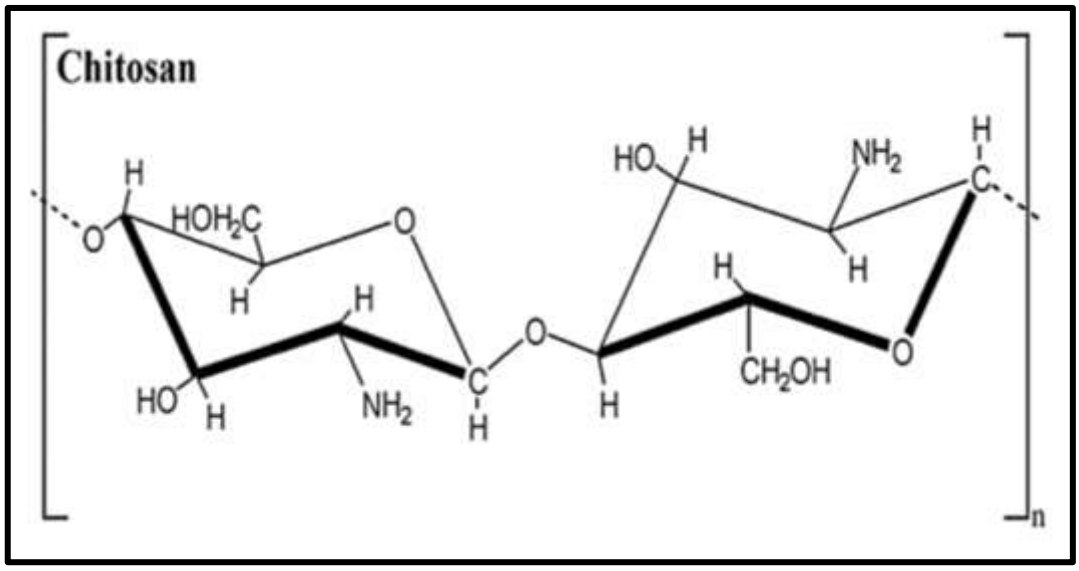


Figure 2.2 Structure of Chitosan

Pure chitosan is devoid of antigenic effects, biocompatible, non-toxic, biodegradable and polar. It has been used to prepare a variety of forms like powders, hydrogels, fibers, membranes, beads and porous scaffolds which have been tested in many medical and biological applications. Tissue engineering applications of chitosan scaffolds have been prepared by the freeze drying and freeze gelation methods and their mechanical and biological properties have been characterized [26].

The property of Chitosan allows it to speedily clot blood and gain approval for use in bandages and other hemostatic agents that are used in tests to quickly stop bleeding and reduce blood loss. The chitosan salts can be mixed with another materials to make them more absorbent (such as mixing with alginate), or to vary the rate of solubility and bio-absorbability of the chitosan salt. Chitosan can be used to deliver a drug to an acidic environment, where the chitosan packaging will then degrade, releasing the drug to the desired environment. One example has been the transport of insulin [27].

Chitosan polysaccharide has been explored for tissue engineering application in recent years. It is derived by the deacetylation of chitin and consists of glucosamine units. Additionally, the rate of gelation of chitosan scaffolds can be controlled using pH. Chitosan has been widely used as material for regenerating skin, bone and nerve tissue and has more recently been studied for use in combination with stem cells. One of the studies states that ability of such 3D scaffolds to promote osteogenic differentiation of mouse mesenchymal stem cells [28]. This study exhibited that the addition of corraline, another seaweed derived material, enhanced osteocalcin release over time, which is important for bone formation. A different method for bone tissue engineering used adipose-derived mesenchymal stem cells seeded inside of chitosan particles, which were then aggregated to form scaffolds. By means of using a particle aggregation method, it was possible to obtain chitosan-based scaffolds with very promising properties for the use in cartilage and osteochondral applications [29].

G.T. Franzesi et al. [29] reported a simple method of micromolding rapidly gelling, based on controlled release of the gelling agent from the mold of chitosan and alginate. This soft lithographic approach is easy to implement and can be used to produce microparticles and micropatterned membranes for a variety of applications, such as scalable cell culture systems, diagnostics, drug delivery, and tissue engineering. *Lie Ma et al.* [30] fabricated scaffolds from collagen and chitosan by freeze drying method with glutaraldehyde to improve their biostability

for skin tissue engineering. They recommended collagen/chitosan scaffold cross-linked by GA is a potential candidate for dermal equivalent with enhanced biostability and good biocompatibility. One of the studies demonstrated use of chitosan-gelatin scaffolds for tissue engineering applications. The combination of gelatin with chitosan on scaffold stiffness properties and degradation kinetics were characterized in this study [31].

Yet another combination is studied by *Yong Zhang et al.* [32]. They have used chitosan scaffolds reinforced by b-tricalcium phosphate (b-TCP) and calcium phosphate invert glass fabricated with a low-cost, bioclean freeze-drying technique via thermally induced phase separation for tissue engineering applications. This study suggests that the desirable pore structure, biodegradation rate, and bioactivity of the composite scaffolds might be achieved through controlling the ratio of chitosan and calcium phosphates or β-TCP and the glass.

W.W. Thein-Han et al. [33] defined a comparative assessment of the structure–property–process relationship of three-dimensional chitosan–nanohydroxyapatite (nHA) and pure chitosan scaffolds in conjunction with their respective biological response with the aim of advancing aspects of bone tissue engineering. Their observations correlated to well-developed structure morphology, physicochemical properties and superior cytocompatibility suggest that chitosan–nHA porous scaffolds are potential candidate materials for bone regeneration although it is necessary to further enhance the mechanical properties of the nanocomposite. *T.H. Ang et al.* [34] also used same combination of chitosan and hydroxyapatite for scaffolds fabrication but used different method i.e. robotic dispensing system for tissue engineering applications.

Similar kind work is carried out by *Lijun Kong et al.* [35] for synthesizing chitosan-hydroxyapatite scaffolds for bone tissue engineering. They suggested that the addition of nano-HA to chitosan scaffold improved its bone bioactivity, which could develop the use of chitosan in bone tissue engineering. *Jieliang Li, Jilun Pan et al.* [36] conjugated fructose onto the inner surface of highly porous chitosan

scaffold prepared by lyophilization for hepatocyte culture. The results suggested that the fructose-modified porous scaffolds would be helpful in development of the artificial liver support system. *Nandana Bhardwaj et al.* [37] examined the porous polyelectrolyte complex scaffolds of silk fibroin and amino polysaccharide chitosan for tissue engineering applications. The results suggested that silk fibroin and chitosan, the blended scaffolds are promising candidates for various tissue engineering applications particularly for cartilage.

2.3.2 Structure of alginate and its structural modifications

Alginate is usually extracted from seaweed, such as brown algae or from some bacterial species. They consist of unbranched, binary copolymers of 1-4 linked β-D- mannuronic acid (M) and α-L-guluronic acid (G) of widely varying composition. The structure is influenced by the seaweed source as well as the growing conditions of the weeds. Alginate has carboxyl end groups hence called as an anionic mucoadhesive polymer. Figure 2.3 represents the structure of sodium alginate [38, 39].

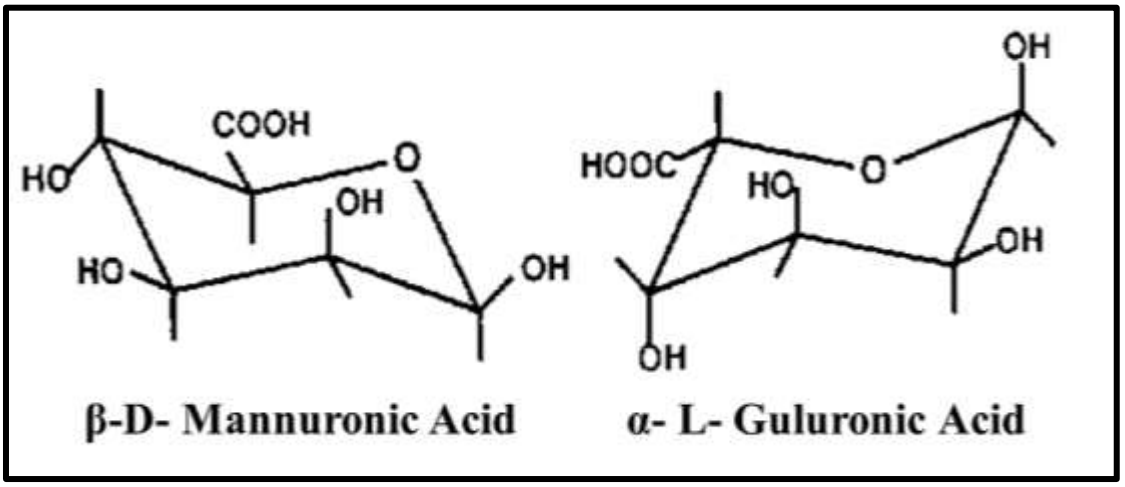


Figure 2.3 Structure of Sodium Alginate

The use of alginate scaffolds in tissue engineering applications is limited, however, owing to their weak mechanical properties, lack of cellular interactions,

and uncontrollable degradation. Scaffolds made from alginate are soft and weak, which may limit their further application as templates for tissue regeneration. Because alginate is hydrophilic, alginate scaffolds have limited protein adsorption capacity; thus, most of cells do not adhere to the scaffold. Furthermore, once the scaffold gets dissolved in the medium, the loss of divalent cations into the surrounding medium causes the ionically cross-linked alginate's uncontrollable degradation. To improve these limitations, we propose using chitosan as a reinforcing material to make porous alginate-chitosan composite scaffolds [39].

In recent years alginate can be combined with variety of biomolecules for enhance its activity. *Taesik Chae et al.* [40] fabricated Hydroxyapatite/alginate nanocomposite fibrous scaffolds via electrospinning method for bone tissue regeneration. Similar combination is tried by *Darilis Sua rez-Gonza lez et al.* [41]. They have developed macroporous, HAP-coated alginate scaffolds using a biomimetic approach. Their results suggested that the scaffolds may ultimately be used in bone tissue engineering applications as a scaffold material to deliver stem cells, and perhaps also biologically active molecules. Again, *A. Bernhardt et al.* [42] synthesized alginate scaffolds with orientated tube-like pores reinforced with HA and covalently cross-linked gelatin. Results suggested that alginate–gelatine–HA scaffolds are promising candidates for application in bone tissue engineering.

Yongxiang Luo et al. [43] developed a composite material consisting of mesoporous bioactive glass (MBG) and concentrated alginate pastes for fabrication of hierarchical scaffolds by 3D plotting. Results have indicated that 3D-plotted MBG incorporated alginate scaffolds with well-ordered nano-pores, controllable large pores, and significantly improved physicochemical, biological and drug-delivery properties could be a platform for bone tissue engineering. *Sang Ho Cho et al.* [44] fabricated porous alginate/polyvinyl alcohol (PVA) hybrid scaffolds as bioartificial cell scaffolds to improve cell compatibility as well as flexibility of the scaffolds. *Jennifer S. Wayne et al.* [45] synthesized Polylactic Acid–Alginate Scaffolds for cartilage tissue engineering applications. This finding

suggested that the constructs prepared from the PLA–alginate amalgam may serve as a means for host cell attachment.

Geun Hyung Kim et al. [46] designed a hybrid (core/shell) scaffold composed of an outer collagen and an inner alginate polymer for skin tissue regeneration. They depicted, this core–shell cryogenic technology can be expanded with various growth factors and peptides in the core area, so that functional scaffolds can be readily designed. *F. Despang et al.* [47] scaffolds with tube-like and regular pores from alginate/calcium phosphate composites and to stabilise them by mineralisation with hydroxyapatite from solution for osteoblast culture. *Yong Bok Kim et al.* [48] described a novel hybrid scaffold comprising microsized struts with a core (PCL)–shell (collagen/alginate) structure. These results indicate that the in vitro cellular activities of the hybrid scaffolds were identical to those of the pure collagen scaffold; moreover, the hybrid scaffold exhibited significantly improved mechanical properties. *V.V. Divya Rani et al.* [49] fabricated a nanocomposite scaffold using alginate with nanoTiO2 needles by lyophilization technique. Such alginate/nanoTiO2 composite scaffold can be used as a better option for tissue regeneration. Similarly, *Chiming Yang et al.* [50] have used another novel combination gelatin-alginate for study the differential invivo and invitro response of bone marrow stromal cells.

2.3.3 Structure of CA Scaffolds and its structural modifications

Chitosan and alginate alone has low mechanical strength and high rates of degradation. Therefore it should be used in composites to improve material properties and reduce degradation rates.

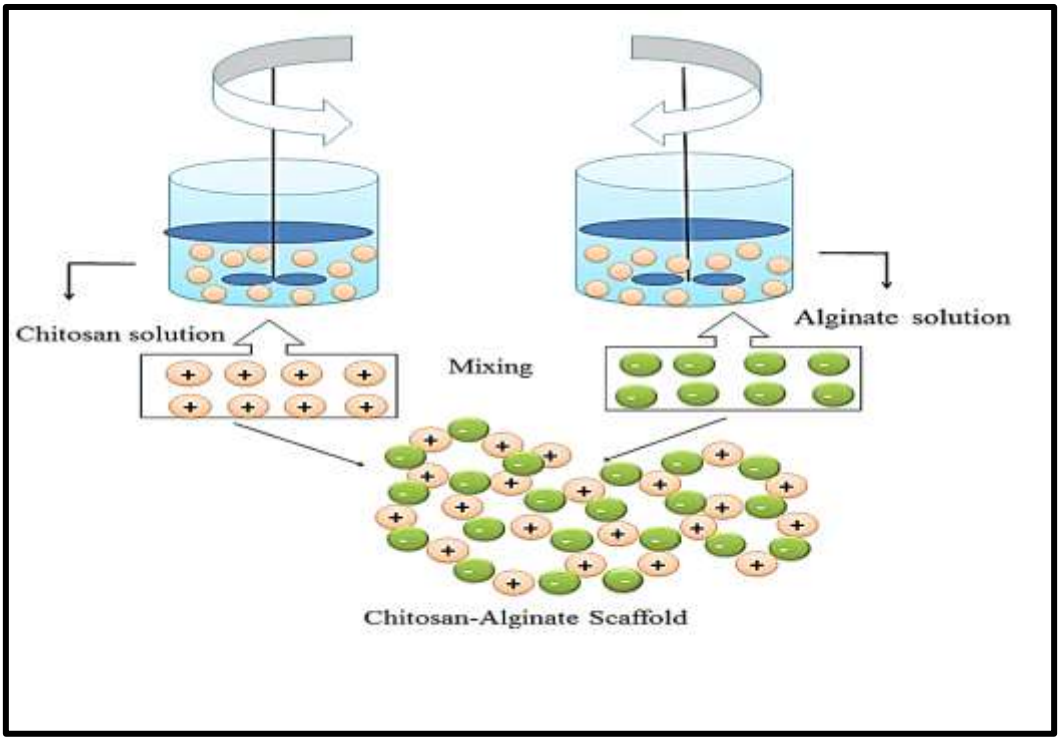


Figure 2.4 Formation of 3D microporous chitosan-Alginate composite scaffold

Upon mixing of chitosan and alginate will form a polyion complex. The carboxyl anion of alginate and amino cation of chitosan can form complexes as explain in Figure 2.4. These complexes form by mixing in the ratio of 1.1 to 1.2 depends on the specific chitosan and alginate being used which develops a heterogeneous structure produces relatively rough surface on the bulky scaffolds as shown in Figure 2.5 [51-53]. The structural properties and yield is depends on the mixing ratio of these two components. Low concentration chitosan and alginate (<3%w/v) are frequently used for beads or films synthesis for drug release especially growth factors release as the neutral gel forming conditions facilitate these protein based growth factor to be incorporated [54], but we are using high concentrations (>3%w/v) chitosan and alginate for making scaffolds. High concentration hybrid chitosan- alginate scaffold will be discussed more in later chapters.

Chitosan-alginate composite do not need certain modifications for better results is its other main characteristic. As both possess opposite charges, interacting with each other forms 3D porous structured scaffolds, they are self-functionalized organic molecules too, hence can get relief from tedious functionalization procedures. Chi-Alg composites have been widely used for drug delivery and protein delivery, wound healing, tendon and ligament tissue engineering and intervertebral tissue engineering.

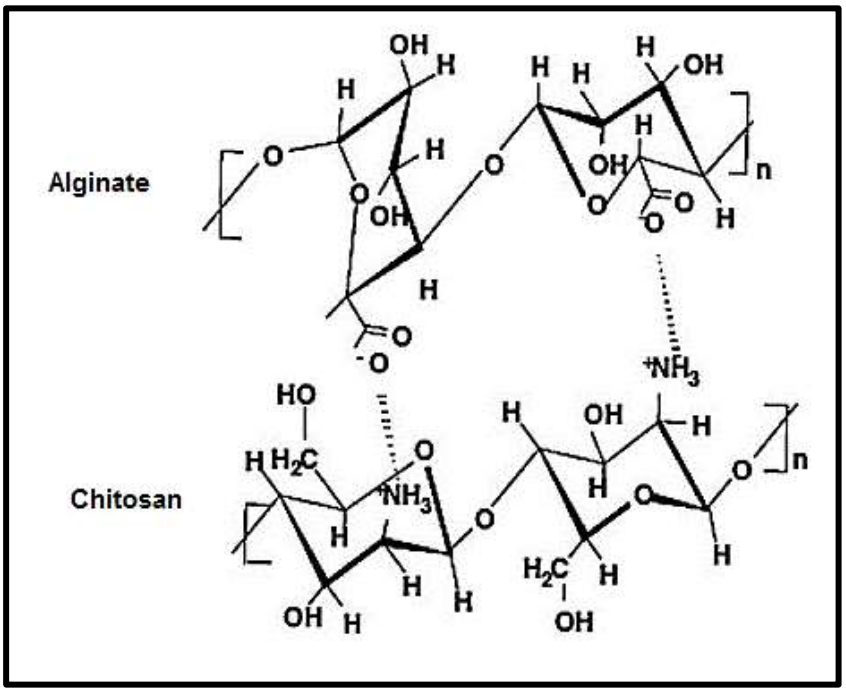


Figure 2.5 Graphical representation of the chemical interaction of Chitosan (Chi) - Alginate (Alg) composite scaffolds

Giovanna Gomez dAyala et al. [55] developed a new calcium sulphate-based composite containing alginate and N-succinylchitosan, as polymeric components for bone regeneration. *Hamideh Amir Afshar et al.* [56] synthesized aminated chitosan/alginate contsining halloysite nanotubes. Their study indicates that amination treatment improves cell adherence and proliferation. Similarly, *Peng Zhao et al.* [57] synthesized photocurable chitosan-gelatin scaffold in sodium alginate hydrogel. These results imply photocurable chitosan- gelatin scaffold in

combination with sodium alginate hydrogel is a promising complex scaffold for cartilage tissue engineering. *Derya Algul et al.* [58] prepared biomimetic three layered scaffold from chitosan and alginate using freeze drying method for treatment of osteochondral defects. The results depicted that biomimeticTLS can be considered to be suitable for osteochondral applications.

Kemal Baysal et al. [59] developed hybrid polymer networks of chitosan with alginate using a crosslinker which enabled the covalent binding of the two macromolecules. This study presents functional hydrogel formation by crosslinked chitosan and alginate, a novel biomaterial which also supports cell growth. *Jen-Ray Chaw et al.* [60] developed new designed nerve conduits with a porous ionic cross-linked alginate/ chitosan composite for nervous regeneration. Moreover, *Nattawut Charoenthai et al.* [61] investigated chitosan as a pelletization aid in extrusion/spheronization using water as granulation liquid. The results indicate that chitosan combined with sodium alginate could be used as a promising alternative pelletization aid to MCC in extrusion/spheronization.

Sung-Ching Chen et al. [62] developed a novel pH-sensitive hydrogel system composed of a water-soluble chitosan derivative (N,O-carboxymethyl chitosan, NOCC) and alginate blended with genipin for controlling protein drug delivery. The results clearly suggested that the genipincross- linked NOCC/alginate hydrogel could be a suitable polymeric carrier for site-specific bioactive protein drug delivery in the intestine. Moreover, *Mei Dai et al.* [63] synthesized chitosan-alginate sponge instead of scaffolds for dermal wound healing in rat. *J.A. Sowjanya et al.* [64] fabricated biocomposite scaffolds by blending chitosan (CS), alginate (Alg) and nano-silica (nSiO2), followed by freeze drying. These results indicate that CS/Alg/nSiO2 scaffolds may have potential applications for bone tissue engineering.

Cristian Tapia et al. [65] performed comparative study of chitosan-alginate and chitosan-carrageenan scaffolds for prolonged drug delivery systems. They have shown that the chitosan–alginate system is better than the chitosan–

carrageenan system as a prolonged drug release matrix system because the drug release is controlled at low percentage of the polymers in the formulation. Again, literature reveals that [34,37,38] alginate-chitosan/hydroxyapatite scaffolds in combination and showed AG-CS PEC scaffold exhibited higher mechanical strength and better thermal stability which can be used for bone tissue engineering. *Yi-Cheng Ho et al.* [35] prepared heparin functionalized chitosan-alginate scaffolds for controlled release of growth factor (bFGF). They also reveal, such scaffolds prevent the growth factor from inactivation.

2.4 Drug delivery applications of CA scaffolds

In these, the cells with growth factor are encapsulated or seeded in the scaffold and administered into the body. Previously, CA polyelectrolyte complexes in various forms have been used to encapsulate and deliver proteins or drugs by manipulating the degree of association between the two polymers functional groups as well as their pH-dependent charge density [66, 67]. For example, CA self-assembling polyelectrolyte multilayer films have been used to immobilize antibodies [67], CA blend gel beads with dual crosslinking were shown to have gastrointestinal site-specific protein release [68] and drug-loaded, polyelectrolyte complexed CA fibers released charged compounds such as bovine serum albumin (BSA), platelet-derived growth factor-bb (PDGF-bb), and avidin over the course of 3 weeks [69].

Also, three-dimensional scaffolds prepared from the polyelectrolyte complexes (PEC) of chitosan and alginate developed for the delivery of bFGF [70]. Similarly, sponges composed of sodium alginate and chitosan were prepared in order to assess the utility of mixed sponges as potential wound dressings or matrices for tissue engineering application. Such sponges were also used for controlled release of paracitamol [71]. *Min Lee et al* [72] showed that CA scaffolds were used as osteogenic protein carriers in the form of microparticles.

2.5 Tissue engineering applications of CA Scaffolds

Previous research regarding the use of organic and inorganic materials, including chitosan and sodium alginate, in scaffolds has been conducted. Yet, little research has been documented on the mechanical stability and strength of scaffolds which composed of differing ratios of chitosan and sodium alginate. Many of times chitosan-alginate in the form of scaffolds, beads, hydrogels, and thin films was used for attachment with various cell types and its interaction was studied. *Amy chen et al*. [73] carried out analysis of chitosan-alginate bone scaffolds. In this experiment, they examined the scaffolds structural integrity as well as adhesion of cells to it under various conditions. *Stephen J. Florczyk et al*. [74] used chitosan-alginate scaffold for studying interactions of prostate cancer cells and lymphocyte *invitro*. *Li Z et al*. [75, 76] used chitosan–alginate scaffolds for studying cartilage as well as bone tissue engineering. Similarly, human embryonic stem cells, hepatocytes and annulus fibrosus cells, glioma tumor cells, cultured on chitosan-alginate scaffolds for variety of applications [77-81].

Some modification is also done that enhanced use of chitosan-alginate scaffold as a culture system for many cell types. *Seog-Jin-Seo et al*. [82] used galactosylated chitosan scaffold and alginate for culturing hepatocytes with NIH 3T3 cells which enhanced liver functions of hepatocytes cells. Some combinations with alginate such as polypyrrole-alginate with chitosan used in bone tissue engineering [83]. Yet another combination with hyaluronate was also studied for cartilage regeneration [84]. *Bibek Chandra sutradhar et al.* [85], showed the effect of chitosan-alginate beads with demineralized bone matrix which enhances chondrogenesis. This study demonstrated that chondrocytes co cultured with DBM in chitosan-alginate beads shows enhanced proliferation while keeping the chondrocytic round morphology showing combined superior biological and mechanical properties over its alginate equivalent, raises the possibility of using chitosan-alginate as an improved alternative to alginate for osteochondral repair and regeneration. *Matthew Leung et al*. [86] studied Chitosan- alginate scaffold

culture system for hepatocellular carcinoma which increases malignancy and drug resistance. They have shown the CA scaffold system is a highly reproducible, versatile model of HCC with direct applications for evaluating tumor behavior and the efficacy of novel anticancer therapies. *Shu wen hu et al.* [87] demonstrated evaluation of bone marrow mesenchymal stem cells seeded into BCII and chitosan-gelatin composite scaffolds and cultured in a dynamic culture system for neocartilage regeneration invitro. *Idiberto Jose et al.* [88] showed Chitosan-collagen scaffolds has been regulate the biological activities of adipose mesenchymal stem cells for tissue engineering. *Dunia M et al.* [89] showed differentiation of mesenchymal stem cells in chitosan scaffolds with double micro and macro porosity. GBMSCs seeded in Chitosan scaffolds are able to attach and gradually proliferate along 4 weeks of culture. *Sabine Neuss et al*. [90] studied assessment of stem cell/biomaterial combinations for stem cell-based tissue engineering. They have used variety of combinations of biomaterials- stem cells, and studied cell adhesion, proliferation, migration, viability properties which can be essential while used as a tissue engineering construct. *M Pandima devi et al*. [91] demonstrated use of a novel wound dressing material fibrin-chitosan-sodium alginate composite sheet, and applied on the clinical wounds of dogs to find its efficacy as wound dressing material and the study is in progress. *Shao X et al,* [92] presented a paper in 52nd Annual Meeting of the Orthopaedic Research Society, entitled developing an alginate/chitosan hybrid fiber scaffold for intervertebral disc annulus fibrous cells. The study demonstrated the feasibility of alginate-based chitosan hybrid scaffold fabrication and support of annulus fibrosus cell growth. *Sung In Jeong et al.* [93] fabricated Chitosan–Alginate nanofibers polyelectrolyte Complexation for use as tissue engineering scaffolds. The nanofibrous scaffolds were able to promote the adhesion and proliferation of cells and they offer great promise for use of scaffolds in tissue regeneration strategies. *Bo He et al.* [94] studied creation and degradation of chitosan-alginate scaffolds for *invitro* cell culture. The results show that cells are viable in the chitosan alginate scaffolds and

that the cells can be reliably released for study after being cultured in the scaffolds. These scaffolds support several cell types and can be a great tool in mimicking in vivo conditions in vitro more closely.

Recently, chitosan/gelatin- alginate scaffolds have been used as a tissue engineering construct which can be directly implanted *invivo* upon seeding stem cells/proginator cells [95]. *Xuan Meng et al*. [96] studied the 3D culture systems for stem cell culture and discuss the relationship between stem cells and 3D growth matrices including the roles of the extracellular matrix, scaffolds, soluble factors, cell-cell interactions and shear stress effects within this environment. *Nicola L. Francis et al.* [97] showed that C/A scaffold is a promising candidate for use as a nerve guidance scaffold, because of its ability to support neuronal attachment and the linearly aligned growth of DRG neuritis. *Therese Andersen et al.* [98] compile information regarding the use of alginate and in particular alginate hydrogels in culturing cells in 3D. *Wujie Zhang et al.* [99] studied proliferation and differentiation of mesenchymal stem cells encapsulated in Alginate-chitosan microcapsule.

2.6 Summary

In summary, biomaterial is defined as a nonviable material used into a medical device, which intended to interact with biological systems; given by European Society for Biomaterials (ESB) in 1976, however, the ESB's current definition is a material anticipated to interface with biological systems to evaluate, augment, treat or replace any tissue, organ or function of the body. This subtle change in definition is indicative of how the field of biomaterials has evolved. Typically, three individuals groups of biomaterials bioceramics, synthetic polymers as well as natural polymers are used in the fabrication of scaffold for

tissue engineering. Each of this individual biomaterial group has specific advantages and needless to say, disadvantages so the use of composite scaffolds those comprised of different phases are becoming increasingly common. Usage of chitosan-alginate scaffolds in drug delivery and tissue engineering applications is presented by this review. Both chitosan-alginate scaffolds are polysaccharides, naturally occurring, biodegradable and biocompatible organic molecules and it is the main reason to use them. When they combined, they possess desirable mechanical strength and stability. Hence, they can be served as a potential material for tissue engineering applications.

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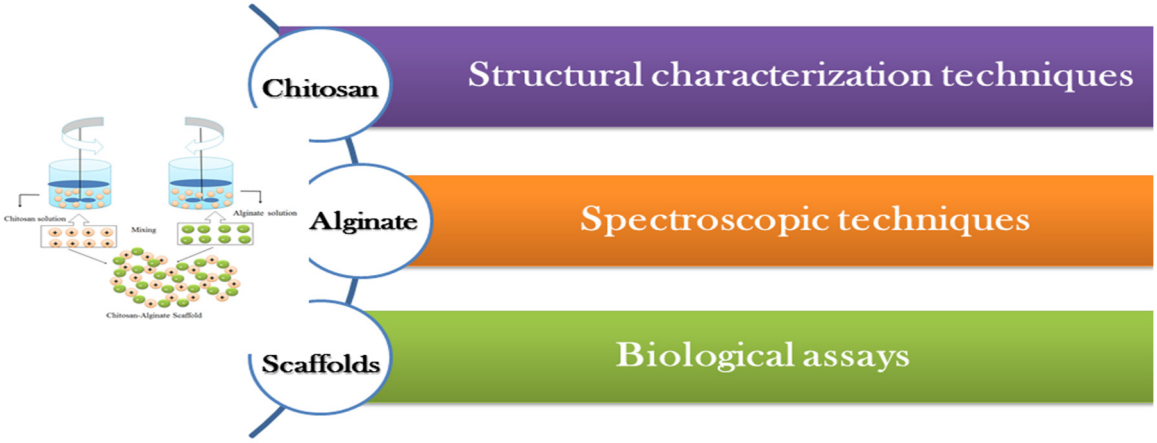
Chapter 3

Experimental Techniques



3.1 Introduction

This chapter focuses on the synthesis of chitosan-alginate scaffolds and experimental techniques employed for characterizations. There are many routes in the synthesis of CA scaffolds. According to the literature survey, several methods have been embraced for the synthesis of CA scaffolds which are reviewed later in this chapter. In current research work, chitosan-alginate scaffolds are synthesized by freeze drying method due to enormous advantages which has been discussed in below. The different analytical techniques used for composition study, elemental analysis, structural and morphological analysis of the prepared samples are described with principle and working.

3.2 Scaffold fabrication methods

In the body, cells and tissue are organized into three-dimensional architecture. By different methodology Scaffolds have to be fabricated to engineer these functional tissue and organs and to facilitate the cell distribution and guide their growth into three-dimensional space. The synthesis techniques for scaffolds fabrication are mainly a freeze drying, particulate leaching, gas foaming and electrospinning. These are discussed as follows:

3.2.1 Freeze drying

The freeze-drying technique is use for fabrication of porous scaffolds [1]. Freeze-drying has emerged as a drying process for converting solutions of labile materials into solids of sufficient stability for distribution and storage in applications such as food science, pharmaceuticals, and enzyme stabilization. Three major steps are involved in freeze drying: the solution is frozen at a low temperature (-70^{o}C to -80^{o}C); the frozen sample is located in a chamber where the pressure is lowered (to a few millibars) through a partial vacuum, known as the primary drying process, in which ice in the material is removed by direct sublimation; and most of the unfrozen water from the material is removed with

desorption in a secondary drying process. In the last two decades, the freeze-drying method has been widely investigated for the fabrication of three dimensional porous scaffolds for tissue engineering. For instance, methacrylamide-modified gelatin-2-hydroxyethyl methacrylate porous scaffolds have been fabricated using the freeze-drying method. Human foreskin fibroblasts seeded onto these novel scaffolds still maintain high (95%) viability after 7 days in culture in vitro. A porous polysaccharide-based scaffold using the freeze-drying method is prepared and demonstrated that pore diameter (55-243 μm) and porosity (33%-68%) in the scaffolds can be regulated by the freeze-drying pressure. Adhesion and proliferation of human mesenchymal stem cells on both porous and nonporous polysaccharide-based scaffolds have also been investigated. Although there are several advantages of the freeze-drying method, including use of water and ice crystals instead of an organic solvent in the scaffold fabrication process, which is more suitable for biomedical applications, it is still a big challenge to engineer scaffolds with hierarchical structures (e.g. vascularized systems) using this approach.

Freeze-drying is conducted by freezing the material followed by reducing the surrounding pressure by applying a vacuum and adding enough heat to allow the frozen water in the material to sublime directly from the solid phase to the gas phase [2, 3]. This technique is applied to a number of different polymers including silk proteins [4, 5], PEG, PGA, poly-l-lactic acid (PLLA), PLGA/poly (propylene fumarate) blends. Emulsification/freeze-drying allows for faster preparation of highly porous structures with high pore interconnectivity's.

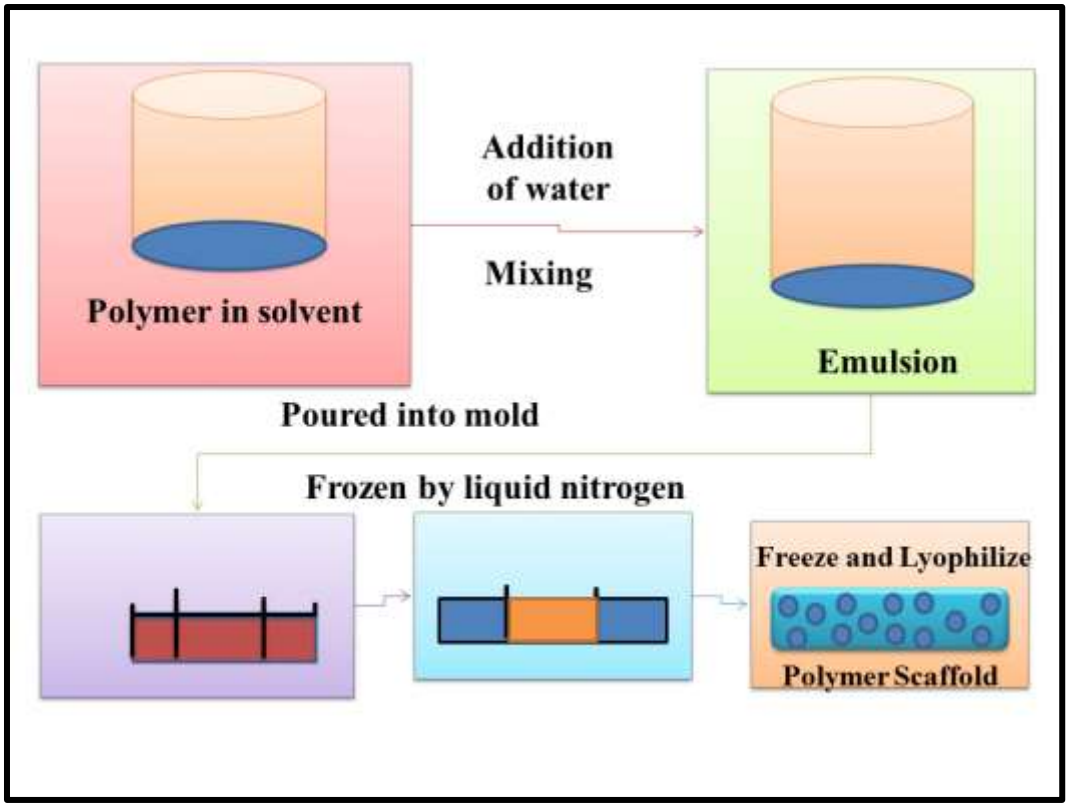


Figure 3.1 Fabrication of scaffold using the emulsification/freeze-drying method

The main advantage of this technique is that it requires neither high temperature nor a separate leaching step. Its disadvantages, however, are limited to small pore size 154 (porosity is often irregular) and a long processing time. The pore size can be controlled by optimizing the freezing rate and pH; a fast freezing rate produces smaller pores.

3.2.2 Particulate-leaching technique

There are many techniques that are widely used to fabricate scaffolds for tissue engineering applications [6, 7] 'Particulate leaching' is one of those. In melt molding–particulate leaching, the polymer is cast into a mold with the embedded solid porogen. Salt, wax or sugars known as porogens are used to create the pores or channels. Here salt is grounded into small particles and those particles that have

desired size are poured into a mold and filled with the porogen. After that a polymer solution is cast into the salt-filled mold. After the evaporation of the solvent, the salt crystals are leached away using water to form the pores of the scaffold.

There are many advantages like large range of pore sizes, independent control of porosity, pore size, ability to tailor crystallinity and highly porous structures. Nevertheless, this method suffers from major drawbacks because of the long period of soaking in water required to leach all of the salt particles. To conquer this drawback new technologies are being developed.

3.2.3 Gas foaming

A "gas foaming" method was developed by *Nam et al.* [8] using an effervescent salt as a gas foaming agent. This technique uses high pressure carbon dioxide gas for the fabrication of highly porous scaffolds. The scaffold's porosity and porous structure depend upon the amount of gas dissolved in the polymer. This process involves exposing highly porous polymer with carbon dioxide at high pressure (800 psi) to saturate the polymer with gas [8]. Cells and nearby tissues can be damaged by the residues that remains after completion of process. This may also denature the biologically active molecules incorporated within the scaffolds. The gas foaming scaffold fabrication techniques does not require the utilization of organic solvents as well as high temperature.

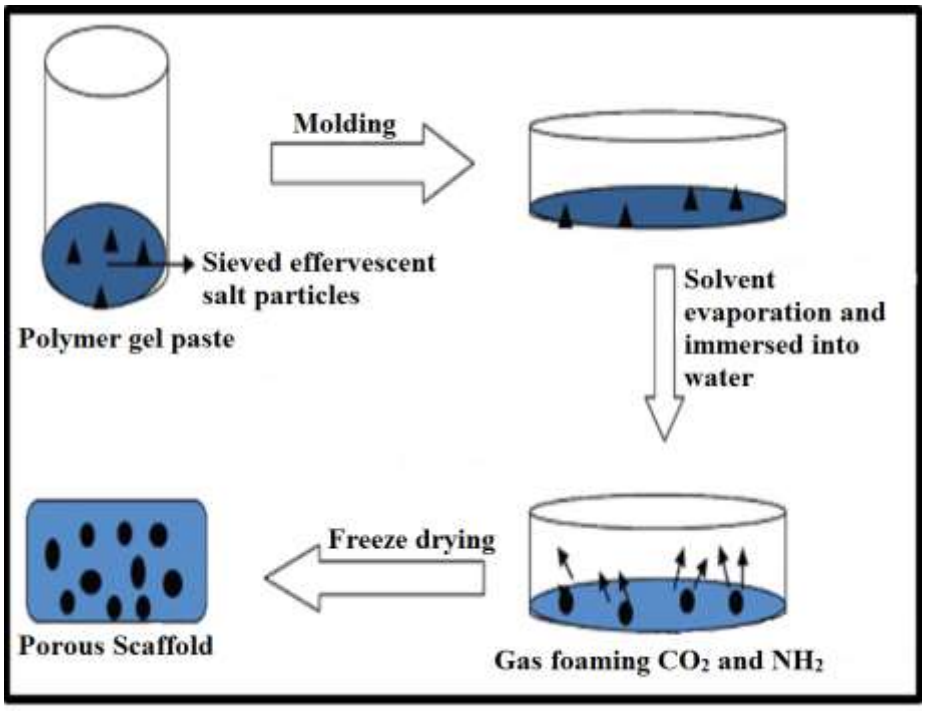


Figure 3.2 Fabrication of scaffold using the gas foaming method [9]

3.2.4 Electrospinning

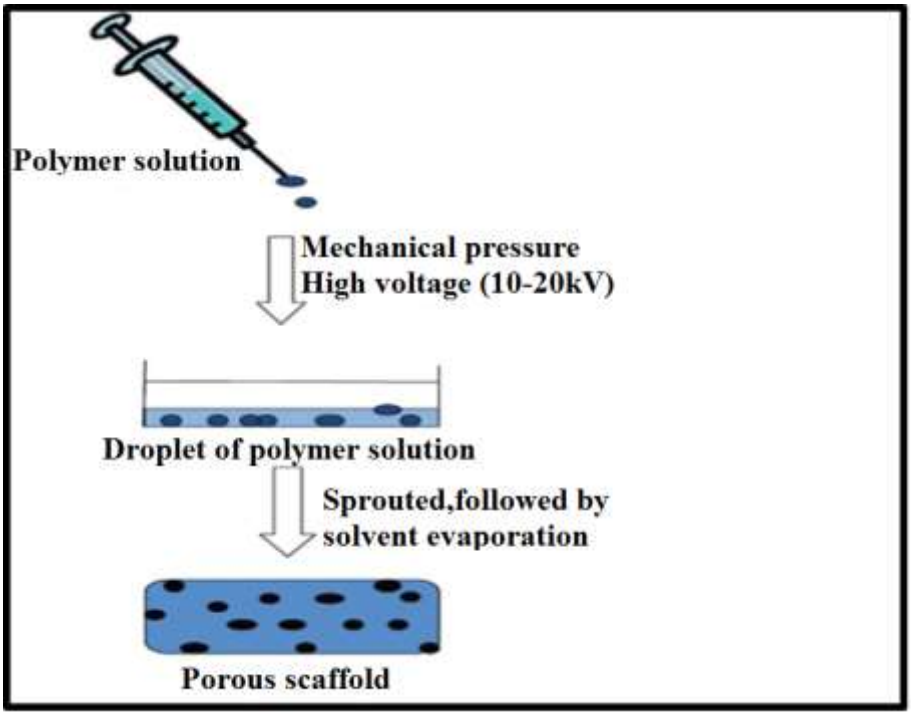


Figure 3.3 Fabrication of scaffold using the electrospinning method [9]

The electrospinning technique for the scaffolds designing exploits the electrostatic force for the production of polymeric fiber ranging from nanoscale to microscale. High intensity electric field between two electrodes having electric charges of opposite polarity controls this process. One electrode is placed in the polymer solution and other is placed in collector. Generally, polymer solution is driven as result in forming a drop of solution. Afterwards, electric field is generated which intends to produce a force, due to this the droplets results to overcome the surface tension of the solution. A jet of polymer is ejected, which forms the fibers, same instant the solvent starts evaporating due to jet formation and continues after the Nano fibers are deposited to collector.

One of the main advantages of this technique is that it can produce the scaffold with main structural feature suitable for growth of the cell and subsequent tissue organization [10-12]. It can forms the ultra-fine fibers with special orientation, high aspect ratio, high surface area and having control over pore geometry. These characteristics are favorable for better cellular growth for in vitro and in vivo because they directly influence the cell adhesion, cell expression and transportation of oxygen, nutrients to the cells. This provides spatial environment for the growth of new tissue with appropriate physiological functions. Cell seeding is the main problem of electrospining technology. This is overawed by sacrificial biopolymer or cryospinning, which allows creating the hole of desired size in electrospun matrices.

Table 3.1 Merits and demerits of different fabrication techniques [13]

Methods	Merits	Demerits	References
Solvent casting/ particulate leaching	Control over Porosity, pore size and crystallinity	Limited mechanical property, residual solvents and porogen	Ma, 2007 [14]; Xiang et al., 2006 [15].

		material	
Gas foaming	Free of harsh organic solvents, control over porosity and pore size	Limited mechanical property, inadequate pore interconnectivity	Ikada., 2006 [16].
Self assembly	Control over porosity, pore size and fiber diameter	Expensive material, complex design parameters	Zhang et al., 2003; 2006 [17, 18].
Electrospinning	Control over porosity, pore size and fiber diameter	Limited mechanical property, pore size decrease with fiber thickness	Liang et al., 2007 [10].
Rapid Prototyping	Excellent control over geometry, porosity, no supporting material required	Limited polymer type, highly expensive equipment	Hutmacher et al., 2000; 2001[19, 20].
Fiber bonding	High surface to volume ratio, high porosity	Poor mechanical property, limited applications to other polymers	Mooney et al., 1996 [21]
Freeze drying	High temperature and separate leaching step not required	Small pore size and long processing time	Boland et al., 2004 [22]; Mandal & Kundu, 2008 (a) [23].

3.3 Structural characterization techniques

In order to investigate different properties of chitosan-alginate scaffolds various characterization techniques are required. Different techniques provide different information some about chemical and physical properties and others about structure and morphology. The different techniques are used in the present work which includes X - ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FT - IR), Field Emission Scanning Electron Microscopy (FE - SEM), EDAX ZAF, Rheology etc.

3.3.1 Scanning electron microscope and Energy Dispersive X-ray Spectroscopy (EDAX)

Scanning electron microscopy (SEM) is a method used for high-resolution imaging of surfaces. The SEM uses electrons for imaging, much as a light microscope uses visible light. The advantages of SEM over light microscopy include much higher magnification (>100,000X) and greater depth of field up to 100 times that of light microscopy.

Schematics 3.4 (a) shows ray diagram for scanning electron microscope. In, actual working principle, SEM generates a beam of incident electrons in an electron column above the sample chamber. The electrons are produced by a thermal emission source, such as a heated tungsten filament or by a field emission cathode. The energy of the incident electrons can be lower up to 100 eV or higher up to 30 keV which depends on the evaluation objectives. The electrons are focused into a small beam by a series of electromagnetic lenses in the SEM column. Scanning coils near the end of the column position the focused beam onto the sample surface. The electron beam is scanned in a raster pattern over the surface for imaging. At a single point, the beam can also be focused or scanned along a line for x-ray analysis. The beam can be focused to a final probe diameter as small as about 10 A^{o}.

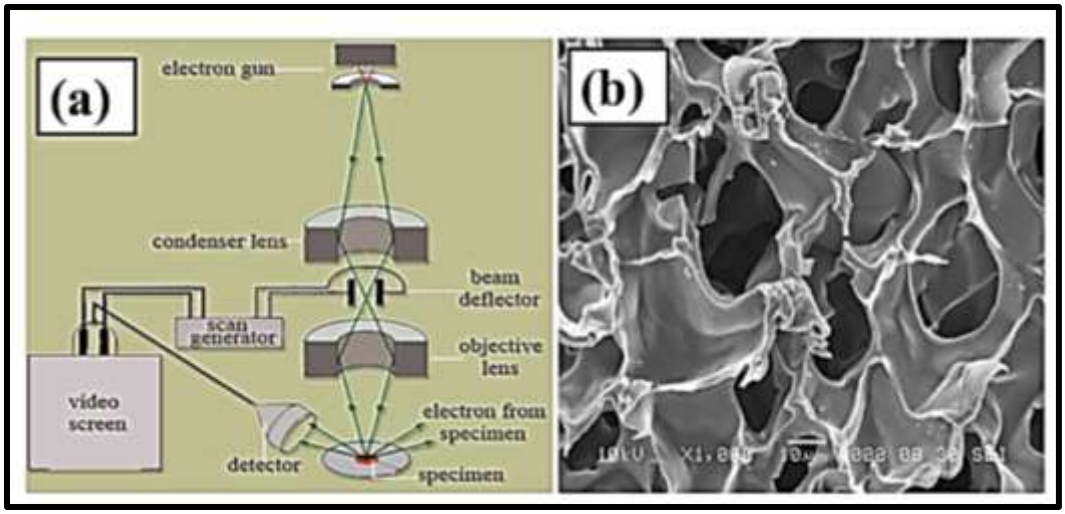


Figure 3.4 (a) A Schematic diagram of Scanning electron microscope [24] (b) SEM images of CA Scaffolds

Energy-dispersive X-ray spectroscopy (EDS, EDX or XEDS), occasionally called energy dispersive X-ray analysis (EDXA) or energy dispersive X-ray microanalysis (EDXMA) is an analytical technique used for the elemental analysis or chemical characterization of a sample. It relies on an interaction of some source of X-ray excitation and a sample. Its characterization capabilities are due in large part to the fundamental principle that each element has a

unique atomic structure allowing unique set of peaks on its X-ray emission spectrum [25].

EDAX systems are typically integrated into either an SEM or EPMA instrument. EDAX systems include a sensitive x-ray detector, a liquid nitrogen gas for cooling and software to collect and analyze energy spectra. During SEM EDAX Analysis, an electron beam is scanned across a sample's surface and the electrons strike and stimulate the sample. Almost instantaneously, as each element returns to its original energy state, it emits X-rays of specific energies and at different wavelengths characteristic of the element. EDAX plots these results with X-ray wavelength on the X-axis and intensity on the Y-axis and labels each

corresponding element. Identification of the elements is done by matching the peak values on the x-axis with known wavelengths for each element to reveal the sample's elemental composition as shown in Figure 3.5.

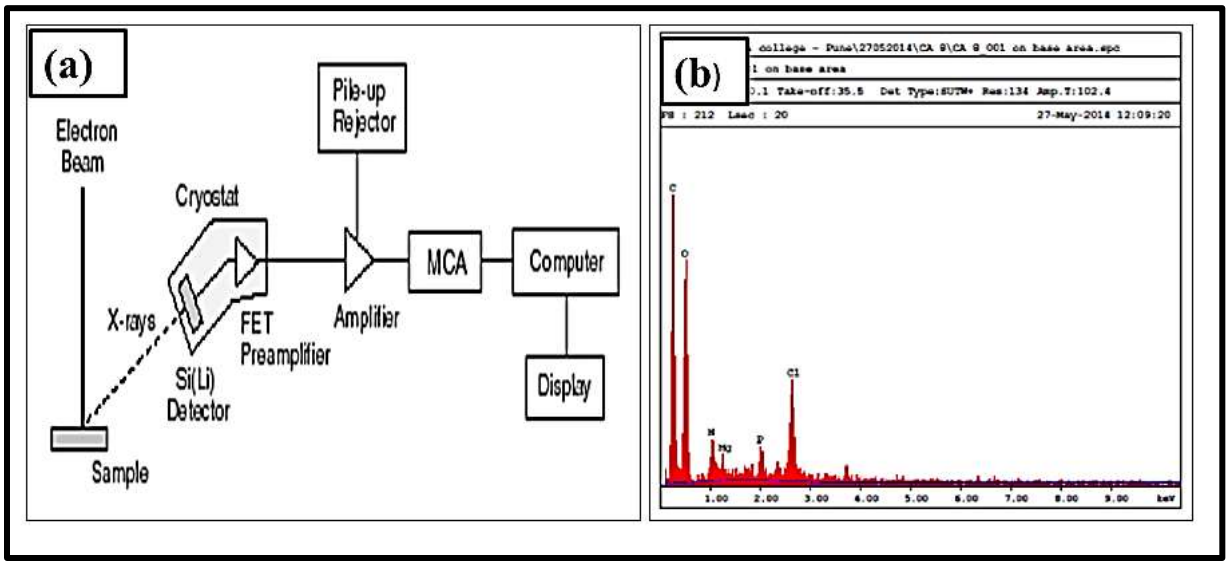


Figure 3.5 (a) A Schematic representation of an energy-dispersive spectrometer, (b) EDAX of chitosan-alginate scaffold [26]

3.3.2 Environmental Scanning Electron Microscopy (ESEM)

The environmental scanning electron microscope or ESEM is a scanning electron microscope (SEM) that allows for the option of collecting electron micrographs of specimens that are "wet" uncoated or both by permitting for a gaseous environment in the specimen chamber.

The ESEM is based on a number of changes to the instrument of conventional SEM, namely, the introduction of differential pumping and new detection systems. A minimum of a pair of apertures with pumping between them is placed at the end of the objective lens, thus separating the electron optics column from the specimen chamber. Gas introduced around the specimen, flows through the bottom aperture first and most of it is removed with a pump before only a small fraction of gas escapes in the column. The latter can easily be handled

by the vacuum system of the SEM. Depending on the type of electron gun used in the generation of the electron beam, additional differential pumping stages may be introduced. ESEM uses a proprietary Environ mental Secondary Detector (ESD) which can function in non-vacuum environment instead of Everhart-Thornley (ET) detector used in SEM [27].

3.3.3 X-ray Diffraction technique

X-ray diffraction (XRD) is one of the most important non-destructive tool to analyse all kinds of matter - ranging from fluids to powders and crystals. From research to production and engineering, XRD is an indispensible method for structural materials characterization and quality control which makes use of the Debye-Scherrer method.

X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate and directed toward the sample. The incident rays and the sample interaction produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law which is given by,

$$2d \sin \theta = n\lambda \qquad (3.1)$$

Where, d is inter planar spacing, θ is diffraction angle, λ is wavelength of X-ray and n is order of diffraction.

The wavelength of electromagnetic radiation is related by this law to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. By scanning the sample through a range of 2θangles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each

mineral has a set of unique d-spacings. Typically, this is achieved by comparison of d-spacings with standard reference patterns [28].

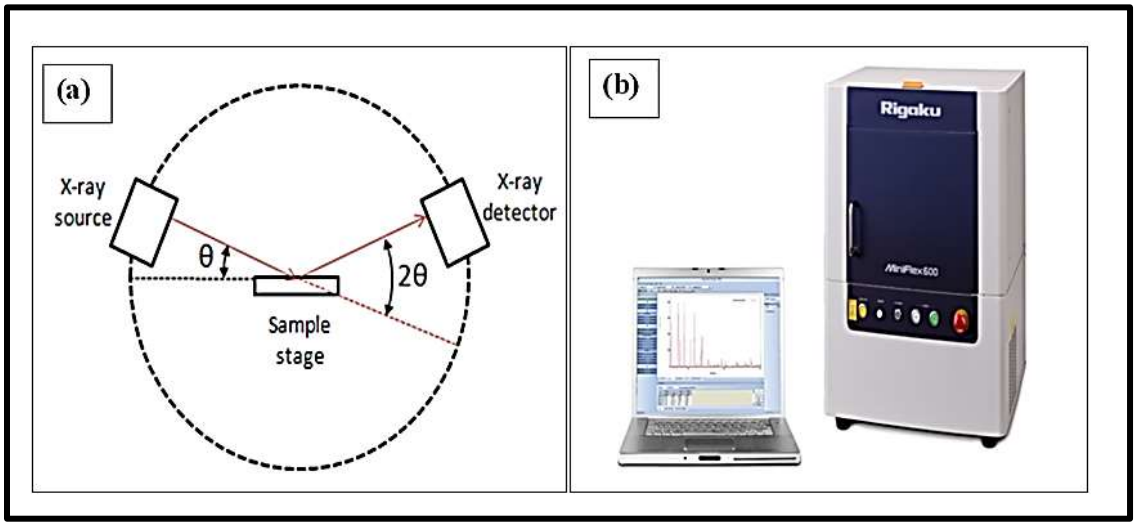


Figure 3.6 (a) Schematic of X-ray diffractometer [29] (b) Benchtop X-ray diffraction instrument [30]

In this technique, material is ground to a fine powder. The fine powder of sample are generally spread uniformly over a rectangular area of a glass slide. The sample usually adhered to glass either using binders like collodion grease or wax. Several types of sample holders namely glass slide, circular disc or thin capillary etc. are used for the different designs of the instrument. The schematic representation of the typical XRD system is presented in the Figure 3.6 (a).

Identification of phases in a sample is calculated from the d spacing using the standard JCPDS powder diffraction file and the reflections can be indexed with Miller indices. However, if the size of the diffracting tiny crystal is small, there is no more complete destructive interference at θ±dθ, which broadens the peak corresponding to diffracted beam in proportion to the size of the tin y crystal and that can be used to calculate the particle size. The relation f or the same is given by Debye Scherrer formula

$$D = \frac{0.9\lambda}{\beta\cos\theta} \qquad (3.2)$$

Where, D is particle size, θ is diffraction angle, λ the wavelength of X – rays and β is Full Width at Half Maxi ma (FWHM). The phase composition, lattice parameter and the mean size of the crystallites of NPs were determined by XRD (RIGAKU Miniflex 600) equipped with a crystal monochromator employing Cu - Kα radiation of wavelength 1.54 Å a n d applied s canning rat e of 3º min - 1 , ranged from 20 to 80°. The patterns were analyzed by X'Pert High score software and compared with standard JCP DS. The average crystallite size was calculated from the broadening of the XRD peaks using the Scherrer's equation.

3.3.4 BET surface area and pore volume analyzer

Surface area and porosity are two important physical properties that affect the quality and utility of solid phase chemicals including agrochemicals, additives and pharmaceutical active ingredients. Differences in between the surface area and porosity of particles within the material, which otherwise may have the same physical dimensions; can greatly influence its performance characteristics. Surface area and porosity have major roles in the purification, processing, blending of chemical products as well as product function, efficacy and stability. For surface area and porosity measurements Gas Adsorption analysis is commonly used. This includes exposing solid materials to gases or vapors at a variety of conditions and evaluating either the weight uptake or the sample volume. Information regarding the physical characteristics of the solid including: skeletal density (ρS), porosity, total pore volume (TOPV) and pore size distribution is provided by analysis of these data. The Brunauer, Emmett and Teller (BET) technique is the common method for determining the surface area of powders and porous materials. BET analysis offers precise specific surface area evaluation of materials by nitrogen multilayer adsorption measured as a function of relative pressure using a fully automated analyzer. The technique encompasses external area and pore area evaluations to determine the total specific surface area in m^2/g yielding important information in studying the effects of surface porosity and particle size in many

applications. To determine pore area and specific pore volume BJH analysis can also be employed using adsorption and desorption techniques. This technique illustrates pore size distribution independent of external area due to particle size of the sample [31,32].

Nitrogen gas is generally employed as the probe molecule and is exposed to a solid under investigation at liquid nitrogen conditions (i.e. 77 K). The surface area of the solid is evaluated from the measured monolayer capacity and knowledge of the cross-sectional area of the molecule being used as a probe. For the case of nitrogen, the cross-sectional area is taken as 16.2 Å2 / molecule.

Specific surface area measuring the external surface area and open pores of macroporous and mesoporous materials, along with pore volume and area distributions that characterise porosity below the effective range of mercury intrusion porosimetry are techniques that can be especially useful for studies of artificial bone materials, catalysts, particle and gas filtration, fuel cell technology, absorbents, sintering studies, zeolites and reactivity studies of materials among a variety of other applications.

3.3.5 Thermal gravimetric Analysis (TGA/DSC)

Thermogravimetric analysis or thermal gravimetric analysis (TGA) is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate) or as a function of time (with constant temperature and/or constant mass loss). TGA can provide information about physical phenomena, such as second order phase transitions, including vaporization, sublimation, absorption, and desorption. Also, TGA can provide information about chemical phenomena including chemisorptions, desolvaion (especially dehydration) decomposition, and solid-gas reactions (e.g. oxidation or reduction) [33].

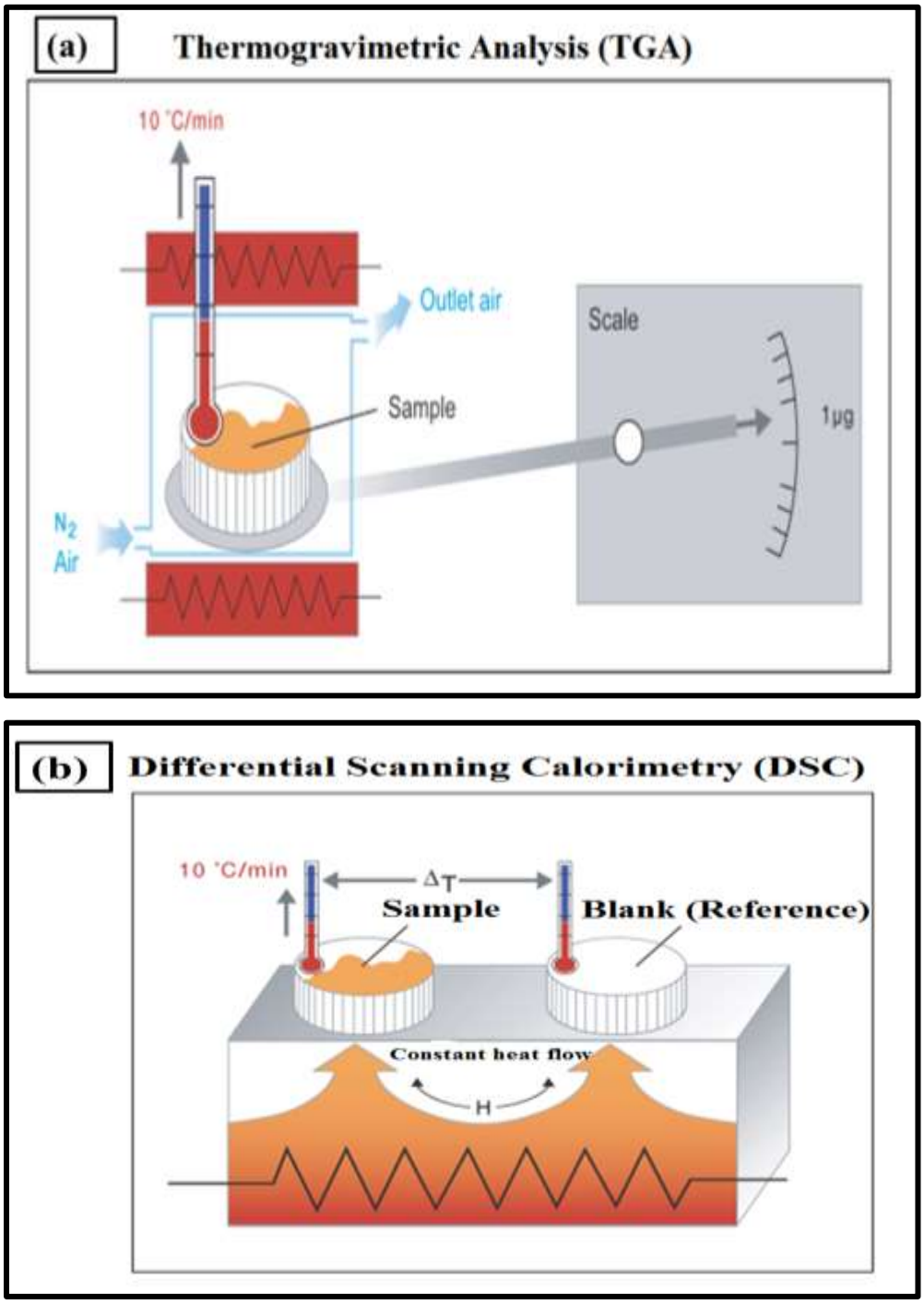


Figure 3.7 Schematic principle of (a)TGA and (b) DSC measurement [34]

The basic principle of TGA is after a sample is heated, its mass changes. This change can be used to determine the composition of a material or its thermal stability, up to 1000^{o}C. Usually, a sample loses weight as it is heated up due to decomposition, reduction, or evaporation. A sample could also gain weight due to oxidation or absorption. While in use, the TGA machine tracks the change in weight of the sample via a microgram balance as shown in Figure 3.8. Temperature is monitored via a thermocouple. The TGA can also track change in weight as a function of time. Data can be graphed as weight percent or time vs. temperature (^{o}C). The data produced by this lab was graphed as weight percent vs. temperature. TGA output curves can be analyzed in a number of ways. If the material in question is stoichiometric, the molar weight of the component being burned off can be ascertained based on the weight percent lost and the total molar weight of the material. In the case of tire tread, a nonstoichiometric material, the decomposition temperature of each component must be considered when interpreting the curves. The rubber in a tire tread has a lower decomposition temperature than carbon black and inert filler, so is the first to decompose [35].

TGA is commonly used to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition, oxidation, or loss of volatiles (such as moisture). Common applications of TGA are (1) materials characterization through analysis of characteristic decomposition patterns (2) studies of degradation mechanisms and reaction kinetics (3) determination of organic content in a sample and (4) determination of inorganic (e.g. ash) content in a sample, which can be beneficial for corroborating predicted material structures or merely used as a chemical analysis. It is an especially useful technique for the study of polymeric materials including thermoplastics, thermosets, elastomers, composites, plastic films, fibers, coatings and paints [36].

3.3.6 Rheological studies

A Rheometer is a laboratory device which is used to measure the way in which a liquid, suspension or slurry flows due to applied forces. It is used for those fluids which cannot be defined by a single value of viscosity and therefore require more parameters to be set and measured than is the case for a viscometer.

A Rheometer measures flow. Specifically, rheometers measure the forces (like shear) associated with a flowing substance. Typically, the flowing substance is much thicker than a liquid (like concrete). A rheometer does not simply measure flow but also measures the effects of flow at different pressures-especially key characteristics of the flow like shear (how difficult it is to change the flow) or how the flow of a substance tends to drag surrounding materials. A set of curves that depict these forces as the pressure on the flowing material changes is the typical output of a rheometer. Rheometers are almost as varied as the substances and forces that they measure [37]. Figure 3.8 (a) shows a schematic representation of the geometry and the moving parts in a controlled-stress instrument.

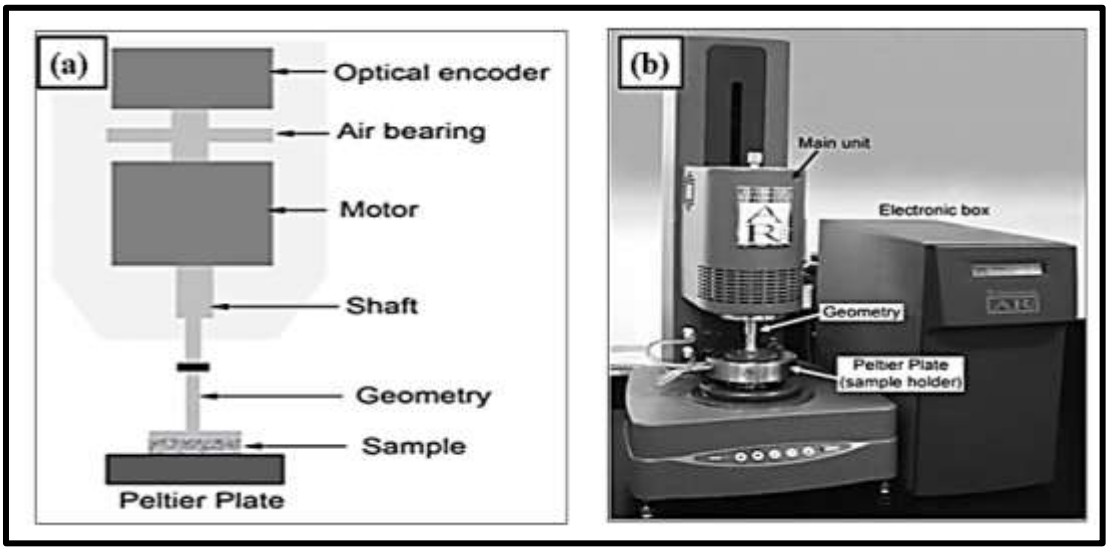


Figure 3.8 (a) Schematic diagram of a stress-controlled rheometer main unit (b) TA Rheometer 2000 controlled-stress dynamic shear rheometer

There can be two types of Rheometers: controlled-stress and controlled-strain. A controlled-stress rheometer implies a torque to either control the stress at a desired level or to drive the strain to a desired amount. In such type of rheometer the material is placed in between two surfaces a bottom surface, which is fixed and a rotating top surface, called the geometry. In the controlled-stress rheometer, the torque or stress is the independent variable that is applied to the geometry. In the controlled-strain rate rheometer the material is placed in between two plates. The bottom plate moves at a fixed speed and the torsional force formed on the top plate is measured. Therefore, for this case, the strain rate is the independent variable whereas the stress the dependent one. Here it is worth noticing that a controlled-stress rheometer is a better approach for determining yield stress. Figure 3.8 (b) shows a TA AR2000 (TA instruments, New Castle Deleware, USA) rotational controlled-stress rheometer. This has three main components: (a) the main unit mounted on a cast metal stand that supports the geometry, (b) an electronic control circuitry contained in a separated box (electronic box) and (c) the sample holder (Peltier plate) [37].

3.4 Optical characterization Techniques

3.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR stands for Fourier Transform InfraRed, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. The sample absorbed some of the infrared radiation and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission creating a molecular fingerprint of the sample. No two unique molecular structures produce the same infrared spectrum just like a fingerprint. This makes infrared spectroscopy useful for several types of analysis [38].

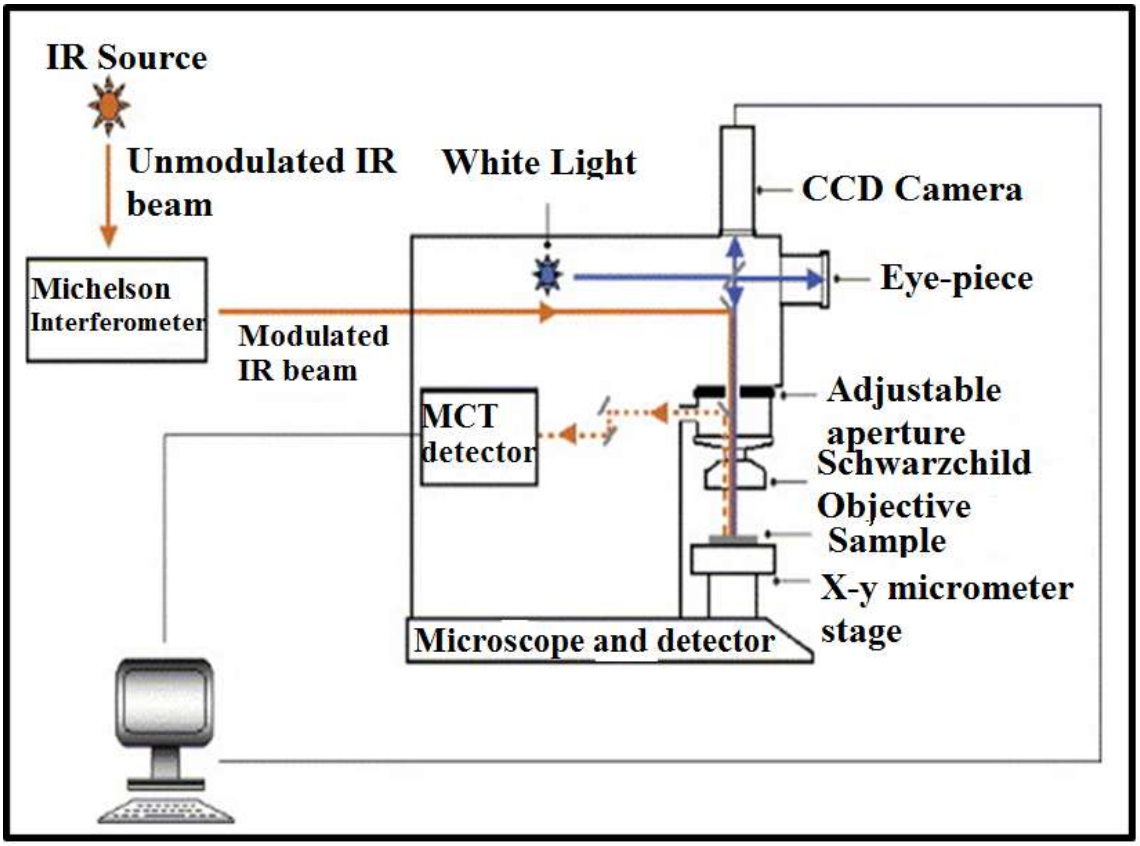


Figure 3.9 FTIR spectrometer [39]

The FTIR spectrometer uses Michelson interferometer to modulate the wavelength from a broadband infrared source. Figure 3.9 shows a typical spectrometer mainly consist of radiation source, interferometer, sample and detector. The intensity of transmitted or reflected light is measured by a detector as a function of its wavelength. The signal obtained from the detector is an interferogram, which must be analyzed with a computer using Fourier transforms to obtain a single-beam infrared spectrum. The FTIR spectra are generally presented as plots of intensity versus wavenumber (in cm^{-1}). Wavenumber is the reciprocal of the wavelength. The intensity can be plotted as the percentage of light transmittance or absorbance at each wavenumber [40].

3.5 Biological Techniques

3.5.1 Biocompatibility study:

3.5.1.1 Cytotoxicity assays

Biocompatibility is associated to the behavior of biomaterials in various contexts. The term refers to the capacity of a material to perform with an appropriate host response in a specific situation. According to IUPAC definition, the term is defined as "Ability to be in contact with a living system without producing an adverse effect" [41]. Scaffolds can be used mostly in tissue engineering field *in vivo* and *in vitro*; therefore, understanding the properties of scaffolds and their effect on the body is vital before clinical use. For this purpose it is essential to study cytotoxicity of scaffolds. A variety of assays are used to study the toxic effects of scaffold on cell cultures, including LDH (lactate dehydrogenase) leakage, a protein assay, the neutral red and MTT 3-(4,5-Dimethyl-2thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay and identification of cytokine/chemokine production.

In present investigation MTT and TBDE (Trypan blue dye exclusion assay) assays have been used to study cytotoxicity. In TBDE assays cell viability analysis is done with trypan blue dye exclusion staining. Cells were routinely counted manually with a hemocytometer. Now a day advanced programmed instrumentation has been introduced to supplement this traditional technique with the efficiency and reproducibility of automated sample handling, computer control and advanced imaging. The conventional method of performing TBDE analysis involves manual staining and use of a hemocytometer for counting. Recent advances in instrumentation have led to a number of fully automated systems that can enhance the output and accuracy of this technique [42].

The MTT assay is a colorimetric assay for measuring cell viability. The reduction of MTT (tetrazolium salts) is now extensively believed as a reliable way to observe cell proliferation. The yellow tetrazolium MTT (3(4, 5-dimethylt

hiazolyl-2)-2,5-di phenyltetrazolium bromide) is reduced by metabolically active cells through dehydrogenase enzymes and make reducing equivalents such as NADH and NADPH. The consequential intracellular purple formazan can be solubilized and measured by spectrophotometrically. The cell proliferation rate can be calculated by using MTT cell proliferation assay. When metabolic events initiate apoptosis or necrosis it results into the reduction in cell viability. The number of stages involved in assay has been minimized as much as possible to expedite sample dispensation. The MTT reagent give ups low background absorbance values in the absence of cells [43].

3.5.1.2 Hemocompatibility assay

The biocompatibility and immunocompatibility of polymeric materials is of fundamental importance for their possible therapeutic uses [44]. Hemocompatibility is a key property of biomaterials that reflects the degree of interaction between material and blood [45]. Materials used in *invivo* applications must be assessed for blood compatibility to establish their safety. In practice, all materials are incompatible to some extent with blood, because they can either disrupt the blood cells (hemolysis) or activate the coagulation pathways (thrombogenicity) and/or the complement system [46].

Hemocompatibility testing, a major part of biocompatibility testing is the evaluation of critical interactions of foreign material with blood to explore possible adverse effects arising from the exposure of the foreign material to blood cells and proteins. Because such adverse effects are frequent and may represent serious health risks, testing of new medical devices, intravenously applied medicines, blood products and biomaterials for hemocompatability is very important [47].

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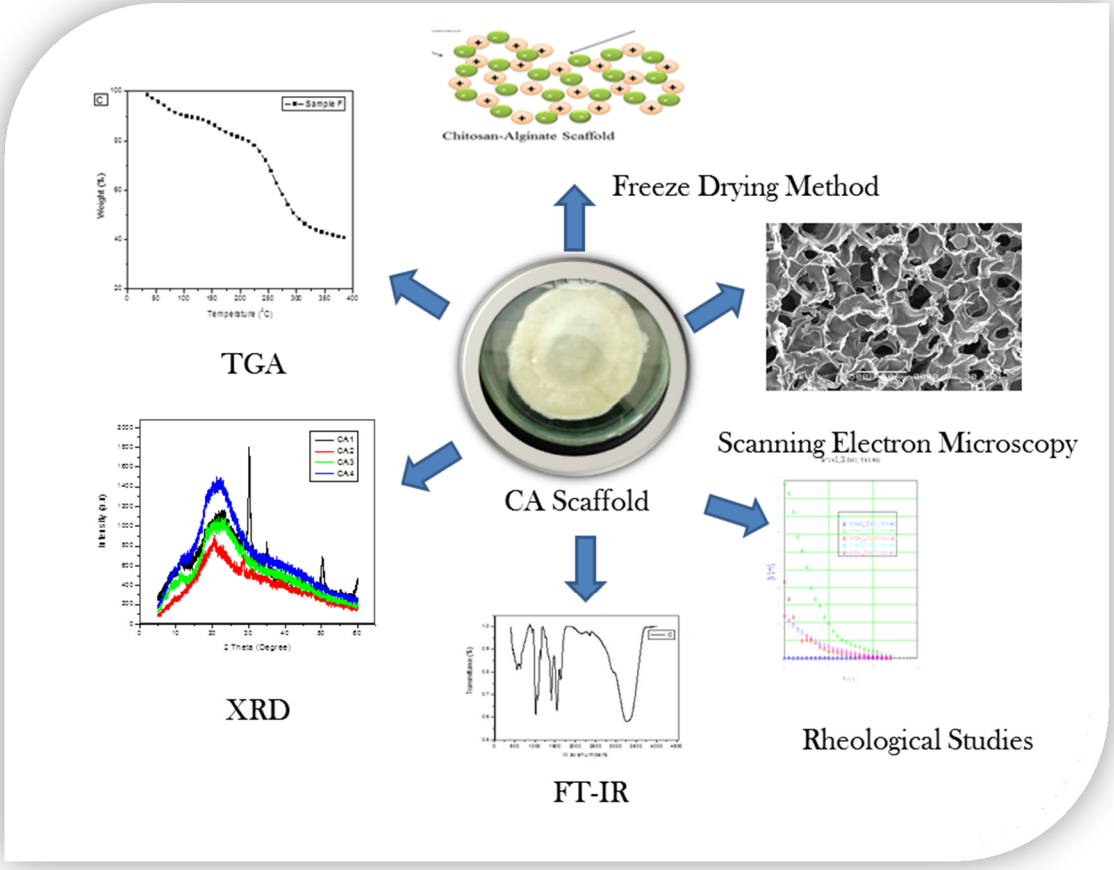
Chapter 4

Synthesis of Chitosan–Alginate Scaffolds and Their Physical Properties



4.1 Introduction

A well-formed three-dimensional scaffold is one of the fundamental tools to guide tissue formation *in vitro* and *in vivo*. Frontiers areas in medicine are changing rapidly from utilizing synthetic implants and tissue grafts to a tissue engineering approach which uses degradable porous material scaffolds integrated with biological cells or molecules to regenerate tissues [1]. Therefore, the selection of scaffold is important to allow the cells to behave in the desired manner to generate tissues and organs of the desired shape and size. The TE scaffold is typically a three-dimensional structure manufactured from synthetic polymers and/or natural biopolymers to provide temporary mechanical, physical and biological support for the embedded cell types [2-5].

Principles of Scaffold Design:

All tissue and organs in the body are three-dimensional structures. In order to repair and regenerate lost or damaged tissue and organs, three-dimensional scaffolds must be designed, fabricated and utilized to regenerate the tissue similar in both anatomical structure and function to the original tissue or organ to be replaced or repaired. Therefore, certain principles of scaffold design must be established to ensure proper tissue regeneration.

1) Tensegrity Concept:

Ingber proposed an important principle in scaffold design based on the tensegrity concept. The tensegrity concept sounds new, but it is not a brand new concept because it has been used for centuries in the area of civil engineering. The core of this concept is that mechanical forces are evenly distributed on all regions of the entire scaffold. Therefore, stable structures that can bear forces uniformly, such as triangles, pentagons or hexagons are preferred structures in a scaffold.

2) Nutrient and Metabolic Concept:

Any living thing needs to consume nutrients and release waste products in order to survive. To retain tissue-engineered grafts alive, the diffusion of nutrients and metabolic products into and out of the three-dimensional scaffold is a key parameter to maintain. Highly porous and interconnected structures have been employed to facilitate the transport of materials through the scaffolds. However, these measures are not sufficient for the regeneration of large tissue and organs, such as the regeneration of liver. Unfortunately, there is not a good answer for this problem yet.

3) Neovascularized Network Concept:

The neovascularized network concept is an advancement of the nutrient and metabolic concept. In order to eventually solve the nutrient and metabolic problems of engineered tissues, well-organized and uniformly distributed blood vessel networks are required. However, the technology to construct sufficient blood vessel networks outside the body has not been established yet. So far, one vital hurdle in the creation of tissue-engineered whole organs is the inability to provide proper vascularization to the newly created tissue or organ. This problem is especially serious in the development of a tissue-engineered liver. The longest possible distance from a cell to blood vessels necessary to keep the cells alive in most healthy tissue is less than 1 mm. Therefore, when designing a tissue or organ, the time frame of blood vessel development must be well thought out. With knowledge of neovascularization obtained from developmental biology and cancer studies well established, this problem may finally be solved. Currently, most scientists are trying to incorporate angiogenic factors into scaffold design in order to encourage the formation of blood vessel networks in the grafts.

The ideal injectable polymer would need to satisfy the following design criteria:

1. It would polymerize in situ in a timely fashion without detrimental effects to the surrounding tissue such as a temperature rise.

2. It should fill defects of various shapes and sizes and have the correct mechanical and physical properties for a particular application.
3. It would maintain these mechanical properties as it degrades.
4. It must degrade into biocompatible degradation products according to a predetermined degradation profile, have a long shelf life, be sterilizable without loss of its properties and be available to the surgeon in the sterile operating field on short notice [6].

The scaffolds for tissue engineering should be made-up from a biocompatible polymer, which does not have the potential to elicit an immunological or foreign body reaction. The chosen polymer can degrade at a controlled rate in concert with tissue regeneration. The degradation products should not be toxic and must be easily excreted by metabolic pathways. Many types of polymeric materials have been castoff for bone tissue engineering [7, 8]. They can be simply categorized as naturally derived materials (e.g. collagen, fibrin, chitosan and alginate) and synthetic polymers (e.g. poly (lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers PLGA). Naturally derived materials have the potential benefit of biological recognition that may positively support cell adhesion and function.

The use of natural origin polymers is one of the present trends in tissue engineering applications because natural origin materials are biocompatible and biodegradable and have been demonstrated to promote healing at a faster rate and more importantly, they are expected to exhibit greater compatibility with human tissues [9]. Chitosan (Chi), an important derivative of chitin, is composed of repeating units of D-glucosamine as well as N-acetyl glucosamine linked in a β (1-4) manner. Chitosan possesses excellent biocompatibility, biodegradation, antimicrobial activity and low immunogenicity. It can be molded into various forms (like gels, membranes, sponges, beads and scaffolds) and it has an exceptional pore forming ability for potential applications in tissue engineering, drug delivery and wound healing

[10,11]. Thus, the inclusion of chitosan in scaffold is beneficial as chitosan is often associated with the structural similarities with glycosaminoglycans an important component of extra cellular membrane. Chitosan is biodegradable to normal body constituents and thus, it is highly useful in wide range of applications in tissue engineering. It binds to mammalian and microbial cells aggressively (antibacterial) [12-14]. Chitosan has been combined with a variety of biopolymers and bioceramic systems like alginate, hyaluronic acid, amylopectin, carbon nanotubes, poly(methyl methacrylate), polylactic acid, growth factors, HA and calcium phosphate [15-22].

On the other hand, the use of alginate scaffolds in tissue engineering applications is limited, [23]. Scaffolds made from alginate are soft and weak, which may limit their further application as templates for tissue regeneration. To overcome these limitations, porous alginate-chitosan composite scaffolds is proposed using chitosan as a supporting material. The composite scaffold, if formed from these two polymers should provide various functional groups like -NH2, -COOH, -OH, etc. which are responsible for cell adhesion and might provide proper chemical cues to cells for proliferation and differentiation. Moreover, the scaffold can be handled easily. In this context, natural biopolymers like chitosan and alginate are used in composites to improve material properties and reduce degradation rates.

Many techniques have been developed to fabricate three-dimensional porous architectures to fill this role, such as by particle leaching [24], phase separation [25] and textile technology. However, scaffolds fabricate by such techniques do not adequately mimic the structure of the natural extracellular matrix in terms of architecture, consequently, new designs and manufacture technologies are required to improve the function and architecture of scaffolds. The different scaffold fabrication methodologies/techniques utilizing several synthetic and natural polymers, including solvent casting, gas foaming, electrospinning, porogen leaching, fiber mesh, fiber bonding, self-assembly,

rapid prototyping, melt molding, membrane lamination and freeze drying has been developed.

This chapter focuses synthesis of chitosan-alginate scaffold by freeze drying method and its physical and biological characteristics. This technique is based upon the principle of sublimation. Polymer is generally prepared by dissolving/suspending polymers/ceramics in water or in an organic solvent followed by emulsification with a water phase. After pouring this mixture into a mold, solvents are removed by freeze-drying and porous structures are obtained. Physical characteristics including SEM, XRD, FTIR and biological characteristics including pore size, stability and degradation and water holding capacity are focused in this chapter.

4.2 Experimental

Chitosan (from shrimp shells) DD (degree of deacetylation) was > 75%, Calcium alginate (alginic acid calcium salt), Lysozyme and Phosphate buffered saline (PBS) were also obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Acetic acid, Sodium hydroxide (NaOH) pellets, Calcium chloride ($CaCl_2$) was purchased from Merck Ltd., Mumbai. All chemicals were of AR grade and used without further purification. All aqueous solutions were prepared in deionized water throughout the experiment.

4.2.1 Chitosan-alginate scaffold synthesis

The chitosan – alginate (CA) scaffolds were prepared in the ratio of 90:10, 80:20, 60:40, 40:60, 20:80, and 10:90 respectively as previously reported [26]. Chitosan was dissolved in 0.1 N glacial acetic acid to form a 1.5% solution. Alginate was dissolved in a solution of 1 M NaOH to form a 2% solution. The chitosan and the alginate solutions were mixed together, and the increase in pH resulted in the gelation of the chitosan phase. Glacial acetic acid was added drop by drop into the polymer mixture to adjust the pH of the mixture to 7.4. The mixture was then homogenized in

a blender for 1h to form a mixture with a 2% final polymer content. The mixture was frozen at -80^{o}C for 24 h, and then lyophilized for 72 h. The dried scaffold was then immersed into a 0.03 M $CaCl_2$ solution for the cross-linking of alginate to occur. The composite was rinsed with dH2O three times to remove unreacted $CaCl_2$.The freeze-drying sublimated the ice crystals into the scaffold, leaving minute pores in the chitosan alginate mixture. Different combinations of scaffold were made such as Chi-alg1 (CA1), Chi-alg2 (CA2), Chi-alg3 (CA3), Chi-alg4 (CA4), Chi-alg5 (CA5), Chi-alg6 (CA6), Chi-alg7 (CA7), and Chi-alg8 (CA8) respectively. The final chitosan-alginate composite was stored in a desiccator prior to evaluation.

4.3 Characterization Techniques

4.3.1 SEM morphology

Morphological analysis was carried out by a scanning electron microscope (SEM) JEOL Model JSM-6360LV at an accelerating voltge10kV and at 500, 1000 and 5000 resolution. The Energy-dispersive X-ray spectroscopy (EDAX) elemental analysis was carried out with the same instrument.

4.3.2 Porosity of CA scaffolds

The pore size of the cross section and the average pore diameter of samples were determined by Scanning Electron Microscope (SEM) images. Dried CA scaffold was soaked overnight in water. Eight pieces of tissue paper were dried overnight in an oven at 55°C and then the weight of tissue papers (W1) was measured. Then water absorbed scaffold was taken from the water medium and the scaffold's diameter and thickness of the scaffold were measured and the volume of the water absorbed scaffold, V1 = (πr^2h) then calculated. The water absorbed scaffold was placed on the top of the tissue papers in a centrifuge tube and then centrifuged at 4,500 rpm for 5 minutes. After that scaffold was removed from the tissue papers and the weight of wet tissue papers, (W2) was measured and then the weight of water in the void space of scaffold, W3 = (W2 –W1) was calculated. The volume of water in the void space, V2

was determined by dividing the weight of water in the void space of scaffold, W3 by the density of water (1.0).

Porosity of water absorbed scaffold (%) = (V2/V1) × 100 (4.1)

4.3.3 Swelling behavior of CA scaffold

The swelling ratio of lyophilized, chitosan, chitosan–alginate, and alginate scaffolds was obtained after 5, 10, 15 days incubation in 1X PBS solution at 37^{o}C. After excess PBS solution was removed from the surface of the scaffolds by gently dabbing with a Kim Wipe, the weights of the swollen chitosan–alginate samples were measured. The samples were then placed at -70^{o}C for 1 day and then lyophilized for 3days. The mass of the dried samples was then measured. The swelling ratio was calculated using the following formula:

Swelling ratio (%) = (Ws-Wd) /Wd X 100 (4.2)

Where Ws and Wd are the weight of samples in the swollen and dry state, respectively.

4.3.4 FTIR

The scaffolds were frozen in liquid nitrogen for 5 min and ground to a fine powder. After, the powder were mixed with KBr powder and compressed into pellets for FTIR examination. The spectrum was analyzed by FT-IR, Bruker, ALPHA 100508, USA spectrophotometer in the range 4000 to 500 cm^{1}.

4.3.5 XRD analysis

The phase composition, lattice parameter and the mean size of the crystallites were determined by XRD (RIGAKU Miniflex 600) equipped with a crystal monochromator employing Cu-Kα radiation of wavelength 1.54 A^{o} and applied scanning rate of 3^{o} min^{-1}, ranged from 20 to 80^{o}. The patterns were analysed by X'Pert High score software.

4.3.6 TGA

The thermal gravimetric analysis (TGA) was used to measure the thermal stability of complexed chitosan–sodium alginate films. TGA SDT Q600 Thermogravimetric Analyzer from TA Instruments Company (Tokyo, Japan) was used. The analyses were made by increasing the temperature from room temperature to 400^{o}C in an inert nitrogen atmosphere with a flow rate of 60 mL/min and warming rate of 10^{o}C/min.

4.4 Results and discussion

4.4.1 SEM Morphology

In tissue engineering, scaffold should have high porosity and inter connected pore structure to enhance a compatible biological condition for cell attachment, proliferation and differentiation. The porous structures of scaffolds are achieved by freeze-drying method. During the freezing process, ice crystals are formed. After removing these crystals by lyophilisation, a porous material is formed. The kind of porosity depends, to a greater extent, on the freezing conditions. The temperature, the thermal gradient and the cooling rate have an effect on the pore structure. In the present study, surface morphology was examined from micrographs taken with a SEM. The scan micrographs of CS, SA and CS-SA scaffolds are shown in Figure 4.1. Scaffolds CA1 and CA2 exhibits flat lamellar phases on which a large number of protruding microfibrils are evident, while scaffold CA3 clearly shows porous smooth surface having highly interconnected pores.

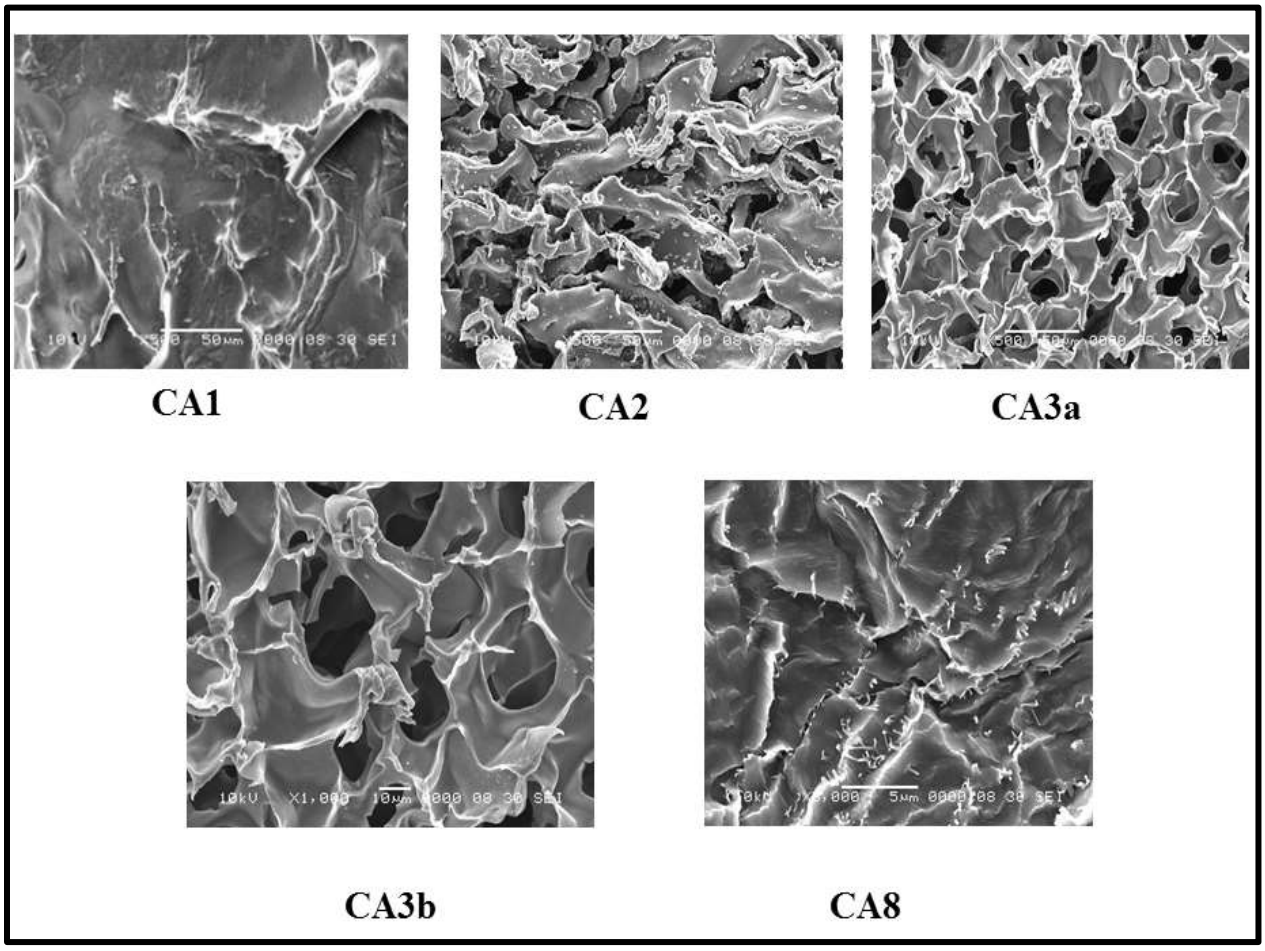


Figure 4.1 SEM pictures of chitosan (CA1), chitosan-alginate (CA2, CA3a, and CA3b), alginate (CA8) scaffolds with X500 and X1000 resolution

Table 4.1 Variation of atomic percentages (At%) of C, O, N, P and Ca atoms in the chitosan-alginate scaffolds

EDAX ZAF Quantification (Standardless) Element Normalized SEC Table : Default				
Element	CA3		CA8	
	Wt %	At %	Wt %	At %
C	59.09	69.00	56.86	65.82
O	28.70	25.16	34.76	30.20
N	4.36	2.66	2.40	1.45
Mg	0.23	0.13	0.66	0.38
P	0.60	0.27	1.15	0.51
Cl	7.03	2.78	4.16	1.63

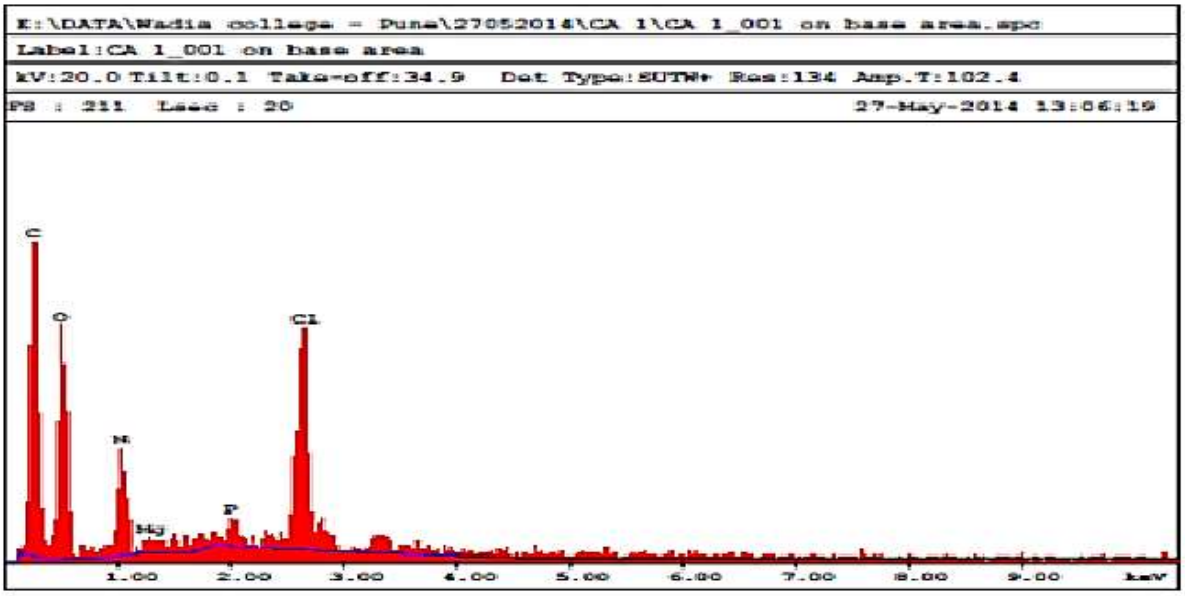


Figure 4.2 Variation of atomic percentages (AT %) of C,O,N,P and Cl atoms in chitosan- alginate scaffolds (CA3)

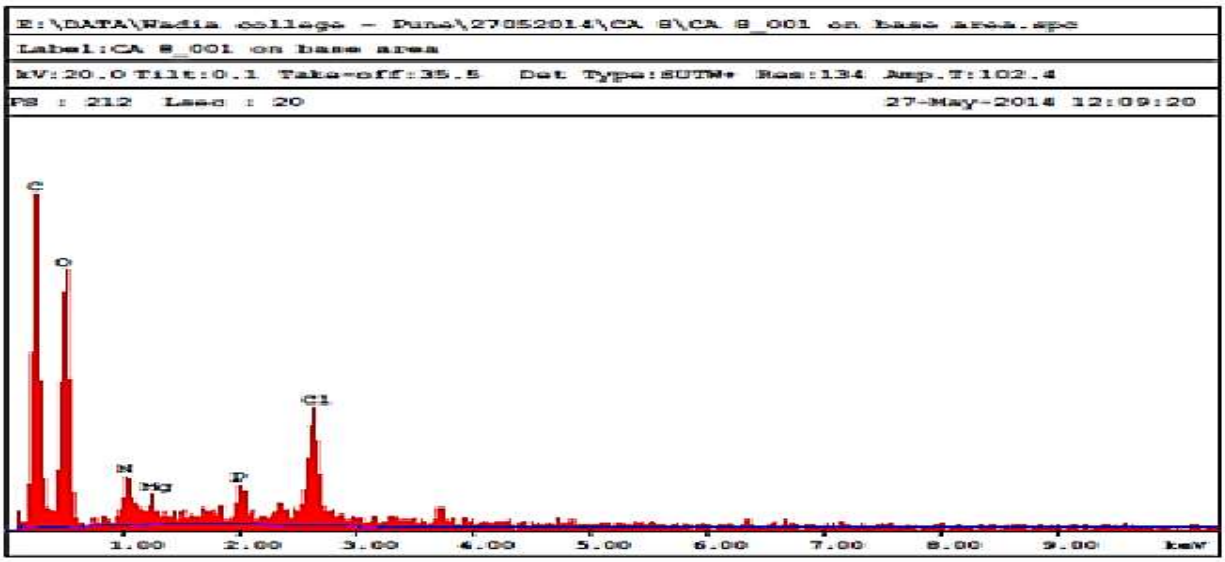


Figure 4.3 Variation of atomic percentages (AT %) of C,O,N,P and Cl atoms in chitosan- alginate scaffolds (CA8)

The elemental composition of the scaffolds (CA3 and CA8) is shown in Table 4.1. Chitosan contains nitrogen in its amine, and acetylamine groups on its backbone which is higher in amount in CA3 as compare to CA8 as CA3 scaffold possessing higher concentration of chitosan than CA8 as shown in Fig 4.2. On the other hand Alginate contains carboxylic group in its structure which is higher in amount in CA8 as compare to CA3 as CA8 possessing higher concentration of alginate than CA3 as shown in Fig 4.3.

4.4.2 Porosity of CA scaffolds

The microstructures, such as pore size, pore distribution, and pore morphology of the scaffolds were observed with a Scanning Electron Microscope (SEM). Porous CA structures are formed by freezing and lyophilisation. During the freezing process, ice crystals are formed. By removing these crystals by lyophilisation a porous material remains. The kind of porosity depends, to a greater extent, on the freezing conditions.

The pore diameter should be in the range of cell diameters to enable cell infiltration and vascularisation of the scaffold. Figure 4.1 shows Scanning Electron

Microscopy (SEM) micrographs of cross – sections of the neutralized Chitosan-Alginate scaffolds.

Table 4.2 – Comparative study of Porosity (%) of chitosan & CA scaffolds

Concentration of CA Scaffold (%)	Porosity of CA scaffolds (%)	Porosity of chitosan scaffolds (%)
CA1	25.11	38.2
CA2	13.55	-
CA3	17.63	25.4
CA4	26.36	35.9
CA5	38.47	45.7
CA6	47.11	55.2
CA7	54.24	63.5
CA8	65.82	73.8

Table 4.2 is the porosities of CA scaffolds at various concentrations chitosan and alginate solution. As shown in the table, porosity increases along with the decrease of chitosan concentration. It is observed that porosities slightly decrease when compared with chitosan scaffolds. It may be due to varying alginate concentrations. Different alginate conc. may influence the results. This high degree of porosity would allow cells to migrate into and populate within the scaffold.

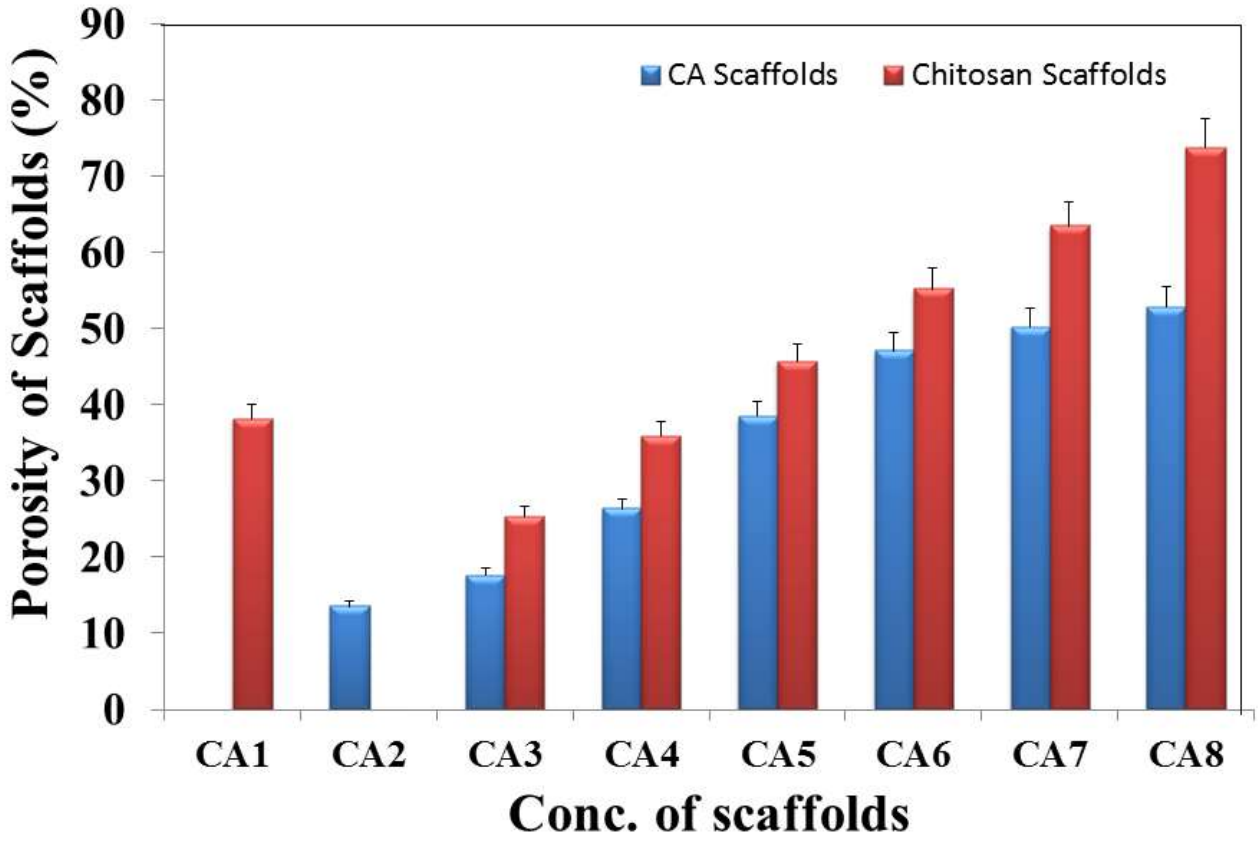


Figure 4.4 Porosity of chitosan, CA scaffolds at various concentration of chitosan and alginate

4.4.3 Swelling behavior of CA scaffold

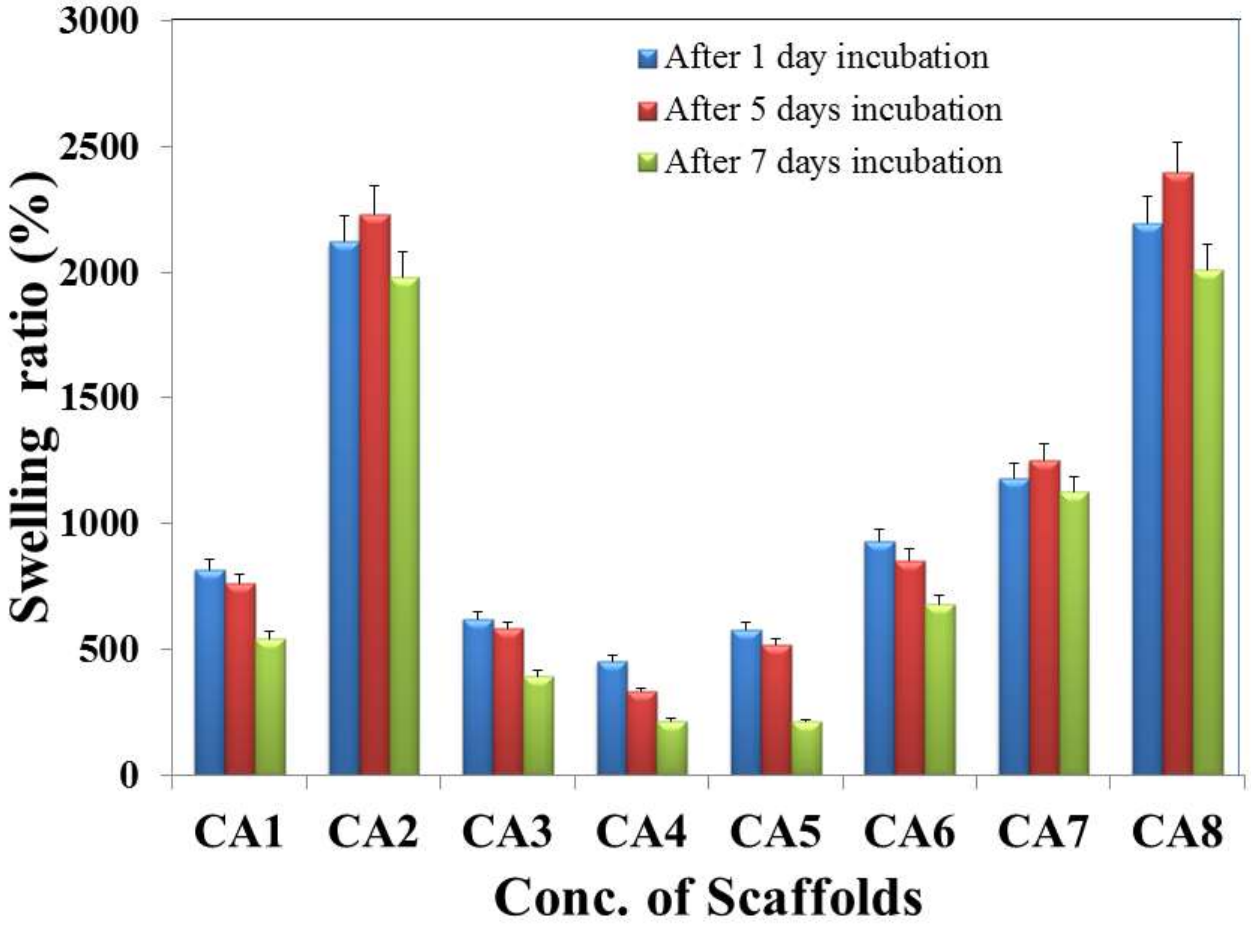


Figure 4.5 Swelling ratios of chitosan-alginate 3D porous scaffolds at various Concentrations

Pores and the small sheet-like structures in scaffold substrate layers supported the absorption of high amount of water. The chitosan used in this study was not a water-soluble salt. Thus, it is expected that the scaffold containing higher percentage of chitosan would have less swelling in an aqueous environment compared to those composed only of water-soluble alginate. The swelling ratio increased as the amount of chitosan decreased (Figure 4.5). The scaffolds containing10%chitosan, 90% alginate, showed a higher degree of swelling than the other chitosan– alginate scaffolds, likely due to the higher amount of hydrophilic alginate present in these scaffolds.

4.4.4 FTIR

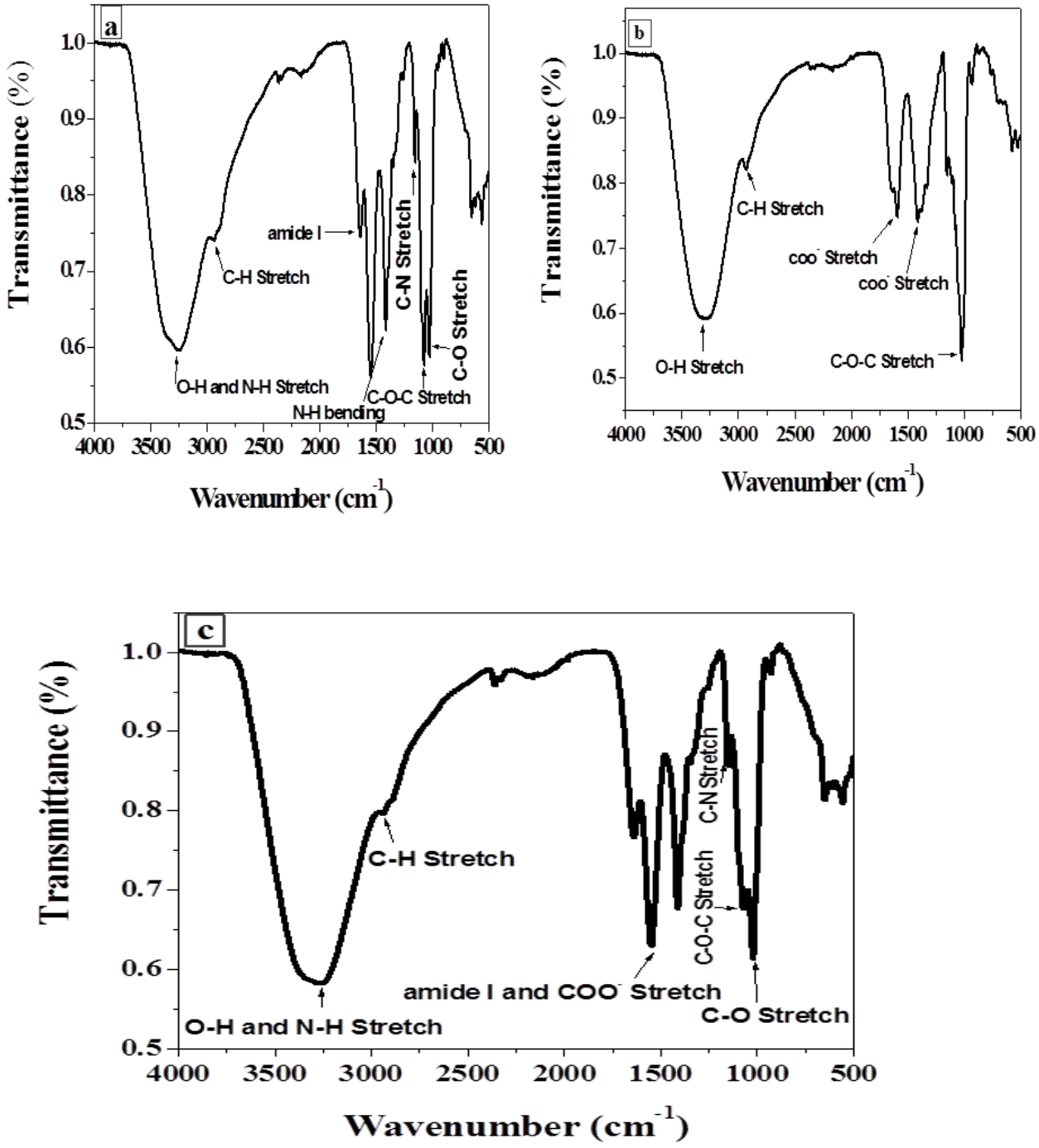


Figure 4.6 FTIR spectra of (a) chitosan (CA1); (b) alginate; and c); chitosan-alginate scaffold.

The significant increase in Young's modulus and yield strength for the chitosan-alginate scaffold can be attributed to the strong ionic interactions between chitosan and alginate to form a chitosan-alginate complex, as confirmed with FTIR analysis shown in Figure 4.6.

In the spectra of CS as shown in Figure 4.6 (a), the broad band at 3247 cm-1 corresponded to the amine and hydroxyl groups; the peak at 2876 cm-1 was caused by -OH stretching; the absorption band of the carbonyl (C=O) stretching of the secondary amide (amide I band) at 1655 cm-1, and the bending vibrations of the N-H (N-acetylated residues, amide II band) at 1599 cm-1 [27]. The peaks at 1488 and 1164 cm-1 belong to the N-H stretching of the amide and ether bonds and N-H stretching (amide III band), respectively. The peaks observed at 1127 and 1083 cm-1 were the secondary hydroxyl group (characteristic peak of -CH-OH in cyclic alcohols, C-O stretch) and the primary hydroxyl group (characteristic peak of -CH2-OH in primary alcohols, C-O stretch) [28].

The bands around 1030 cm-1 (C-O-C stretching) presenting in the IR spectrum of sodium alginate are attributed to its saccharide structure given in Figure 4.6 (b). In addition, the bands at 1785 and 1488 cm-1 are assigned to asymmetric and symmetric stretching peaks of carboxylate salt groups [29]

So in the IR spectrum of chitosan-alginate scaffolds, shown in Figure 4.6 (c) observe the asymmetrical stretching of -COO- groups shifted to 1731 cm-1 and the symmetrical stretching of -COO- groups shifted to 1488 cm-1 [30]. In addition, the absorption band at 1542 cm-1 of chitosan shifts to 1596 cm-1 after the reaction with alginate, the stretching vibration of -OH and -NH2 at 3247 cm-1 shifts to 3490 cm-1 and becomes broad. These results indicate that the carboxylic groups of alginate associate with ammonium groups of CS through electrostatic interactions to form the polyelectrolyte complex. Results indicate that carboxylate group (COO^-) of alginate majorly react with protonated amino (NH_3^+) group via electrostatic attraction to form interpolyelectrolyte complex. Additionally, interactions between amide bonds on

chitosan and the protonated carboxylate groups on alginate could also form intermolecular hydrogen bonds between the polymers.

4.4.5 XRD

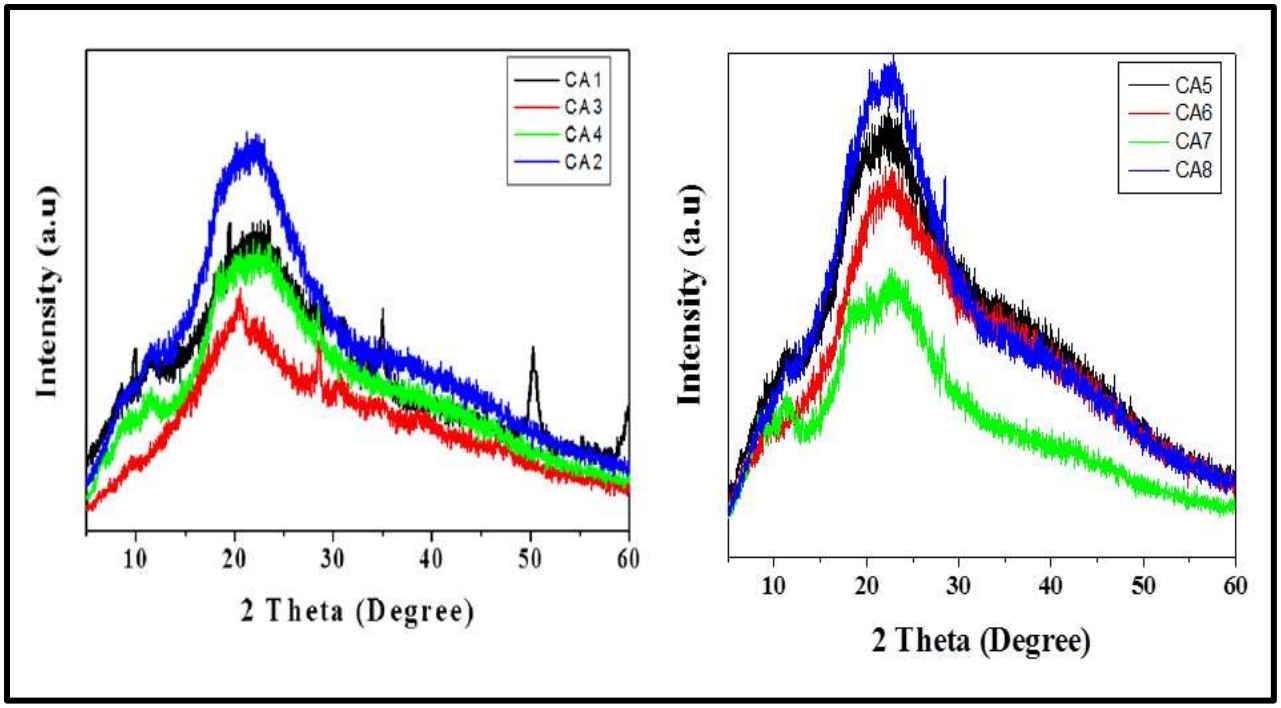


Figure 4.7 XRD patterns of pure Chitosan, alginate and cross linked CA Scaffolds (CA1-CA8) have been studied in the 2θ range of 10^o to 60^o

Figure 4.7 shows the X-ray diffraction pattern of pure Chitosan, Alginate and CA Scaffolds. The XRD analysis was used to study the crystallinity of the prepared samples. The peaks appeared at 2θ=10°, 19^o, 35^o and 50° which match well with the literature values [31] for pure chitosan confirms the semicrystalline nature. The XRD pattern of Alginate polymer alone obtained an amorphous structure. No specific peaks were observed in pure alginate, indicating an amorphous-like structure (CA2). Above graph shows XRD spectra of cross linked Chitosan with alginate which has no broad peaks, indicates that the sample is going from crystalline to amorphous nature. It is

well known that the rigid crystalline structure of pure Chitosan is stabilized mainly by intra and intermolecular Hydrogen bonds [32]. When alginate is cross-linked with Chitosan, protonated hydrogen bonding involving the NH2 groups in chitosan is disrupted, so the rigid crystalline structure weakens. Graphs represent the crystallinity disappearing gradually and the amorphous character of the CA Scaffold increasing as increasing alginate concentration and decreasing chitosan concentration.

4.4.6 TGA

During an overheating process, polymers undergo molecular deterioration which results in thermal degradation. At high temperature, components of polymer start to break down and react among each other. Consequently, it changes the properties of polymer. Thermal degradation cause the polymer to loss its mechanical property. For this reason, TGA is used to determine the degradation temperature (Td) of polymer and the percentage of residue left at a certain temperature. Degradation of polymer has very complex mechanism which involves the processes such as dehydration, chain scission and deacetylation [33].

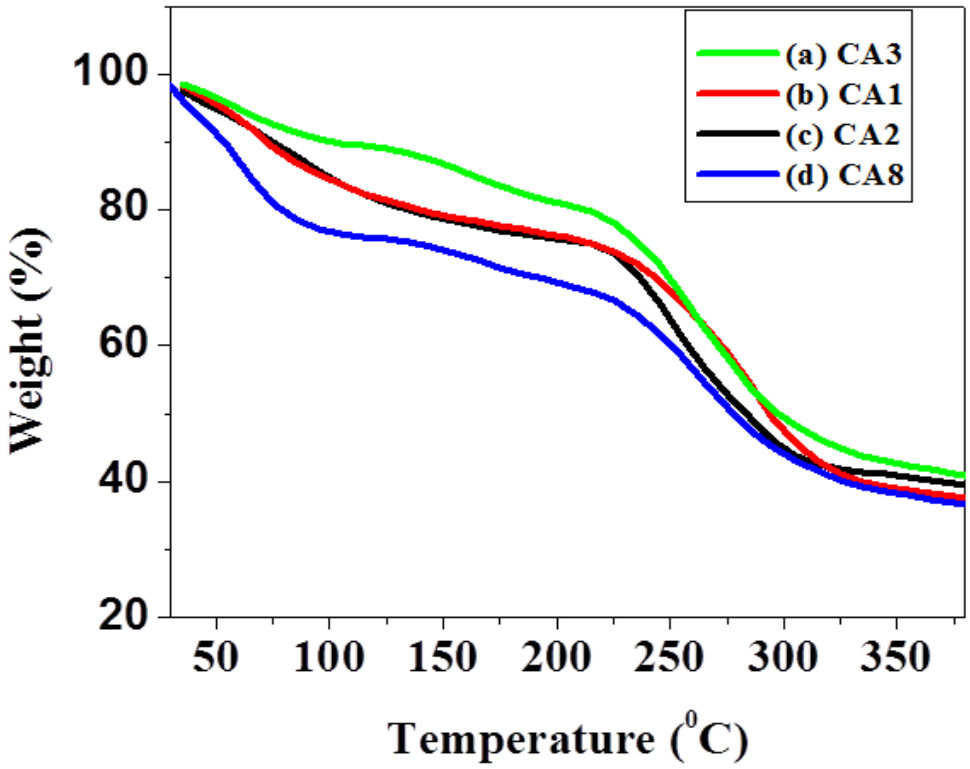


Figure 4.8 Thermal decomposition curves of chitosan-alginate complexed samples (a) CA3; (b) Pure chitosan (CA1); (c) Pure alginate (CA2); (d) CA8

Thermal decomposition process of complexed materials and their pure counterparts was assessed by thermogravimetric analysis (TGA). The thermal decomposition curves were provided as the inflection points depicting the peak maximum to evaluate the degradation characteristics (Table 4.4). Simultaneously, percentage mass loss during thermal decomposition was investigated (Table 4.5). Furthermore, exemplary thermal decomposition curves of sodium alginate-chitosan complexed samples (Chi-Alg 1 and Chi-Alg 2) along with pure chitosan-alginate are presented in Figure 4.8.

Table 4.4 Thermal decomposition temperatures (based on inflection points) of pure polyelectrolytes: sodium alginate (Alg), chitosan (Chi) and chitosan-alginate polyelectrolyte complexes (Chi-alg 1 and Chi-alg 2)

Sample	Inflection Point 1 (°C)	Inflection Point 2 (°C)	Inflection Point 3 (°C)	Inflection Point 4 (°C)
CA1	79.95	220.38	337.00	389.46
CA2	87.21	223.61	308.00	389.10
CA3	117.88	205.04	347.49	389.47
CA8	102.14	218.36	343.86	389.92

Table 4.5 Percentage mass loss and residue of pure polyelectrolytes: sodium alginate (Alg), chitosan (Chi) and chitosan-alginate polyelectrolyte complexes (Chi-alg 1 and Chi-alg 2) during thermal degradation

Sample	Mass Loss 1 (%)	Mass Loss 1 (%)	Mass Loss 1 (%)	Final Residue (%)
CA1	11.98	13.46	34.70	37.25
CA2	12.55	13.42	30.81	39.04
CA3	10.66	8.67	37.85	40.29
CA8	23.03	8.97	29.00	36.33

The obtained results showed that all four samples undergo a three stage thermal-degradation process. The first stage of decomposition process occurred between 23-31^{o}C, which is due to the loss of mass through vaporization of volatile components, such as free water present in the coating. It was observed that composite scaffold with the highest amount of chitosan (CA3) needed a higher temperature to release absorbed water than sample with a lower concentration of this polysaccharide (CA8). This is a result of stronger immobilization of water molecules amongst chitosan chains. Both chitosan-sodium alginate scaffold showed lower free water mass lost at the first step

of decomposition than uncomplexed chitosan and sodium alginate samples. This may be associated to the lower concentration of free water in the complexed material. Considering the structure of chitosan and alginate, it can be seen that water molecules can be bound by three polar groups: amine, carboxyl, and hydroxyl are in chitosan, sodium alginate, and both polymers' structure, respectively [34]. When a reaction between the amine groups of chitosan and the carboxylic groups of sodium alginate occurs, the interactions of polar groups with those functional groups of polysaccharides are impeded and fewer groups of polysaccharides can react with water molecules, resulting in lower water absorption capacity. The second peak (responsible for major weight loss) showed release of water bounded to the functional groups of both polymers, which was not completely removed in the first step of dehydration. Also, degradation of carboxylic groups present in the sodium alginate structure appeared at the temperature range 86–103^{o}C. The thermal stability of materials is determined by major mass loss, after which thermal degradation starts. The thermal stability of chitosan increased due to complexation with sodium alginate. The greatest changes were detected after exceeding 200^{o}C. The last inflection point of the temperature range 307-348^{o}C was characteristic for deacetylation and partial depolymerization of the chitosan chain. The final residue at the maximum analysis temperature (400^{o}C) for all complexed samples was about ~40% of their total weight (Table 4.5).

4.5 Conclusions

Scaffolds provide sites for cells attachment, proliferation, differentiation and migration by up regulating and down regulating the synthesis of protein and growth factors. They provide mechanical support, deliver inductive molecules or cells to the repair site. Here, chitosan-alginate composite scaffolds were prepared by simple freeze drying method forming a 3D microporous structure which was confirmed by SEM technique. These CA scaffolds exhibited uniform pore formation, so that cells

can adhere easily onto the CA scaffolds. Physical and biological parameters have been studied in this chapter. Porosity or pore size of synthesized scaffolds compares with the chitosan scaffolds. It seems porosity of CA scaffolds quite lesser than chitosan scaffolds due to interference of sodium alginate. Degradation of CA scaffolds can be studied by using lysozyme enzyme. The results shows that CA6 scaffold degrades faster as compare to other combinations of chitosan and alginate which containing lower chitosan conc. and moderate alginate conc. As increase alginate conc. affects degradation rate. The scaffolds containing10% chitosan, 90% alginate, showed a higher degree of swelling than the other chitosan– alginate scaffolds, likely due to the higher amount of hydrophilic alginate present in these scaffolds. FTIR spectrum confirms strong ionic interactions between chitosan and alginate to form a chitosan-alginate complex. Also, XRD patterns confirms amorphous nature of CA scaffolds. TGA confirms thermal stability of chi-alg scaffolds.

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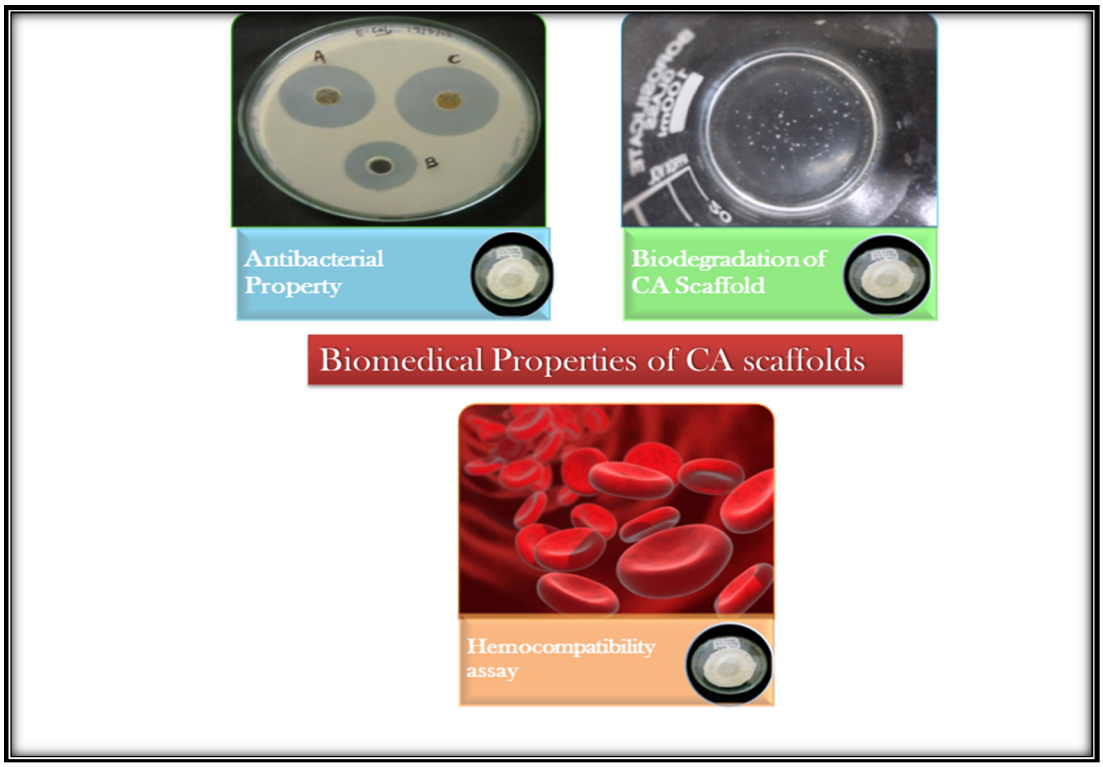
Chapter 5

Biomedical Properties of Chitosan-Alginate Scaffolds



5.1 Introduction

In the mid-1980s, tissue engineering has continued to evolve as an exciting and multidisciplinary field aiming to develop biological substitutes to restore, replace or regenerate defective tissues. Cells, scaffolds and growth-stimulating signals are generally referred to as the tissue engineering triad, the key components of engineered tissues. Scaffolds, typically made of polymeric biomaterials, provide the structural support for cell attachment and subsequent tissue development. However, researchers often encounter an enormous variety of choices when selecting scaffolds for tissue engineering [1]. In order to induce cell adhesion, proliferation and activation, scaffolds must possess requirements such as intrinsic biocompatibility and proper chemistry to induce molecular biorecognition from cells. Materials, scaffold mechanical properties and degradation kinetics should be adapted to the specific tissue engineering application to guarantee the required mechanical functions and to accomplish the rate of the new-tissue formation [2]. Based on the fabrication process, scaffolds with different architecture can be produced with random or tailored pore distribution.

- **Scaffold requirements**

Variety scaffolds produced from different biomaterials and manufactured using a plethora of fabrication techniques have been used in the field in attempts to regenerate different tissues and organs in the body. Irrespective of the tissue type, when designing or determining the suitability of a scaffold a number of key considerations are important for use in tissue engineering:

(i) Biocompatibility

The very first criterion of any scaffold for tissue engineering is that it must be biocompatible, cells must adhere, function normally and migrate onto the surface and eventually through the scaffold thereafter begin to proliferate before laying down new matrix. After implantation, the scaffold or tissue engineered construct must elicit a

negligible immune reaction in order to prevent it causing such a severe inflammatory response that it might reduce healing or cause rejection by the body.

(ii) Biodegradability

The objective of tissue engineering is to allow the body's own cells, to eventually replace the implanted scaffold or tissue engineered construct. Scaffolds and constructs are not intended as permanent implants. The scaffold must therefore be biodegradable so as to allow cells to produce their own extracellular matrix. The byproducts of this degradation should not be toxic and able to exit the body without interference with other organs. In order to allow degradation to occur in tandem with tissue formation, an inflammatory response combined with controlled infusion of cells such as macrophages is required.

(iii) Mechanical properties

Ideally, the scaffold must have mechanical properties consistent with the anatomical site into which it is to be implanted and from a practical perspective, it must be strong enough to allow surgical handling during implantation. Although this is important in all tissues, it provides some challenges for cardiovascular and orthopedic applications specifically. Developing scaffolds with adequate mechanical properties is one of the great challenges in attempting to engineer bone or cartilage. The implanted scaffold, for these tissues, must have sufficient mechanical integrity to function from the time of implantation to the completion of the remodeling process. A further challenge is that healing rates vary with age; for example, in young individuals, fractures normally heal to the point of weight-bearing in about six weeks, with complete mechanical integrity not returning until almost one year after fracture, but in the elderly the rate of repair slows down. This is also taken into account when designing scaffolds for orthopedic applications. However, as the field being evolved, it could be argued that trying to develop scaffolds with mechanical properties similar to bone and cartilage is too much focused. Many materials have been produced with good mechanical properties but retaining a high porosity and many materials which

have demonstrated potential *in vitro* have failed when implanted *in vivo* due to insufficient capacity for vascularization. It is state that a balance between mechanical properties and porous architecture sufficient to allow cell infiltration and vascularization is key to the success of any scaffold.

(iv) Scaffold architecture

In tissue engineering the architecture of scaffolds used is of critical importance. Scaffolds must have an interconnected pore structure and high porosity to ensure cellular penetration and adequate diffusion of nutrients to cells within the construct and to the extra-cellular matrix formed by these cells. Furthermore, to allow diffusion of waste products out of the scaffold, a porous interconnected structure is required and the products of scaffold degradation should be able to elicit from the body without interference with other organs and surrounding tissues. The issue of core degradation, arising from lack of vascularization as well as waste removal from the center of tissue engineered constructs, is of major concern in the field of tissue engineering. Another key component is the mean pore size of the scaffold. Cells interact primarily with scaffolds via chemical groups (ligands) on the material surface. Scaffolds synthesized from natural extracellular materials (e.g. collagen) naturally possess these ligands in the form of Arg-Gly-Asp (RGD) binding sequences, whereas scaffolds made from synthetic materials may require deliberate incorporation of these ligands through, for example, protein adsorption. The ligand density is influenced by the specific surface area i.e. the available surface within a pore to which cells can adhere. This depends on the mean pore size in the scaffold. The pores thus need to be large enough to allow cells to migrate into the structure, where they eventually become bound to the ligands within the scaffold but small enough to establish a sufficiently high specific surface, leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold. Therefore, for any scaffold, a critical range of pore sizes exists which may vary depending on the cell type used and tissue being engineered.

(V) Manufacturing technology

In order for a particular scaffold or tissue engineered construct to become clinically and commercially viable, it should be cost effective and it should be possible to scale-up from making one at a time in a research laboratory to small batch production. The development of scalable manufacturing processes to good manufacturing practice (GMP) standard is critically important in ensuring successful translation of tissue engineering strategies to the clinic. Another key factor is determining how a product will be delivered and made available to the clinician. This will determine how either the scaffold or the tissue engineered construct will be stored [2].

5.2 Experimental

5.2.1 Antibacterial property of CA scaffold

Biodegradable polymeric materials play a major role in medical and pharmaceutical domains because various biomedical devices made with these materials have contributed enormously in human health such as tissue engineering scaffolds [3], drug delivery systems and wound dressing [4]. However, a universal challenge of applying these devices *in vivo* is the infection occurred by microbial-contamination. Serious infection complications include tissue destruction, premature device failure, and the spread of the infection to other areas [5]. In addition, the proliferation of microorganism stimulates the cascade of body defensive responses that can be life-threatening [6]. Bacterial infection is also a major obstacle for wound healing, especially chronic wound healing [7]. Generally, biomaterials used in Food and Drug Administration (FDA) approved wound dressings and other implants include naturally derived materials (e.g., collagen, chitosan and alginate) and synthetic polymers [e.g., polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA)]. However, these commonly used biomaterials do not possess intrinsic antibacterial properties. In recent years, tremendous efforts have been made to prevent and control biomaterials or implant related infections. However, traditional methods

mostly rely on the incorporation of antimicrobial drugs/nanoparticles/peptides into the device matrix or a coating of antibiotics on the device surface with limited effectiveness [8-10]. The main challenge is the rapid loss of antibiotics and the compromise of device or material functionalities including mechanical properties, degradation rate and biocompatibility [11, 12].

Recently, use of biodegradable polymers which can act as antibacterial agent paying very much attention in the field of tissue engineering to bypass above mentioned criteria. The exact mechanism of antibacterial activity is still unclear. The antibacterial mechanism of Chitosan is, generally considered due to its positively charged amino group at the C-2 position of the glucosamine residue, which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms [13]but when alginate is combined with chitosan it will enhance antibacterail property. In this chapter, we studied antibacterial property of chitosan-alginate scaffold by Agar plate diffusion assay and Antibacterial activity assay using ELISA plate reader.

5.2.1.1 Antibacterial activity assay

5.2.1.1a) Agar plate diffusion assay:-

Antimicrobial activity test of the CS, AG and CS-AG hybrid scaffolds was carried out by using agar plate diffusion method. The 0.1gm sample of different scaffolds was absorbed in sterilized phosphate buffered saline (PBS) and evaluated for antimicrobial activity against Gram-positive (*S. epidermidis*) and Gram-negative (*E. coli*) bacterial test strains. Freshly grown colonies (3-5) of bacterial strains were inoculated separately into 25ml nutrient broth medium (10^8CFU/mL). Then broth medium containing bacterial strains was dispersed onto agar plate and scaffolds were transferred on such agar plates. The plates were incubated at 37^oC for 24 h. The inhibitory effect was measured based on clear zone surrounding the scaffold.

5.2.1.1b) Antibacterial activity assay using ELISA plate reader:

Penicillin was used as positive antibiotic controls for *E. coli* and *S. epidermidis*. Standard chitosan and alginate (Himedia) powder were used as a standard controls for antibacterial activity against both *E. coli* and *S. epidermidis*. All three compounds were dissolved in solution of phosphate buffer saline (PBS) and 0.01% acetic acid respectively.

Overnight cultures were diluted with nutrient broth to give microbe concentration of approximately $5x10^5$ CFU/mL. Bacterial suspension (100 µL) was dispersed into each well of a sterile 96 –well polypropylene microtiter plate (Fisher). The blank wells contained nutrient broth alone. Antibiotic solution (11 µL; 20,40,60,120,160 µg/mL) and scaffold concentration (11 µL ; 10 µg/mL) were added to 100 µL bacterial suspension in triplicates to evaluate their antibacterial activity. The pH of the mixtures in the wells was at 7.0, which insured that the bacterial growth was not affected by pH.

Immediately after the addition of antibiotic controller scaffold samples, microtiter plates were gently mixed. Zero hour readings of OD_{570nm} were obtained with ELISA plate reader. Microtiter plates were incubated at 35^oC and OD_{570nm} readings were obtained periodically during the 24h incubation. To insure the accuracy of the OD readings, the contents of the wells were mixed with a micropipette before obtaining the readings to disperse the precipitate formed at the bottom of the wells due to the microbial growth. The average absorbance values of the triplicates were obtained and plotted against time.

After 24h of incubation in the microtiter plates, the number of colonies in each well was determined. The contents of the wells were serially diluted with nutrient broth and plated on nutrient agar to obtain colony counts. Each diluted sample was plated in duplicates for each well. Values for CFU/ml were back calculated according to the number of colonies on the plates after overnight incubation at 35^oC.

5.2.2 Biodegradation of CA scaffolds

Biodegradable and biocompatible polymeric scaffolds have been recently introduced for tissue regeneration purpose. Biodegradation is the crucial parameter of scaffolds to study when applied *in vivo*. The biodegradation of scaffolds provides space for tissue growth and matrix deposition. More and more frequently, chitosan is used in this type of application, in view of its rich biochemical importance. Biodegradability, biocompatibility, non-toxicity, hemostatic, antibacterial, regenerative and antioxidant properties of chitosan have already been reported [14-16] as well as ability to form 3D structures for tissue engineering [17]. In addition, sodium alginate is a natural, biodegradable, inexpensive polymer, whose hydrogel is structurally similar to extracellular living tissue and is an excellent carrier of biologically active substances allowing its use in medical, pharmaceutical and food applications [18, 19]. However, alginate and chitosan are susceptible to various types of degradation 1) Chemical degradation by acids and alkali, 2) usage of certain microorganisms and 3) enzymes to degrade biopolymers are classified as the biodegradation method for biopolymers. Among those, we especially used third method i.e. use of enzymes. By using acid and alkali, may harm other surrounding tissues. When concerns about microorganisms, they may enhance immune response when applied *in vivo*. Therefore, use of enzymes is more feasible than chemical degradation and use of microorganisms.

Enzymatic cleavage of individual chitosan by lysozyme, papaine, α-amylase and chitinase is already known [20-22]. Hydrolysis of alginate, by alginate lyase, was also reported [23], but simultaneous effect of lysozyme on alginate/chitosan scaffolds is novel. Lysozyme is one of enzymes present in the human body that can hydrolyze the β (1-4) linkages between N-acetylglucosamine and glucosamine in chitosan according to the distribution of N-acetyl group [24, 25]. Many researchers have reported the degradation of chitosan by lysozyme [24, 26-31]. Similarly, lysozyme enzymes break down the glycosidic linkage of the alginate network. And it is

observed that, during the degradation process they didn't generate any harmful substances.

5.2.2.1 Stability and biodegradation method

The initial dry weight of scaffolds was determined and recorded as W1. The scaffolds were sterilized in PBS buffer at121^{o}C for 15 minutes. After cooling, the free solution was removed from the sterilized scaffolds and incubated in 7ml of sterilized PBS buffer containing 10µg/ml lysozyme. All incubations were done in 6 well plates at 37^{o}C in a CO_2. Media were replaced weekly with freshly prepared lysozyme solution. After fourteen days of incubation the samples were removed from the degradation media, washed with distilled water and freeze-dried. The weight of the freeze-dried scaffolds was recorded as W2.The percentage degradation of the scaffolds was calculated using equation given below;

$$\text{Degradation weight (\%)} = (W1-W2)/W1\times100 \qquad (5.1)$$

5.2.3 Rheological Study

Rheology is the science of deformation and flow of matter under controlled testing conditions. In case of chitosan-alginate scaffolds, it is necessary to study viscoelastic property as they forms hydrogel. It can be studied before lyophilization process. CA scaffolds may deform under the influence of an external force or stress such as biological fluids like blood when scaffolds are used for *in vivo* applications. For this reason, rheology is considered to the study of stress-strain relationships in materials. Viscoelasticity constitutes one of the basic physical characteristics of polymeric materials [32].

5.2.3.1 Rheological Experiment

Rheological experiments were performed using a controlled stress rheometer (AR2000, TA Instrument Ltd.). 2 cone steel plates (20 mm diameter) were used and the 500-lm gap was filled with tested colloidal gel. A solvent trap was used to prevent evaporation of water. The viscoelastic properties of the sample were determined at

20C by forward and backward stress sweep experiments. The viscosity (g) was monitored while the stress was increased and then decreased (frequency ¼ 1 Hz) in triplicate with 10 min between cycles. The gel recoverability was assessed using defined time breaks between cycles. All samples were analyzed in triplicate [33].

5.2.4 Hemocompatibility assay

Estimation of hemocompatibility was done using protocol given by *Pal et al.* [34]. Fresh human blood was collected in 15ml centrifuge tube containing sodium citrate (10:1). The blood was then diluted with normal saline (8ml blood + 10ml saline). To study hemolysis, sample were cut into 5mm x 5mm size and placed in a tube containing normal saline solution and incubated for 30 minutes at 37^{o}C. After the incubation, diluted blood was added to the tube and incubated for 60 minutes at 37^{o}C. The positive control contained diluted blood added to sodium carbonate solution which caused hemolysis and the negative solution had blood in normal saline solution. Following the incubation, all the tubes were centrifuged for 5 minutes at 3000 rpm. The supernatant was transferred to the cuvette and readings were taken at 545nm.

Percentage hemolysis was calculated with the following formula;

$$(\%)\ Hemolysis = \frac{OD\ (test) - OD(Negative\ control)}{OD\ (Positive\ Control) - OD\ (Negative\ Control)} \text{X}100 \qquad (5.2)$$

5.3 Results and discussion

5.3.1 Antibacterial property of CA scaffold

In terms of surrounding clear zone, the 8 scaffold samples (CA1-CA8) showed antimicrobial activity against *E. coli* and *S. epidermidis* as shown in Table 5.1.Porous biohybrid CA1, CA3 and CA8 scaffold exhibited excellent antibacterial activity against *E. coli* where as CA3 and CA8 showed excellent antibacterial activity against *S. epidermidis*. Figure 5.1 and 5.2 shows inhibitory zone against pathogenic bacteria *E. coli* and *S. epidermidis* by CA composite scaffold.

Table 5.1 Antimicrobial activity of chitosan - alginte (CA) scaffold and antibiotic as a control.

	Diam of inhibition zone (mm)			**Diam of inhibition zone (mm)**	
Sample	*E. coli*	*S. epidermidis*	sample	*E. coli*	*S. epidermidis*
CA1	**50**	38	CA5	44	42
CA2	43	33	CA6	42	41
CA3	**46**	**46**	CA7	45	45
CA4	43	45	CA8	**52**	**47**

	Diam of inhibition zone (mm)	
Antibiotic conc.(µg/ml)	*E.coli*	*S.epidermidis*
20	24	30
40	25	40
60	29	42
120	30	46
180	32	48

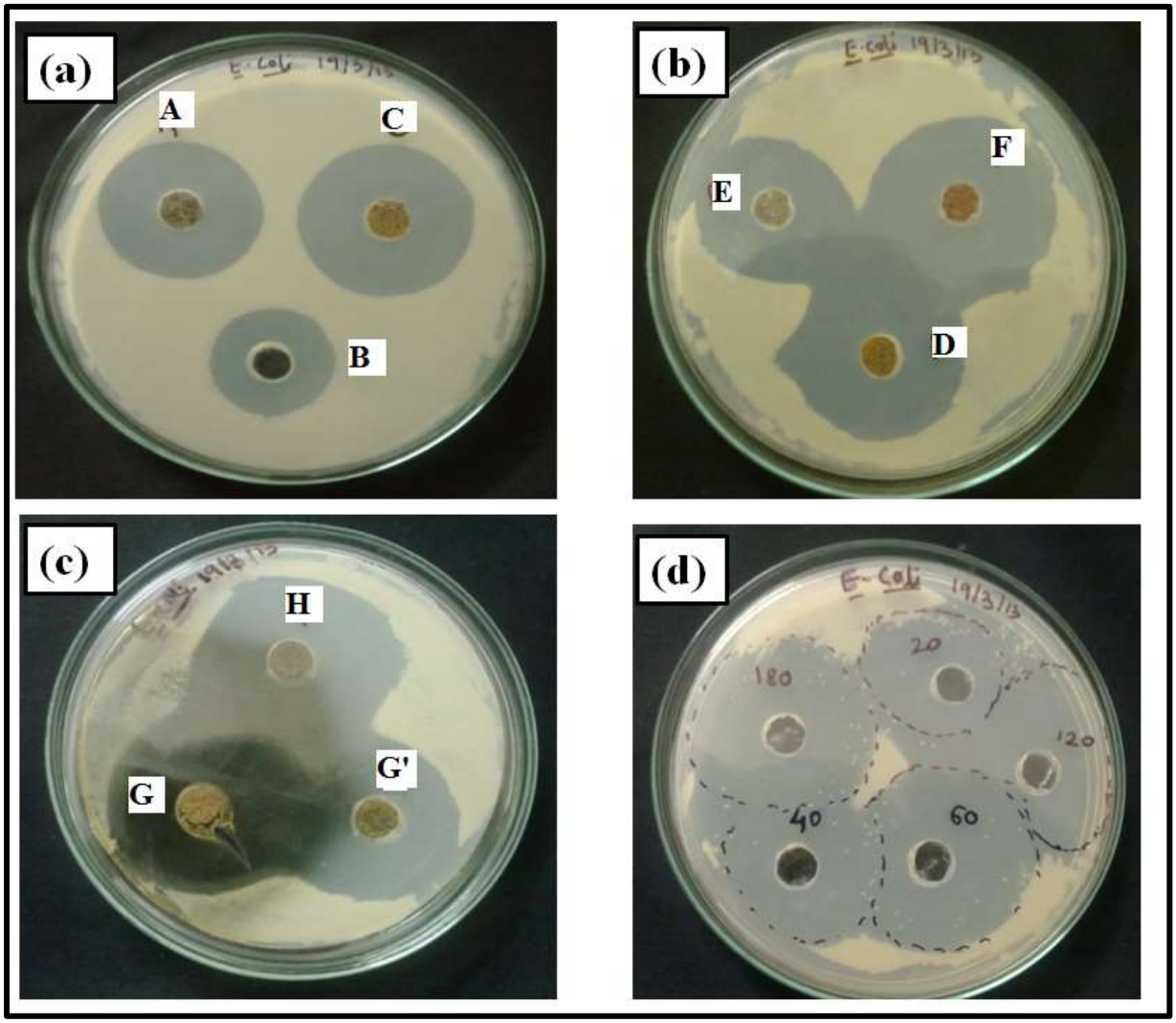


Figure 5.1 Inhibitory zone against pathogenic bacteria (a,b,c) *E. coli* and (d) control antibiotic penicillin.(scaffold samples- A to H i.e.CA1-A,CA2-B,CA3-C.....CA8-H)

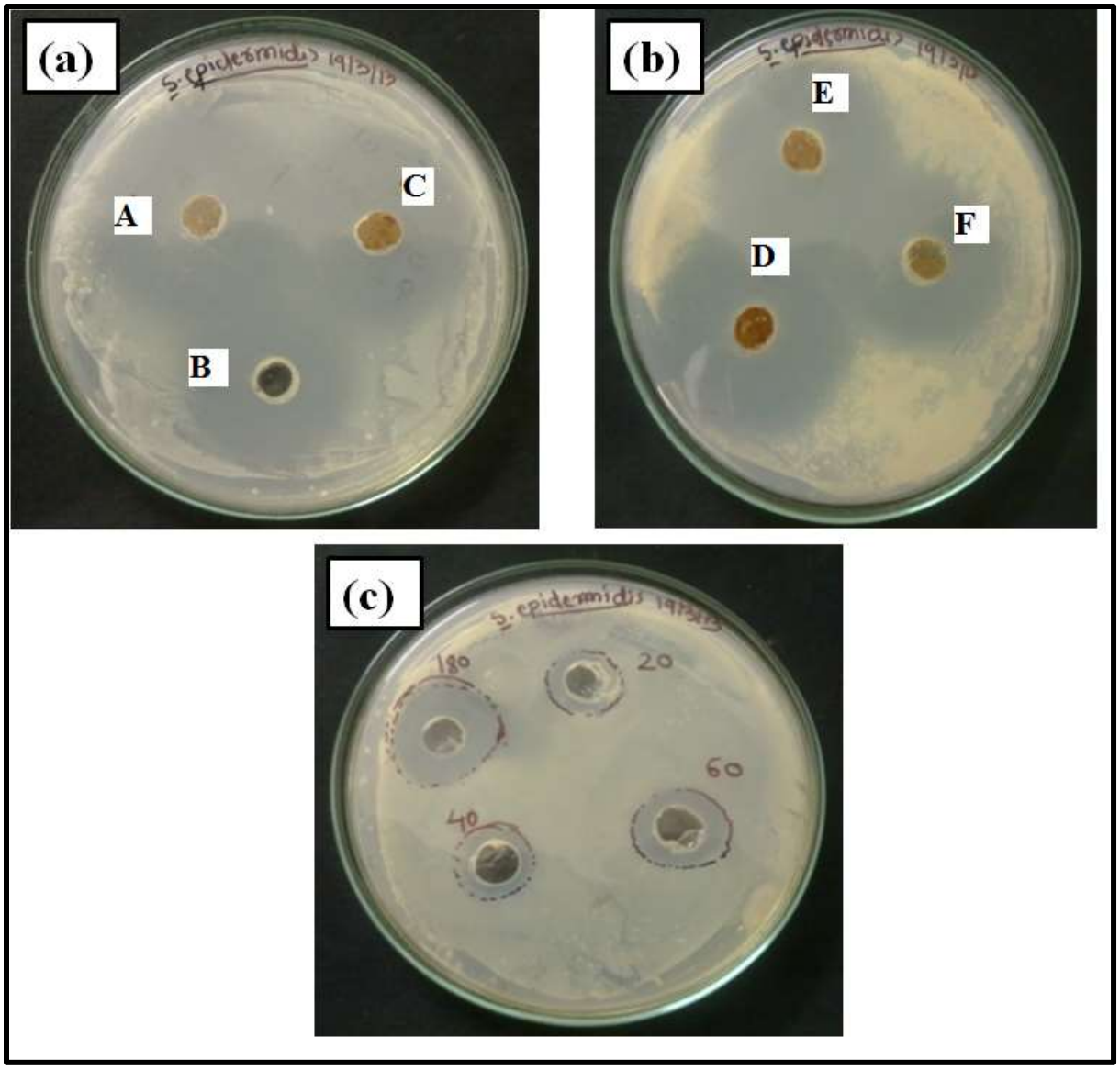


Figure 5.2 Inhibitory zone against pathogenic bacteria (a,b) *S. epidermidis* and (c) control antibiotic penicillin.(scaffold samples- A to H i.e.CA1-A,CA2-B,CA3-C.....CA8-H)

Figure 5.3 Appearance of microtiter plate containing scaffold samples, Pathogenic bacteria *E. coli* and *S.epidermidis,* positive control-antibiotic penicillin and nutrient broth as an negative control

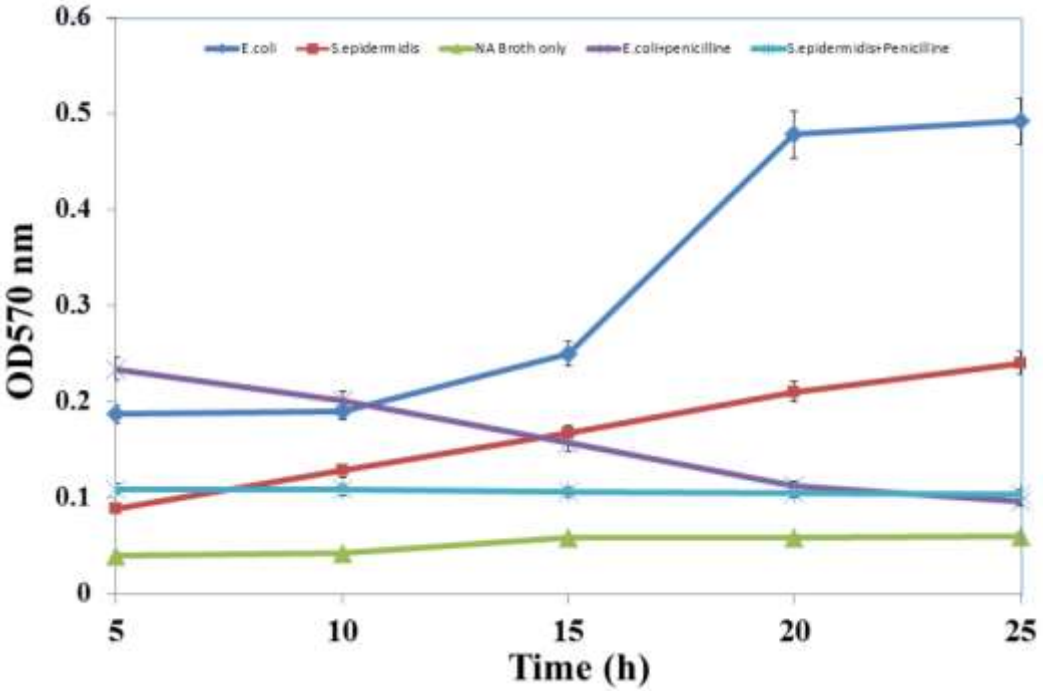


Figure 5.4 Control runs for *E. coli* and *S. epidermidis*: Microbial growth in the absence of antibacterial substances, inhibition of *E. coli* and *S. epidermidis* in the presence of penicillin, NA broth alone as a control of sterile techniques (data points are the average values of triplicate OD readings at 570nm)

Growth curve of *E. coli* reached higher OD (cell density) values than *S. epidermidis* in the absence of antibiotics and scaffold samples as given in Figure 5.4. The growth of *E. coli* was inhibited by antibiotic penicillin but it had little to no effect on *S. epidermidis* growth.

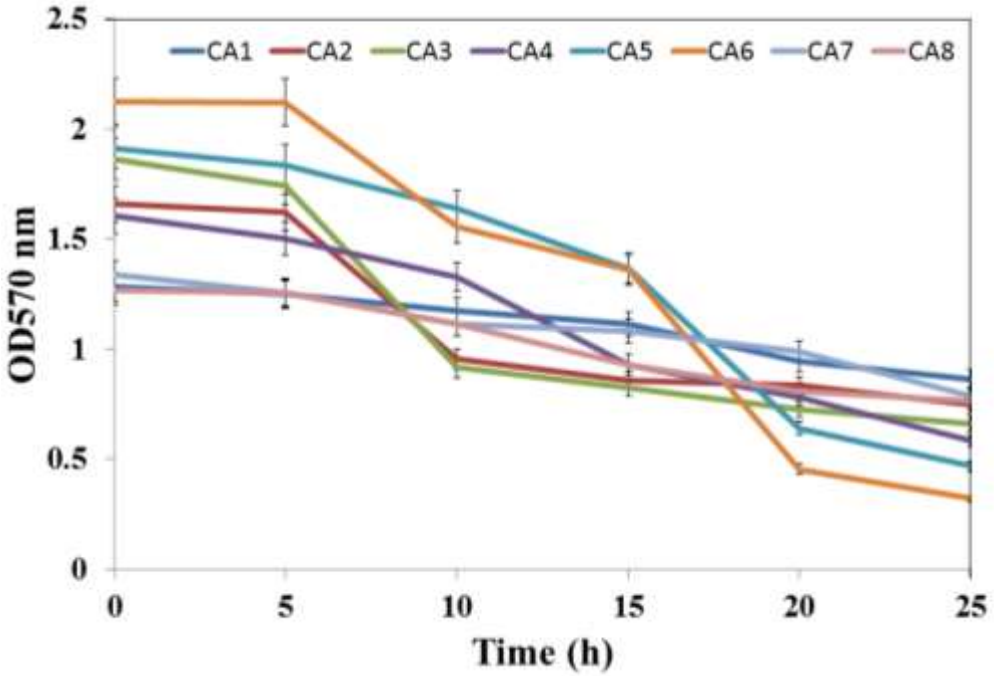


Figure 5.5 Effect of scffold samples (CA1 to CA8) on *E.coli* growth. (Data points are the average values of triplicate OD readings at 570nm)

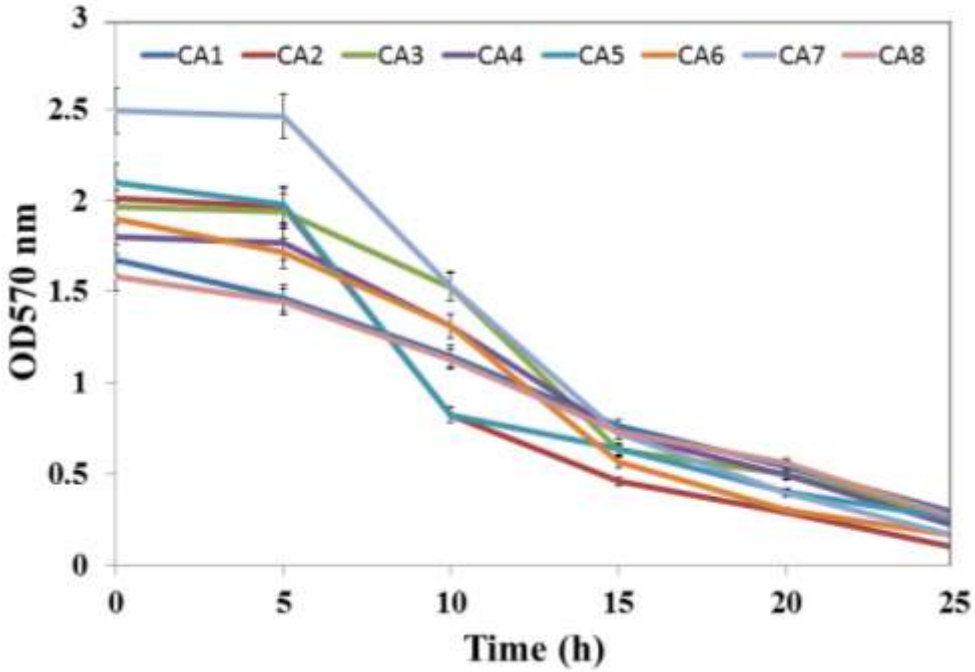


Figure 5.6 Effect of scffold samples (CA1 to CA8) on *S. epidermidis* growth. (Data points are the average values of triplicate OD readings at 570nm)

Table 5.2 Colony counts of *E. coli* and *S. epidermidis* after 24h of incubation with/without antibacterial solutions

Samples	*E. coli* (CFU/ml)	*S. epidermidis* (CFU/ml)
Without antibacterial solution	(164± 7) x 10^7	(156± 7) x 10^7
With Penicillin	(55±5) 10^2	(43±6) x 10^2
CA1	(70±5) x 10^3	(66±4) x 10^3
CA2	(45±3) x 10^3	(38±8) x 10^3
CA3	(65±5) x 10^3	(55±2) x 10^3
CA4	(13±7) x10^3	(08±6) x 10^3
CA5	(65±2) x 10^3	(43±9) x 10^3
CA6	(23±6) x 10^3	(21±4) x 10^3
CA7	(68±5) x 10^3	(53±7) x 10^3
CA8	(22±8) x 10^3	(18±2) x 10^3

All scaffold samples demonstrated a strong antibacterial effect against *E. coli* as shown in Figure 5.5, but it is more effective against *S. epidermidis* as compare to *E. coli* as given in Figure 5.6. The results clearly show that, as the incubation period increases, there were noticeable differences in the extent of microbial growth. After 24h of incubation the bacterial solutions were diluted with NA broth serially and were plated on NA agar plates. The colony counts (CFU/ml) after the overnight incubation of the plates at 35^{o}C are shown in Table 5.2.

Results of present study show that scaffold derived from chitosan and alginate contained antibacterial activity. This antibaceterial activity appears to be against both Gram-positive and Gram-negative bacteria. The electrostatic interaction of chitosan having greater the number of cationized amines, the higher will be the antimicrobial activity [35, 36]. The antibacterial mechanism of Chitosan is generally considered, due to its positively charged amino group at the C-2 position of the glucosamine residue which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms [37], but when alginate is combined chitosan it will enhance antibacterail property. One of the proposed mechnism is that, Utilizing the electrostatic interaction between the carboxyl group on alginate with the amino groups that are located on chitosan results in the two polymers forming strong electrostatic interactions in the cross linking process. However, the polymer is not precipitated in this process [38] leading to the antibacterial action more efficiently.

Attachment of *Escherichia coli* NCIM2064, *staphylococcus epidermidis* NCIM2493 on CA Scaffolds

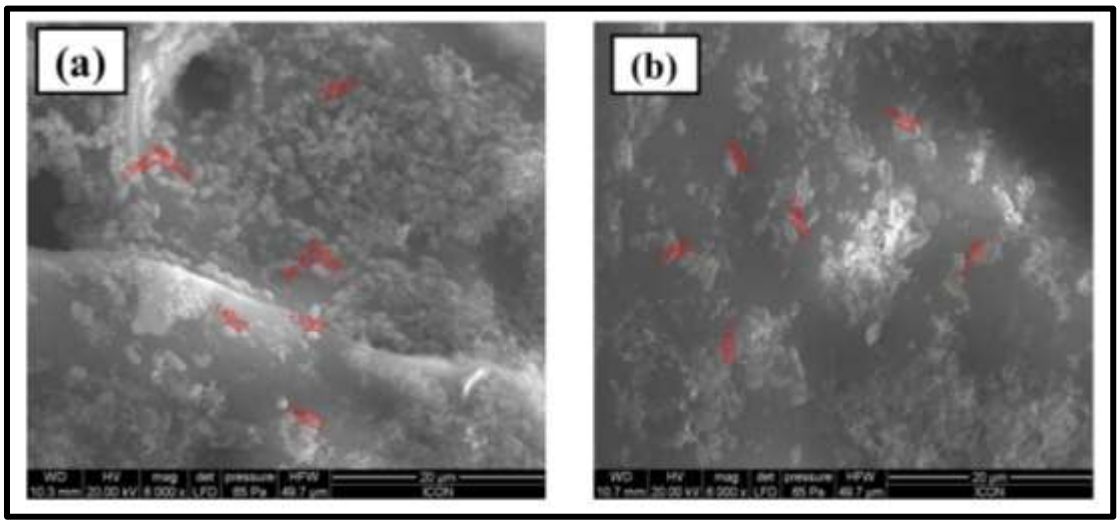


Figure 5.7 E-SEM micrograph of a scaffold CA6 and CA8 exposed in vitro to (a) *Escherichia coli (*NCIM2064) and (b) *staphylococcus epidermidis* NCIM2493 for 24h

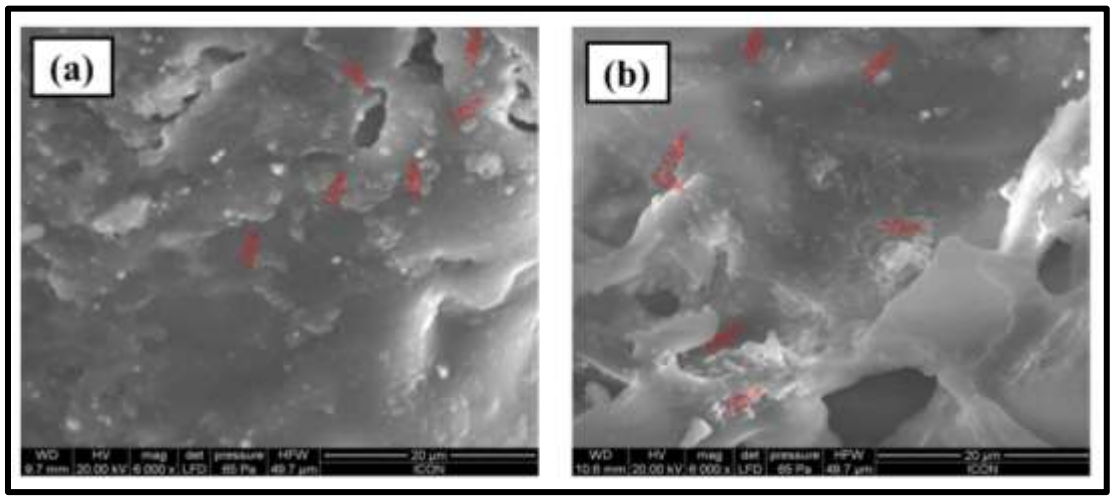


Figure 5.8 E-SEM micrograph of a scaffold CA6 and CA8 exposed in vitro to *(a) Escherichia coli (*NCIM2064) and (b) *staphylococcus epidermidis* (NCIM2493) for 48 h

SEM analysis of the samples of 24h-48h post incubation showed only bacterial adhesion on CA scaffolds shown in Figure 5.7 and 5.8. Within 24h incubation, no significant changes occur in viability of bacterial cells. In addition, no relation could

be found between bacterial adhesion and scaffold's surface reactivity. After being immersed for more than 24h, it is observed reduce microbial count when seeded on scaffold which is supported by following hypothesis.

Gram-negative bacteria possess an outer membrane (OM) that contains lipopolysaccharide (LPS), which provide the bacterium with a hydrophilic surface. The lipid components and the inner core of the LPS molecules contain anionic groups (phosphate, carboxyl), which contribute to the stability of the LPS layer through electrostatic interactions with divalent cations [38].The cell wall of Gram-positive bacteria comprises peptidoglycan (PG) and teichoic acid (TA). TA is an essential polyanionic polymer of the cell wall of Gram-positive bacteria, traversing the wall to contact with the PG layer. They can be either covalently linked to N-acetyl muramic acid of the peptidoglycan layer (wall teichoic acids) or anchored into the outer leaflet of the cytoplasmic membrane via a glycolipid (lipoteichoic acids, LTA) [39].

Despite the distinction between Gram-negative and Gram-positive bacterial cell walls, antibacterial modes both begin with interactions at the cell surface and compromise the cell wall or OM first. For Grampositive bacteria, LTA could provide a molecular linkage for chitosan at the cell surface, allowing it to disturb membrane functions [40]. LPS and proteins in the Gram-negative bacteria OM are held together by electrostatic interactions with divalent cations that are required to stabilize the OM were affected by chitosan-alginate scaffold resulting in disruption of cell wall finally cell death.

5.3.2 Biodegradation of CA scaffolds

The ultimate goal for the application of chitosan 3D porous scaffolds in tissue engineering were the hope that it could disintegrated naturally along the cells growth. As a result, the time to degradation would affect the condition of the cells growth.

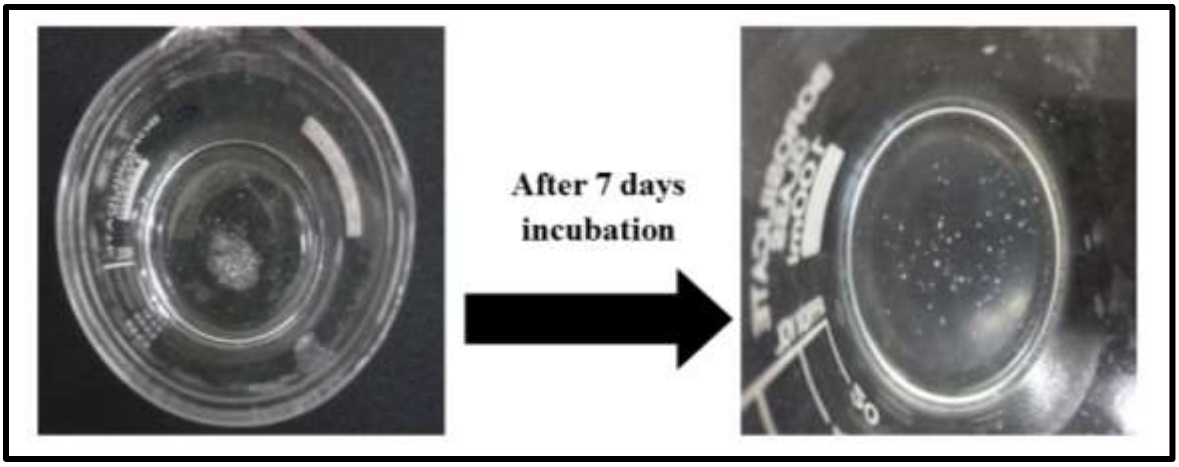


Figure 5.9 *In Vitro* degradation of CA6 scaffold after 7 days of incubation

In vitro biodegradation is a crucial parameter to be considered in bone tissue engineering. The biodegradation of scaffolds provides space for tissue growth and matrix deposition. Lysozyme could hydrolyze the bindings between N-acetylmuramic acid and N-acetylglucosamine in some bacterial cell wall. Therefore, the lysozyme was used as degradation enzyme to investigate through time course of degradation conditions of CA 3D porous scaffolds. Table 5.3 shows the degradation weight (%) of CA scaffolds with various CA concentrations.

Table 5.3 - Degradation weight of CA scaffolds after 7 days incubation

Concentration of CA (%)	Degradation weight of CA scaffold after 7 days (%)
CA – 1	15
CA – 2	32
CA – 3	28
CA – 4	16
CA – 5	14
CA - 6	4
CA – 7	10
CA – 8	12

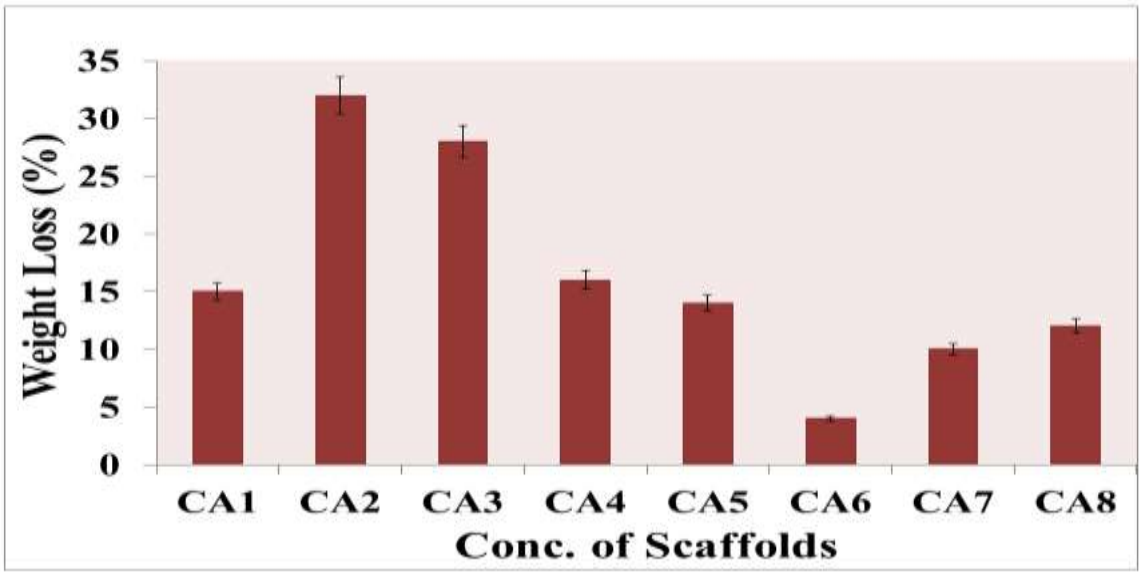


Figure 5.10 Graphical representation of degradation rate of chitosan scaffold after 7 days (%), showing higher degradation rate of CA6

In the degradation process of CA scaffold by lysozyme, the enzyme must enter the scaffolds and react with the chitosan and alginate polymers. The scaffolds all have pore sizes of about 30-70 μm, which provides a large enough area for lysozyme to enter, but the morphology of the scaffold substrate layers is different in each one. Therefore, CA6 scaffold degrades faster as compare to other combinations of chitosan and alginate which containing lower chitosan conc. and moderate alginate conc. But as conc. of alginate increases, its affects degradation rate (CA7 & CA8). It is also observed that scaffolds containing higher chitosan concentration taking time to degrade as compared with CA6-CA8 combinations as shown in Figure 5.10.

5.3.3 Rheological studies of CA Scaffolds

CA scaffolds shows shear thinning with recoverable stiffness. Rheological studies were employed to probe the differences in viscoelasticity of colloidal gels. Mass ratios of 4% chitosan and 3% alginate yielded the highest viscosity gel and improved reversibility compared to other ratios as presented in Figure 5.11. As

expected, colloidal gels containing more positively-charged particles (CA3) exhibited higher viscosity; while colloidal gels composed of excess negatively charged particles (CA8) exhibited more fluidity. Pure suspensions of chitosan and alginate exhibited minimal shear-thinning behaviour (C100 and A100). Delaying shear cycles for more time may enhance the recovery of gel viscosity. All the results suggested that the colloidal gels were desirable for injectable applications.

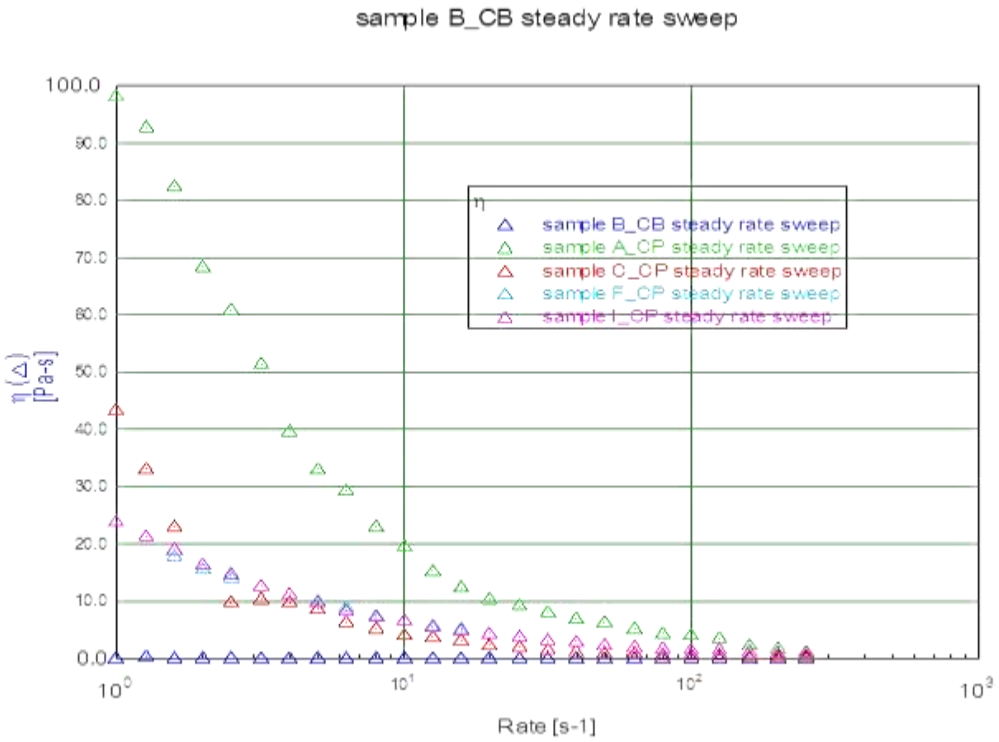


Figure 5.11 Viscosity and shear-thinning behavior of colloidal gels mixed at different ratios for accelerating (solid symbols) and decelerating (open symbols) shear force

For colloidal gels, the strength of the cohesion depends upon the interparticle interactions such as electrostatic forces and van der Waals attractions [41]. These interparticle interactions were controlled by the composition of the colloidal gels, such as concentration and ratio of the two oppositely charged particles. chitosan and alginate scaffolds self-assembled through interparticle interactions resulting in a stable 3D porous network. Under static conditions, the viscosity and structure of

colloidal assemblies leading to a stable structure exhibiting high viscosity at equilibrium. If the particle-particle equilibrium is disrupted, for example by external force applied to disrupt the interparticle interactions, the colloidal system will demonstrate shear-thinning behaviour. Once the external force is removed, the strong cohesive property of the colloidal gel is recovered and the 3D porous structure reconstructed. This reversibility makes the gel an excellent material for applications in molding, extrusion, or injection of tissue scaffolds and drug delivery systems.

5.3.4 Hemocompatiblity Assay

Thought the scaffolds are found to be porous, it is important that the scaffolds should not alter the integrity of blood when they come in contact with it. Hence, hemocompatiblity of CA scaffolds was analyzed and is shown in Table 5.4. The percentage hemocompatiblity was found to be less than 5% suggesting that all the four scaffolds were highly compatible with human blood.

Percentage Hemolysis

Highly hemocompatible (< 5% hemolysis)

Hemocomparible (5-10% hemolysis)

Non- hemocompatible (> 20% hemolysis)

Table 5.4 Hemocompatibility assay of CA Scaffolds

Sample	O.D at 545 nm	% hemolysis	Remarks
Positive control	0.654		
Negative control	0.021		
CA1	0.0231	0.331	Highly Hemocompatible
CA3	0.035	2.21	Highly Hemocompatible
CA6	0.023	0.315	Highly Hemocompatible
CA8	0.022	0.158	Highly Hemocompatible

5.4 Conclusions

Basic properties of Chitosan- Alginate scaffolds have been studied in this chapter viz. antibacterial activity, biodegradation and rheological studies. These scaffolds were exhibit a strong antibacterial activity against *E. coli* and *S. epidermidis* investigated by antibacterial activity assay and evaluated reduced microbial count by obtaining SEM images of scaffold exposed *in vitro* to *Escherichia coli (*NCIM2064) and *staphylococcus epidermidis (*NCIM2493) strains. Degradation of CA scaffolds can be studied by using lysozyme enzyme. The results shows that CA6 scaffold degrades faster as compare to other combinations of chitosan and alginate which containing lower chitosan conc. and moderate alginate conc. Rheology is the very important parameter to study in case of such injectable scaffolds. It explains shear thinning behavior of scaffolds under stress. Also, CA scaffolds are highly compatible with human blood. These preliminary studies prove the feasibility of chitosan-alginate scaffold and it can serve as a potential material for tissue engineering applications.

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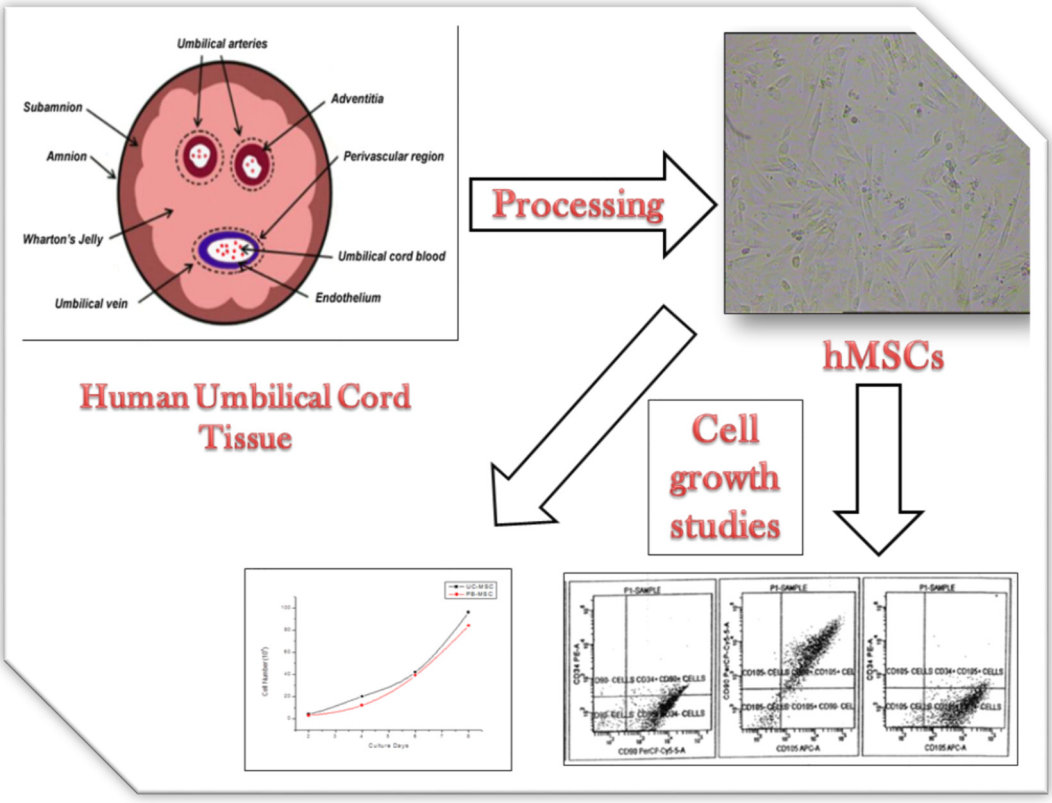
Chapter 6

Isolation of Mesenchymal Stem Cells from Human Umbilical Cord Tissue



6.1 Introduction

"Stemness" refers to the differentiating capabilities of cell into multiple cell types. In other words stem cells are uncommitted cells found within differentiated cells. Recently stem cells have generated more public as well as professional interest than almost any topic in biology. Stem cells are the basic cells for every organ, tissue and cell in the body. They are like a blank microchip which can eventually be programmed to perform any number of specialized tasks. Stem cells are undifferentiated cells that do not yet have a specific function [1, 2]. Under proper conditions stem cells begin to develop into specialized tissues and organs. Additionally, stem cells are self-sustaining and can replicate themselves for long periods of time. Stem cells have the remarkable potential to develop into many different cell types in the body as described in Figure 6.1. Serving as a sort of repair system for the body, they can theoretically divide without limit to replenish other cells as long as the person or animal is still alive [3]. When a stem cell divides each new cell has the potential to either remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell or a brain cell [4].

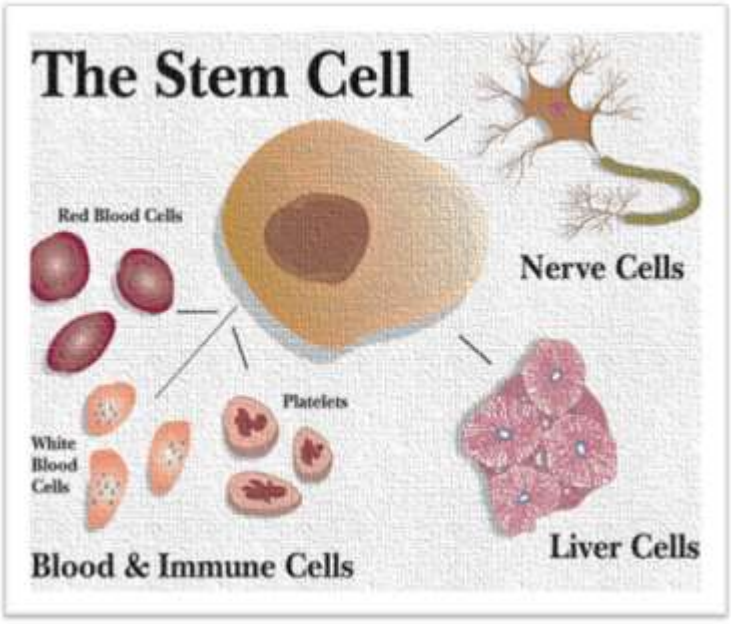


Figure 6.1 One of the property of stem cell – Differentiation [5]

Stem cells can also be found in very small numbers in various tissues in the adult body. For example, bone marrow stem cells are found in the marrow of the bone and they give rise to all specialized blood cell types. Adult stem cells are called as multipotent stem cells and are typically programmed to form different cell types of their own tissue. Adult stem cells have not yet been identified in all vital organs. In some tissues like from brain, though stem cells exist, they are not very active and thus do not readily respond to cell injury or damage. Scientist are now also exploring ways in which they can induce the stem cells already present to grow and make the right cell types to replace the damaged ones.

Stem cells can also be obtained from sources like the umbilical cord of a newborn baby. This is an accessible source of stem cells, compared to adult tissues like the brain and bone marrow. Although scientists can grow these cells in culture dishes, they can do so only for a limited time. Recently, scientists have discovered the existence of stem cells in baby teeth and in the amniotic fluid- the "water bath" that surrounds an unborn baby- and these cells may possess the potential to form multiple cell types. Research to characterize and study these cells can also have the potential to form multiple cell types. Research to characterize and study these cells is very promising but at a very early stage [6-9]. In this chapter, we focused culturing of mesenchymal stem cells from umbilical cord tissue especially from Wharton's jelly.

6.1.1 Structure of umbilical cord

The umbilical cord is a conduit between the developing embryo or fetus_and the placenta. It is called the navel string, birth cord or funiculus umbilicalis. During prenatal development, the umbilical cord is physiologically and genetically part of the fetus and, (in humans), normally contains two arteries (the umbilical arteries) and one vein (the umbilical vein), buried within Wharton's jelly. The fetus with oxygenated, nutrient-rich blood from the placenta is supplied by the umbilical

vein. Conversely, the fetal heart pumps deoxygenated, nutrient-depleted blood through the umbilical arteries back to the placenta.

The umbilical cord develops from and contains remnants of the yolk sac and allantois. It is therefore derived from the zygote. It forms by the fifth week of fetal development, exchanging the yolk sac as the source of nutrients for the fetus. The cord is not directly connected to the mother's circulatory system, but instead of that it joins the placenta, which transfers materials to and from the mother's blood without allowing direct mixing. T.S of umbilical cord is explained in Figure 6.2. The length of the umbilical cord is approximately equal to the crown-rump length of the fetus throughout pregnancy. The umbilical cord in a full term neonate is usually about 50 centimeters (20 in) long and about 2 centimeters (0.75 in) in diameter [10].

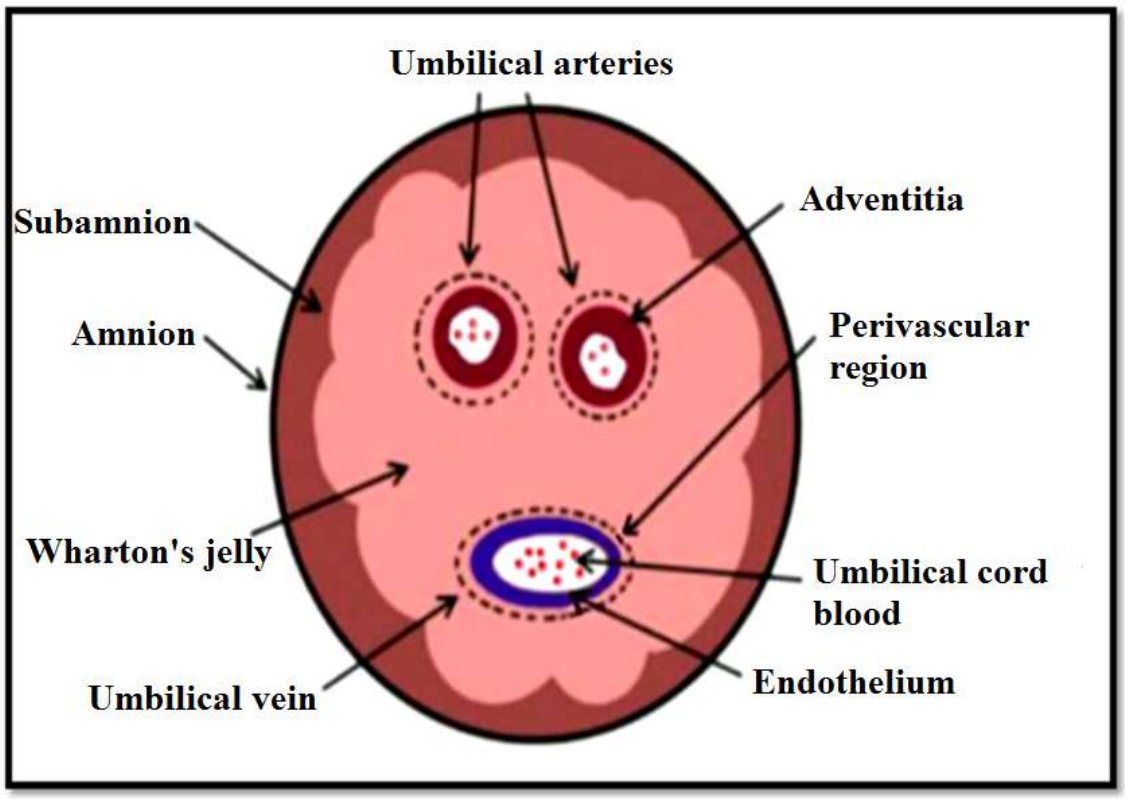


Figure 6.2 cross sectional diagram of Human Umbilical Cord [11]

Properties of Stem cells:

The classical definition of a stem cell requires that it possess following properties

- Self-renewal
- Potency
- Plasticity
- Unspecialized

Self renewal : While maintaining the undifferentiated state, it is the ability to go through numerous cell divisions.

Potency : The capacity to differentiate into specialized cell types. This needs stem cells to be either totipotent or pluripotent to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes referred as stem cells. Stem cells are capable of dividing and renewing themselves for long periods, which do not normally replicate them. Stem cells may replicate many times or proliferate.

- **Totipotent** stem cells further differentiated in embryonic and extra embryonic cell types. Such cells can construct a complete, viable, organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced due to the first few divisions of the fertilized egg are also totipotent.
- **Pluripotent** stem cells are the descendants of totipotent cells and can differentiate into nearly all cells, i.e. cells derived from any of the three germ layers.
- **Multipotent** stem cells can differentiate into a number of cells, but only those of a closely related family of cells.
- **Oligopotent** stem cells can be differentiates into only a few cells, such as lymphoid or myeloid stem cells.

- **Unipotent** cells can give rise to only one cell type, their own, but have the property of self-renewal which distinguishes them from non-stem cells (e.g. muscle stem cells).

Plasticity: The property of stem cell to transdifferentiate into multiple cell lineages. The stem cell has the property to differentiate into multiple cell types. Flexibility and reversibility of tissue and lineage specification *(tissue plasticity)* and of properties within a tissue *(within-tissue plasticity)* have major implications with regard to concepts of stem cell function.

Unspecialized: One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

6.1.2 Types of Stem Cells

The mammalian stem cells are broadly classified as Embryonic, Adult and Fetal stem cells. Embryonic stem cells are isolated from the inner cell mass of blastocysts and Adult stem cells are found in adult tissues [12]. In a developing embryo, stem cells can differentiate into all of the embryonic tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing cells, but also maintain the normal turnover or regenerative organs such as blood, skin or intestinal tissues. Several examples now exist of some adult stem cells with pluripotent flexibility, including cells from bone marrow, peripheral blood, the inner ear, umbilical cord blood, nasal mucosa, amniotic fluid and the placental amniotic membrane [13, 14].

- **Embryonic stem cell**

Embryonic Stem cell are derived from the epiblast tissue of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryos. A blastocyst is an early stage embryo approximately four to five days old in humans and consisting of 50-150 cells. ES cells are pluripotent and give rise to all derivatives of the three

primary germ layers: ectoderm, endoderm and mesoderm during development. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. These stem cells are derived from the inner cell mass of the blastocyst at a stage before it would implant in the uterine wall. The embryonic stem cell is pluripotent and can self- replicate, it can give rise to cells derived from all three germ layers. ES cells are considered the archetypal pluripotent stem cell; they proliferate extensively in culture and, based on their normal function during development or results from reinsertion into another embryo, have the potential to form any tissue. Although this potential is attractive for treatment of degenerative disease, the results to this point have been modest, and there are still many scientific hurdles to overcome before ES cells might be used clinically, including generation of functional differentiated cells, tumor formation, and immune rejection [14, 15].

- **Adult stem cells**

This stem cell is undifferentiated (unspecialized) cells which are found in a differentiated (specialized) tissue. It can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated. Traditional dogma maintains that there are few adult (tissue or postnatal) stem cells present in the body and that they are difficult to isolate and grow in culture and extremely limited in their capacity to generate new cell types, being limited to forming more cells from their tissue of origin. However, an explosion in publications in the last few years is overturning this dogma and showing a remarkable flexibility for these cells. In a 2001 publication, evidence was presented that a single adult bone marrow stem cell could contribute not only to marrow and blood but also to formation of liver, lung, digestive tract, skin, heart, and muscle. Several examples of some adult stem cells now exist with pluripotent flexibility, including cells from bone marrow, peripheral blood, the inner ear, umbilical cord blood, nasal mucosa, amniotic fluid and the placental amniotic membrane [15].

Based on the cell lineage, they are classified as

- Hematopoietic stem cell
- Mesenchymal stem cell
- Endothelial progenitor cell
- Side population cell

❖ **Hematopoietic Stem Cell**

A hematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood and can undergo programmed cell death, called apoptosis-a process by which cells that are detrimental or unneeded self-destruct. As is true for bone marrow, the CD34+ cells are a mixture of stem cells, progenitors, and white blood cells of various degrees of maturity. Stem cells that form blood and immune cells are Hematopoitic Stem cell. They are responsible for the constant renewal of blood. HSC can give rise to all blood cell types including myeloid (Monocytes and Macrophages, neutrophile, basophiles, eosinophiles, erythrocytes, platelets,dentritic cells and lymphoid lineages (T cell,B cell ,NK cell) as shown in Figure 6.3 [15]. They are non-adherent, rounded, with a rounded nucleus and low cytoplasm to nucleus ratio. Two types of HSC have been identified

LONG TERM HSC: Proliferate for lifetime of an animal.

SHORT TERM HSC: Proliferate for a limited time.

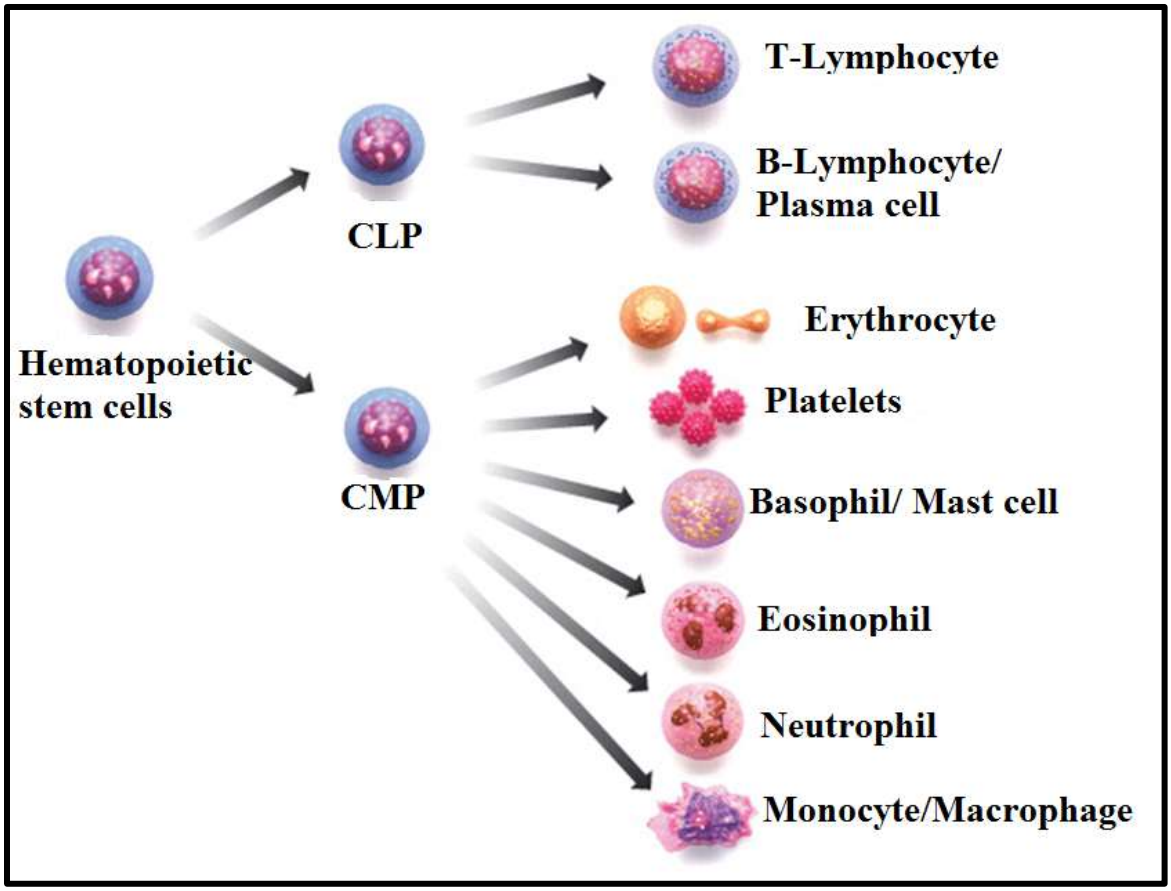


Figure 6.3 Differentiation of Hematopoietic stem cells [16]

❖ **Mesenchymal stem cell**

The term *mesenchymal stem cell* (MSC) refers to adult mesenchymal progenitor cells with the potential to produce progeny that differentiate to produce a variety of mesenchymal cell types (e.g., fibroblasts, muscle, bone, tendon and ligament adipose tissue) as shown in Figure 6.4. Mesenchymal stem cells (MSC) are a type of multipotent adult stem cell. MSCs are immature and like haematopoietic cells, which we find in umbilical cord blood, have a good capacity to renew themselves and differentiate continuously into specialized cells of the various human tissues. MSC's were originally described as early as the 1960's in animal experiments on embryo's, but it was not until the 1980's that the concept of common MSC's in adult tissues was confirmed. Source and availability of MSC's has, however, taken some time to work out. While some MSC's can be

found in many organs, those organs are not realistic targets for harvesting them without resulting organ damage. MSC's have also been sourced in bone marrow (1- 4 per 100,000 nucleated cells), or, in a smaller numbers, in the umbilical cord blood itself. They are also present, in much smaller concentrations in many adult human organs and neonatally in foetal liver and amniotic fluid. Compared to these sources, however, MSC's can be found in more considerable numbers in Wharton's jelly, the matrix of the umbilical cord and in the placental tissues. The added advantage of Wharton's Jelly is also that there is no risk in the harvesting procedure [17].

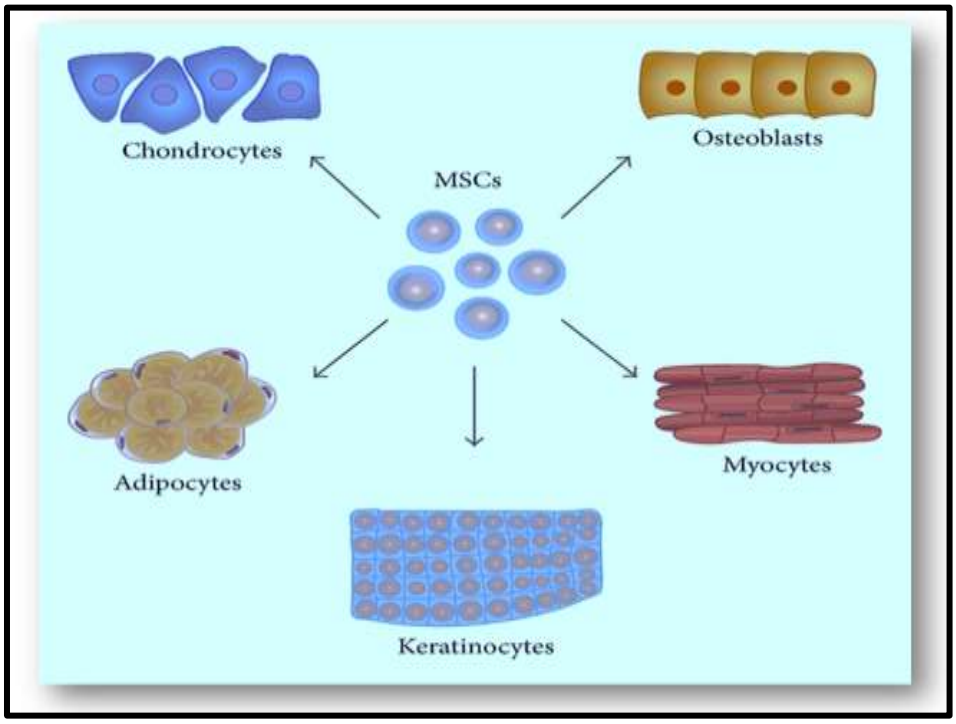


Figure 6.4 Multilineage potential of MSCs (mesenchymal stem cells), such as bone-marrow-derived mesenchymal stromal cells and adipose derived stromal cells, has the potential to differentiate into various lineages. It has been demonstrated that MSCs can undergo osteogenesis, chondrogenesis, adipogenesis, and myogenesis [18]

❖ **Endothelial Progenitors Cells**

Differentiation of organs depends on signals derived from developing vasculature. Embryonic endothelial progenitor cells (EPCs), angioblasts, arise from migrating mesodermal cells and have a precursor in common with hematopoietic stem cells (hemangioblasts, HSCs). These cells appear together during formation of blood islands and the yolk sac capillary network with the EPCs located peripherally to the HSCs. EPCs respond to fibroblast growth factor-2, and vascular endothelial growth factor (VEGF). Activation of vasculoneogenesis in the adult in response to hyperplasia, injury, or tumor growth involves both endothelial cells *in situ* and circulating EPCs from the bone marrow. Bone marrow–derived EPCs may be mobilized by growth factors such as granulocyte macrophage colony-stimulating factor and VEGF. The therapeutic use of EPCs became feasible when it was shown that statins activate EPCs and enhance angiogenesis in vivo. In addition, mobilized EPCs may be expanded in vitro and used for transplantation enhancement of angiogenesis. Gene therapy to enhance circulation in premature atherosclerosis (Buerger disease) may be accomplished using phVEGF [17, 18].

❖ **Side Population Cells**

SP cells are first described by Goodell in 1996. It is a sub-population of cells that is distinct from the main population on the basis of the markers employed cells in a side population has distinguishing biological characteristics. For eg: They may exhibit stem cell-like characteristics, but the exact nature of this distinction depends on the markers used in identifying the side population. SP cells is a small subpopulation of cells with enriched stem cell activity that shows a distinct "low" Hoechst 33342 dye staining pattern. Later studies attributed this phenotype to expression of ABCG2 (ATP- binding cassette (ABC) transporter subfamily G member 2). Recent studies on testicular stem cells indicate that more than 40% of the SP was undifferentiated spermatogonia while other differentiated fractions were represented by only 0.2%. Recently, the side population phenotype

introduced as a reliable marker to identify subpopulation of cells with stem/progenitor cell properties in various tissues including organs (eg: Heart). SP cells express a transport protein that allows them to actively exclude the Hoechst 33342 dye. FACS analysis of these cells reveals that they constitute a discrete population of cells with emission data that differ from that of the other cells. In dot plot of emission spectra they appear at the side of the rest of the cells. These side populations have shown the capacity to function as stem cells in the tissue which they were isolated and may be able to transdifferentiate [18, 19].

6.2 Experimental

All materials were purchased from Himedia unless otherwise stated. A proliferation medium consisting of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG, Himedia Pvt.Ltd), 20% fetal bovine serum (FBS), 100 U/mL penicillin and 100µg/mL streptomycin was used for culturing. Trypsin, Trypan blue, Phosphate buffered saline (PBS) (Himedia Pvt.Ltd) used for dissociation of cells from culture vessel. Cultures were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2.

6.2.1 Cell culture

6.2.1.1 Isolation of Umbilical cord-derived MSCs (UC-MSCs)

Umbilical cord-derived MSCs (UC-MSCs) were isolated from umbilical cords obtained after normal full-term delivery. Six umbilical cords, app. length 20 cm, were stored aseptically in cold PBS with 100 µg/ml Pen/Strep (Himedia Laboratories), within 6-24 hours from partum. The umbilical vein and arteries were dissected from the tissue, which was then cut into fragments (2-3 mm^3). Several pieces of tissue were placed in a Petri dish with a low amount of GM to allow attachment to the plastic and later on were covered completely with culture medium. GM was changed every second day. After 7 days, when the cells grew out of the explanted tissue, the cord fragments were removed and attached cells

were cultured until reaching confluence. MSCs were successfully isolated from two out of six specimens [20]. After reaching confluence, cells were subcultured routinely in GM, using Trypsin/EDTA solution (Hi-media). The populations of MSCs were expanded for up to 10 passages for UC-MSCs.

6.2.1.2 Isolation of Umbilical cord blood-derived MSCs (UCB-MSC's)

Dilute cord blood with PBS with a 3:1 ratio (3 parts cord blood to one part PBS).Isolate mononuclear cells (MNCs) by density gradient centrifugation at 400xg for 30 minutes at room temperature using Ficoll-Paque. Transfer MNCs to new centrifuge tube and add PBS with a 1:3 ratio (1 part MNCs to 3 parts PBS).Again centrifuge at 400xg for 10 minutes at room temperature. Remove supernatant and resuspend cells by adding culture medium and plate. Incubate MNC's at 37°C, 5% CO_2 incubator overnight [20,21].

6.2.2 Cell proliferation and viability

6.2.2.1 Cell growth assays

Cell growth assays were performed as described [21,22]. Short term cell growth assays were performed by seeding MSCs, passage (P) 4 to 6, in 6 well plates at 4×10^4 cells/well and incubating them in GM at standard culture conditions. At days 2, 4 and 7, the cells were detached, counted and their viability determined by Trypan blue exclusion test (Invitrogen,Carlsbad, CA, USA). These assays were repeated at least 3 times for MSC. To determine the long-term population doubling times MSCs were plated in duplicate in 6 well plates at 1×10^4 and 2×10^5 cells/well. At confluence, the cells were detached, counted and reseeded at the initial cell density. This procedure was repeated at every passage for 24days. The population doubling times (PDT) were calculated according to the formula PDT = (T–T0) lg2/(lgNt–lgN0), where T0 and T are starting and ending time of cell culture respectively while N0 and Nt represent the cell number at the start and the end of each culture [21].

6.2.2.2 Colony-Forming Units-Fibroblastic (CFU-F) assay

CFU-F assays were performed by plating MSCs (P3 to P6) in 6 well plates at 10, 50 and 100 cells/well in GM, in two replicas. After 14 days under standard culture conditions, the cells were fixed with ice-cold methanol and stained with 0.3% crystal violet. The number of visible colonies (more than 50 cells) was counted [22].

6.2.2.3 Flow cytometry analysis

After the mesenchymal cells were extracted from the umbilical cord, they were cultured in DMEM supplemented with glucose (4.5 g/l) and 10% FBS for 5-7 days. Next, the cells were trypsinized and suspended in DMEM at a concentration of 5×10^6/ml, and then a 1-ml sample was incubated for 45 minutes at 4°C with 150 µl of various nonlabeled mouse antihuman antibodies followed by fluorescein isothiocyanate (FITC)–conjugated anti-mouse immunoglobulin G (IgG) antibodies (10 mg/ml) for 1 hour at room temperature. Finally, they were washed twice with phosphate-buffered saline (PBS), pH 7.4; centrifuged; and fixed in 1.5 ml of 4% paraformaldehyde. Control samples were incubated with PBS instead of primary antibody. A FACS scan machine (BD FACS Aria) was used to analyze antibody binding [23].

6.3 Results and discussion

6.3.1 Isolation and culture of different MSCs

Depending on the starting amount and type of tissue used to establish the primary cultures, cells that morphologically resembled MSCs could be seen as early as 7 days post-plating for the UC-MSCs up to 15 days post-plating for the PB-MSCs and the confluence could be reached within 10-15 days [24]. Although during the initial culture period some morphological heterogeneity in the adherent fraction could be seen, as the cultures were passaged, morphological homogeneity was gradually achieved, cells were fibroblast-like and no spontaneous differentiation was noticed as shown in Figure 6.5.

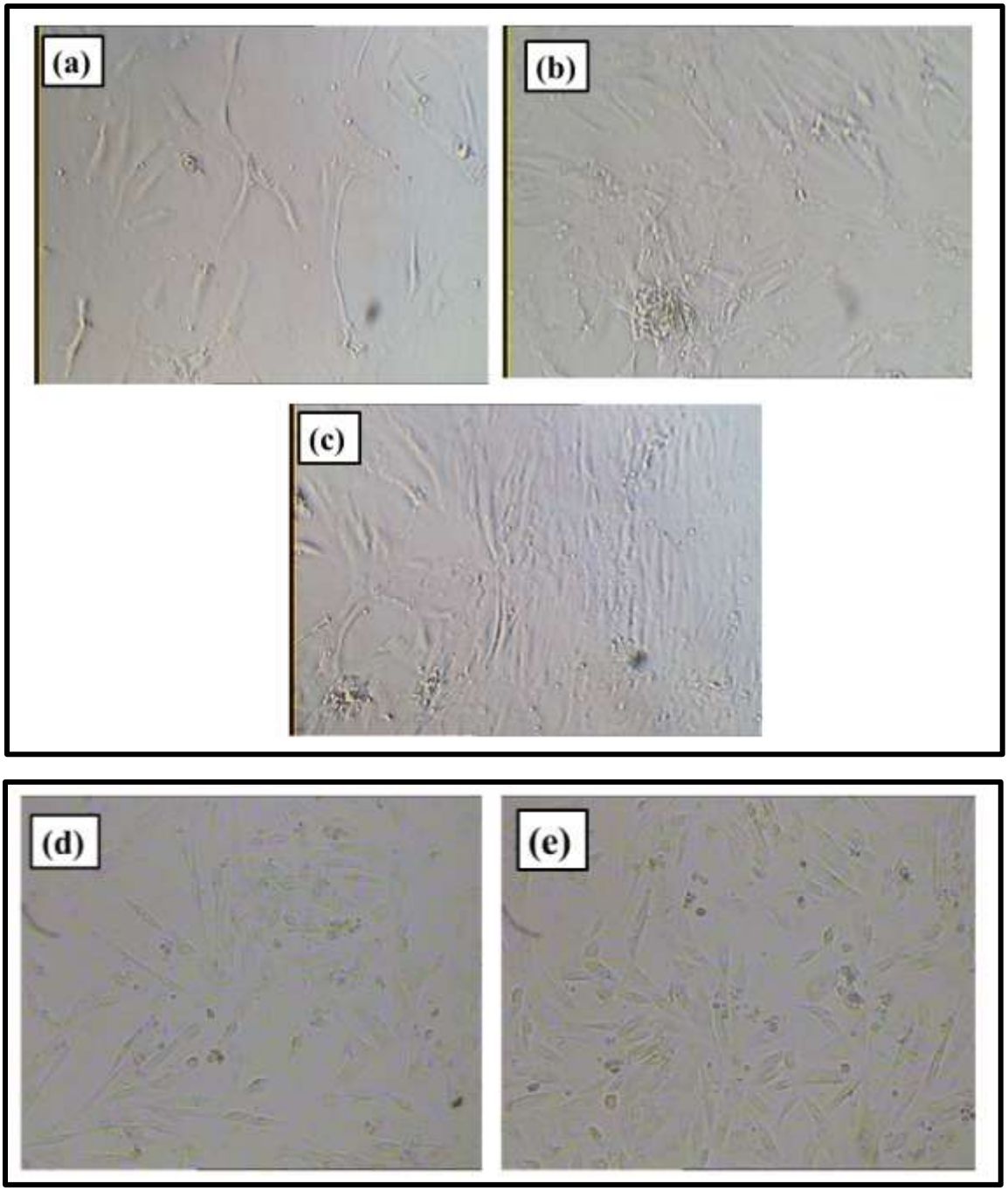


Figure 6.5 Morphology of UC-MSCs cultures. Inverted microscopic images of UC-MSCs (a, b, c) monolayer cultures at passage P0, P1 and P2 And UB-MSC's (d, e) at passage P1 and P2. In monolayer culture, the cells assumed a polymorphic, fibroblast-like morphology which was maintained throughout the passaging process.

6.3.2 Growth characteristics of different MSC populations

When the clonogenic capacity of MSCs was analyzed using CFU-F assay, results demonstrated the presence of clonogenic cell populations with fibroblast-like morphology in both MSCs examined, but with different frequencies ranging from 25% up to 95%. Common feature among tested cells was that MSC populations showed decreases in CFU-F frequencies as the initial plating density was increased, with the lower cell plating density having the highest CFU-F numbers plotted in Figure 6.6. However, the colony-forming efficiency, defined as the ratio of the number of colonies to the number of cells seeded [25, 26].

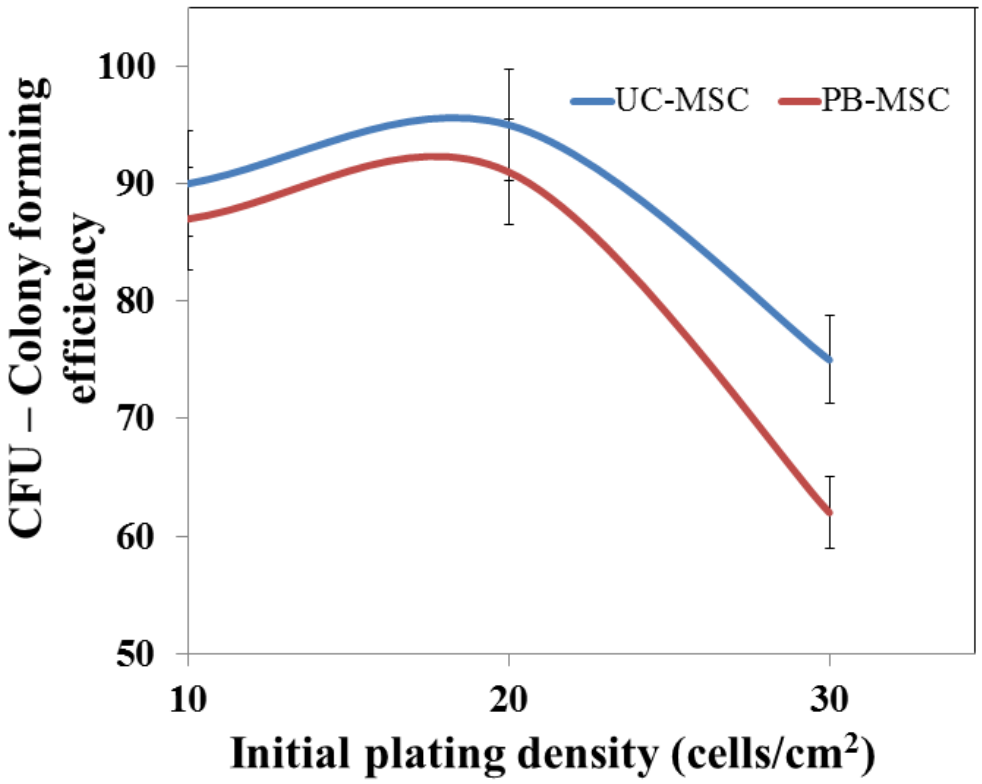


Figure 6.6 CFU-colony forming efficiency of UC-MSCs had up to 2-fold higher CFU-F capacity than the PB-MSCs

Culture kinetics of different MSCs is shown in Figure 6.7.

When the growth kinetics of MSCs was compared by seeding 4th to 6th cell passage the differences related to the tissue of origin could not be observed. As shown in Figure 6.7, short-term proliferation assay demonstrated similar and extremely high proliferation rate of both PB-MSCs and UC-MSCs, as the cell number of these MSC populations increased almost 40-fold at day 7 after plating. To confirm expandability of the cells we evaluated the long-term proliferation ability in which the cells were repeatedly seeded at different plating densities to minimize any effect of density-dependent cell growth.

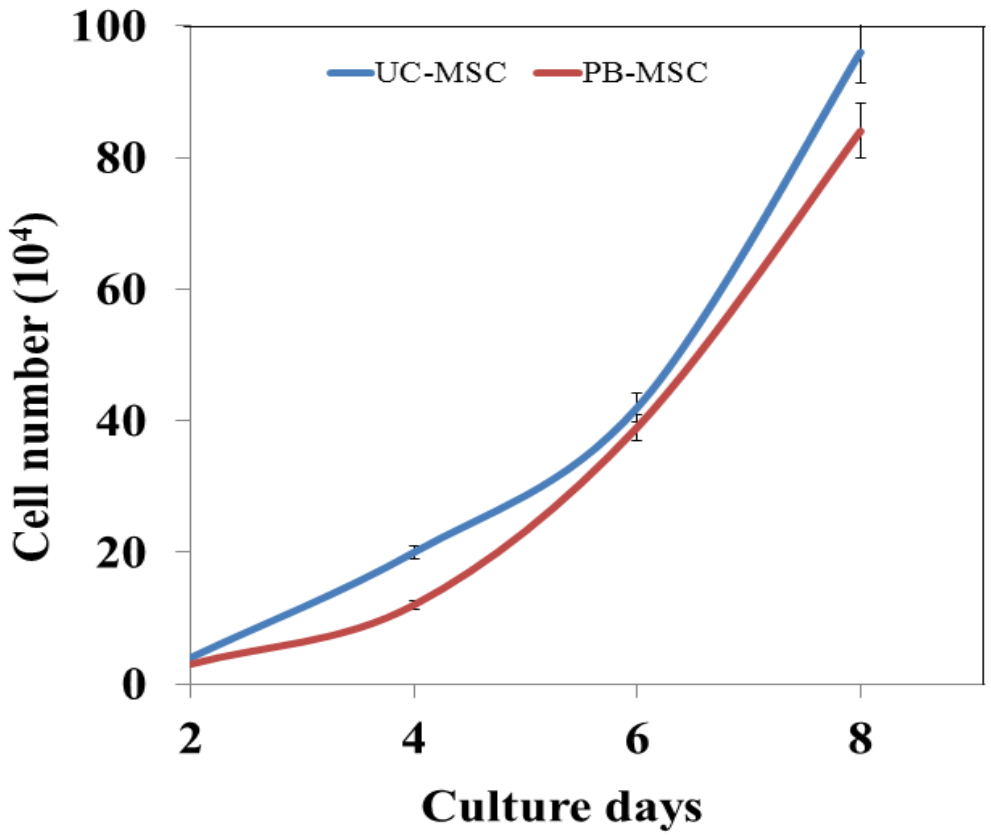


Figure 6.7 The PDT values obtained for both MSC populations were consistent with the results obtained within the initial expandability studies since PB-MSCs and UC-MSCs had short PDTs of app. 27 h and 35 h for 5×10^4 cells plated respectively

6.3.3 Enumeration of FACS report with markers CD34, CD90 and CD105 for huMSC and at different passages

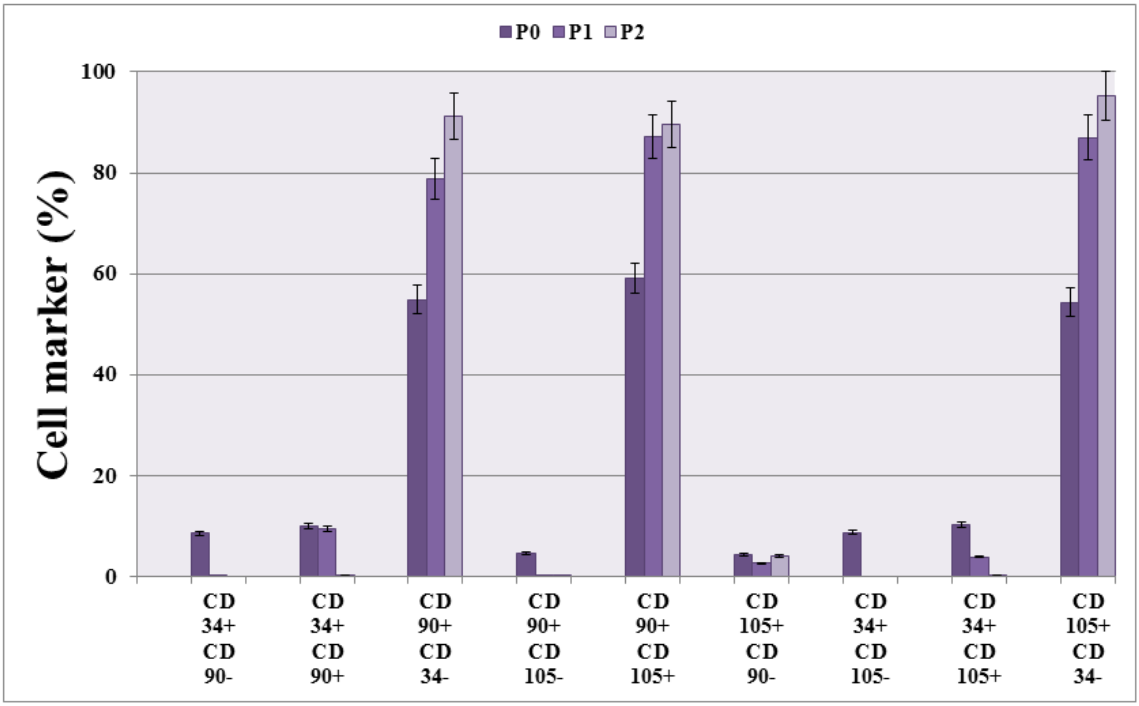


Figure 6.8 Enumeration of FACS report with markers CD34, CD90, CD105, and HLA-DR for different passages (P1 and P2)

The culture expanded hUMSC (P1-P2) in this study expressed typical mesenchymal stem cell markers (CD90, CD105), but they express low level of hematopoietic marker (CD34), and side population marker (HLA-DR). This is evident that early passage started expressing homogenous mesenchymal stem cells and reduced in HSC and came to zero at P1 [27-29]. In our work, CD90+CD34- were found to be 78.8 % at P1 and 91.1% at P2. Similarly, CD105+CD34- was found to be 87% at P1 and 95.2% at P2.The double positive population CD 90+CD 105+ cells found to be 87.1 % at P1 and 89.6 % at P2. This shows that homogenous mesenchymal population is increasing at early passage P1. Studies done earlier by many researcher in homogenous population of MSCs in cord tissue which gives a different percentage of cells in different passages [30, 31], states that homogenous profile MSC is obtained only at P3 or P4, however, in present

work, results of homogenous profile of these markers of CD 34-, CD90+, CD105+ at early P1. Thus it is clear that early passage P0 or P1 function also can be used for regenerative therapy.

6.4 Conclusions

Umbilical cord blood can be viewed as the most promising source of stem cells for research and clinical applications. It is abundant supply, immunological immaturity and high plasticity made it superior to other sources of stem cells. This chapter represents successful isolation of mesenchymal stem cells from umbilical cord. After isolation culturing and passaging were carried out to get uniform monolayer of these MSCs which shows fibroblast like morphology. Growth kinetic studies reveals, long term proliferation ability of isolated cells in culture. And, flow cytometry studies confirms that isolated cells are nothing but MSCs. Therefore, stem cells are capable of giving rise to various mesenchymal and non-mesenchymal cell lineages including bone, cartilage, fat and neurons. Thus, Cord tissue stem cells are candidates to develop stem cell-based therapies for a wide variety of diseases including cardiovascular, ophthalmic, orthopedic and neurological applications.

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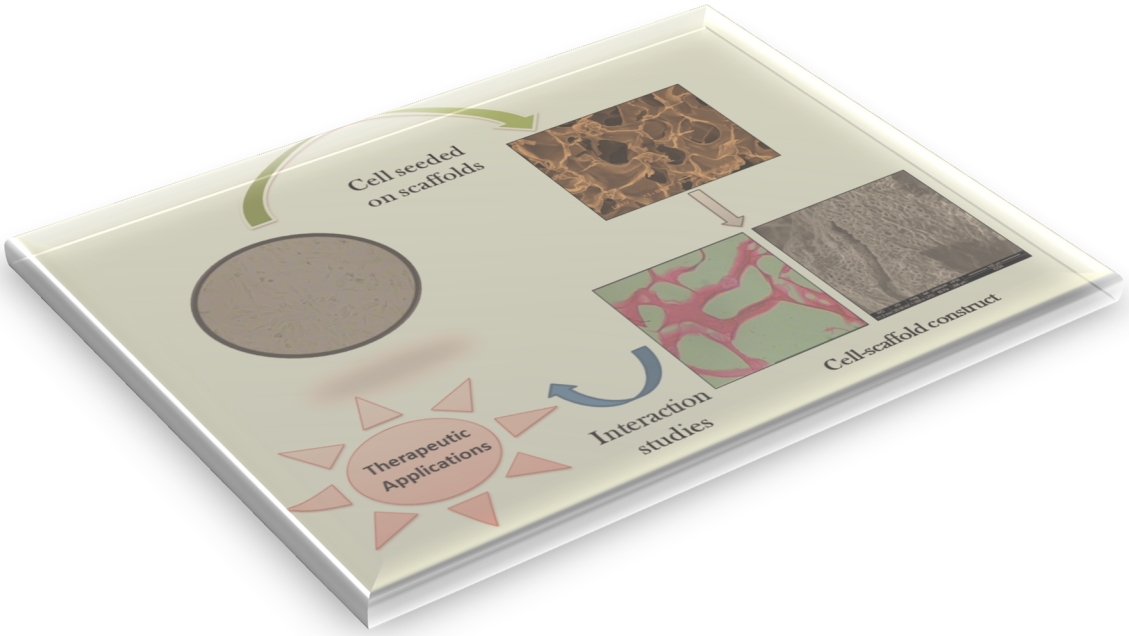
Chapter 7

Seeding of Mesenchymal Stem Cells on Chitosan-Alginate Scaffolds for Their Therapeutic Applications



7.1 Introduction

Current tissue engineering strategies are focused on the restoration of damaged tissue architecture by transplantation of cells in combination with a suitable three-dimensional scaffold [1]. Research within the last few years shows substantial gains in the use of an artificial extracellular matrix (scaffold) to support tissue regeneration. In order to obtain new tissue growth the scaffold must exhibit large surface area, porosity, appropriate mechanical properties and enhanced cell adhesion [2-6]. A scaffold for tissue engineering can be looked at as a surrogate ECM [7]. Scaffolds are currently made from a bioabsorbable material which degrades as the cells grow in and repair the tissue and therefore do not require a second surgery for scaffold removal [8].

To be usable in this strategy, the biomaterials must be non-toxic and highly biocompatible. Moreover, they should be degraded in vivo as they support the loaded stem cells to proliferate, differentiate and secrete beneficial bioactive molecules e.g. growth factors and extracellular matrix components. In this way, the long-term presence of foreign materials and the consequent inflammatory responses can be prevented [9]. As outlined above, building an ideal scaffold requires that multiple, even conflicting criteria to be met. Up to now no biomaterial has yet satisfied all the properties which are necessary to generate such a clinically usable stem cell delivery system. In this study we are trying to fulfill all the properties of an ideal scaffold which is made up of chitosan and alginate, and to study its fine interaction with mesenchymal stem cells. Cell scaffold interaction is well explained in Figure 7.1.

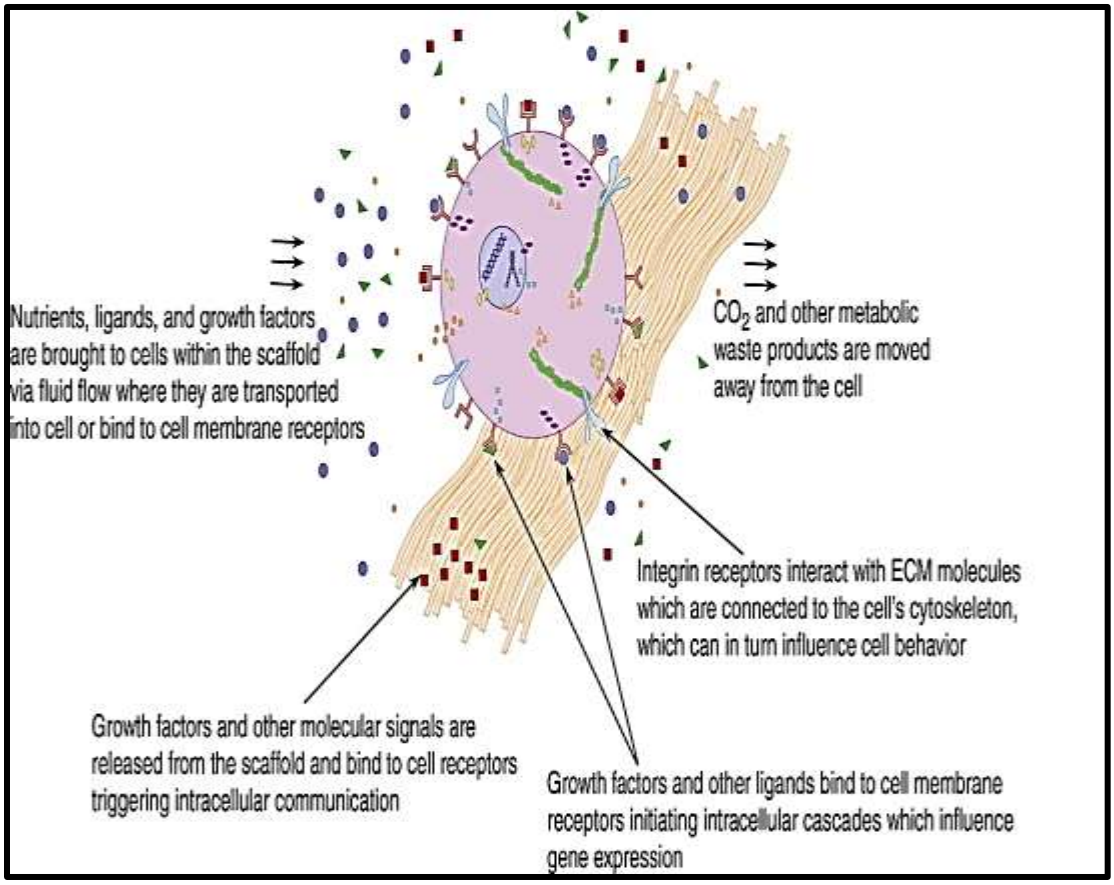


Figure 7.1 Diagram demonstrating the relationship between cells and scaffolds [10]

The microenvironment created around a cell adherent to a tissue-engineering scaffold is complicated. Nutrient transport brings growth factors, ligands, and other signals that can bind to cell receptors. The degrading scaffold can likewise release chemical messengers that bind to membrane receptors and influence intracellular communication and cellular processes such as gene transcription. Cells also attach to the scaffold via integrin receptors. Integrin receptors are closely connected to the cell's cytoskeleton and relay further information to the cell thereby affecting cell function [10].

MSC are characterized by their ability under appropriate stimuli differentiate into lineages of mesenchymal tissues including bone, cartilage, muscle and fat [11]. In addition, MSC have the potential to differentiate into other

types of tissue forming cells such as hepatic [12], cardiac [13] and neural [14] and so on. Initially MSC were characterized in bone marrow. The isolation method was based on the ability of the bone marrow-derived fibroblast-like cells to adhere on the plastic substrate of the cell culture plate [15]. The ease of isolation and the high expansion potential in vitro make MSC attractive as a model system for studies of cell differentiation and potentially useful for cell and gene therapy.

To date, the most common source of MSC is bone marrow. However, the number of bone marrow derived MSC significantly decreases with age [16]; in addition, aspiration of bone marrow from donor is an invasive and painful procedure. This has led many researchers to search for alternative sources of MSC. Recently, several reports have demonstrated a successful isolation of MSC from subcutaneous adipose tissue [17], umbilical cord blood endothelial and subendothelial layers [18-19], Warrton's jelly [20] and chorionic villi of human placenta [21]. Umbilical cord as a source of MSC is particularly attractive for two reasons. First, umbilical cords in delivery rooms are still regarded as a medical waste and therefore are abundantly available for scientific needs. The second and most important consideration is that the proliferation and differentiation potential of MSC decreases with donor's age [16]. In this respect, umbilical cord is an excellent alternative source for MSC.

MSC seeded onto scaffolds can be used for various biomedical applications in vivo as well as in vitro. One of the most common application is regeneration of damaged tissues which includes bone (Figure 7.2), cartilage, liver, pancreas tissue engineering. Similarly, cell seeded scaffolds can be used in skin regeneration and wound healing applications.

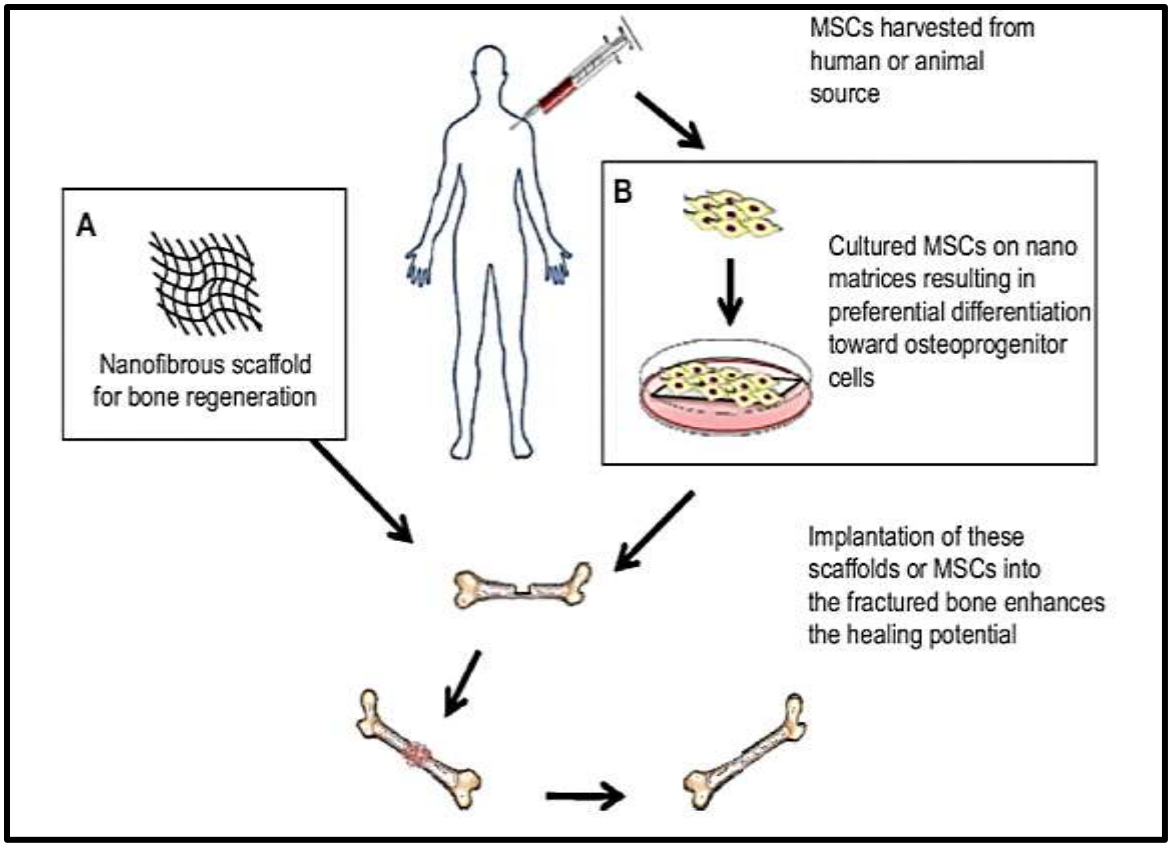


Figure 7.2 Schematic representation of bone regeneration. (a) Nanofibrous scaffolds, (b) Culturing MSC's on nanomatrices [22]

Another application of cell seeded scaffold in cardiac tissue engineering is explained in Figure 7.3. huMSC can be easily obtained from the umbilical cord tissue. These cells have certain plasticity and reduced risk of rejection because they show low immunogenicity. Their injection in animal models has been found to improve their left ventricular function. Chitosan-based polymer was capable of scavenging the reactive oxygen species produced by the ischemic conditions and recruit key chemokines for stem cell homing such as SDF-1. As a cell delivery system with mesenchymal stem cells, this material was capable of improving the microenvironment for the cells when injected in the infarcted myocardium of rats, improving their survival and engraftment. Alginate-chitosan scaffolds have been loaded with placental growth factor (PlGF) to increase the left-ventricular function

and vascular density in rats. Also, Alginate has been used as an injectable material in recent and old infarcts in rats, and it was observed that its injection augmented the scar thickness and limited systolic and diastolic dysfunction [23]

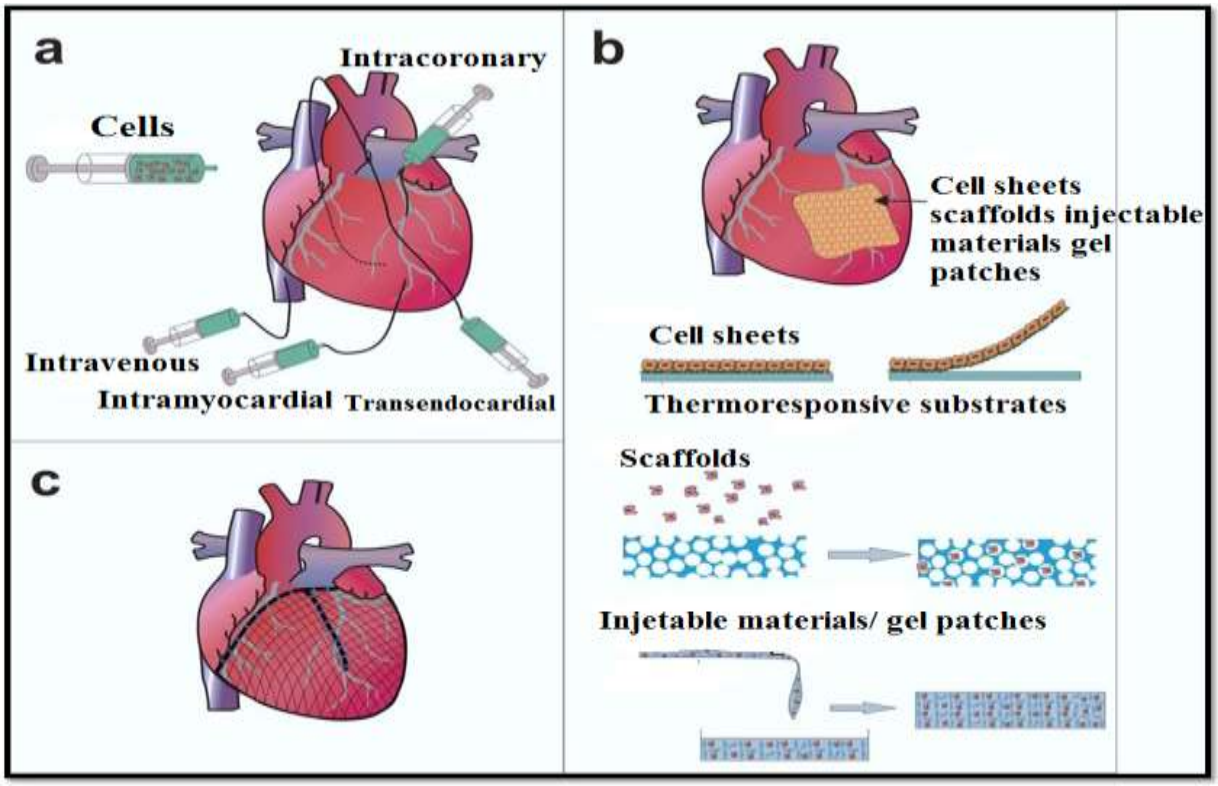


Figure 7.3 (a) Classical cell therapy in the heart (b) Tissue engineering approaches with cell sheets, scaffolds or injectable materials (c) Ventricular restrain device [23]

Furthermore, cell seeded scaffolds having yet interesting applications invitro viz; study the effect of novel formulations of drugs on to cell-scaffold construct, and investigating effect of hormones and growth factor regulators on the same [24]. In this chapter, a successful establishment of MSC-like cell culture from the umbilical cord tissue is reported. Also, a microfibrous three-dimensional scaffold was used to investigate its fine interaction with mesenchymal stem cells. Our results demonstrate that the obtained MSC-like cells could be rapidly expanded in culture. Importantly, MSC-like cells retained their proliferative

potential after seeded onto Chitosan-Alginate Scaffolds. Thus, MSC-like cells derived from the umbilical cord is a promising experimental model and a useful tool for future cell and gene therapy applications.

7.2 Experimental

All materials were purchased from Himedia unless otherwise stated. Chitosan (from shrimp shells)DD(degree of deacetylation)was > 75% ,calcium alginate (alginic acid calcium salt) was also obtained from Himedia Laboratories Pvt. Ltd., Mumbai, Acetic acid(Merck Ltd.,Mumbai), NaOH pellets, Haematoxylin and Eosin, alcohol grades (Merck Ltd., Mumbai) were used as such. Reagents such as cell culture medium and fetal bovine serum were obtained from Himedia Laboratories Pvt. Ltd, Mumbai. Trypsin, Phosphate buffered saline (Himedia Pvt.Ltd) used for dissociation of cells from culture vessel. Cultures were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2.

7.2.1 Seeding of stem cells on scaffolds

The scaffolds were sterilized by autoclaving and presoaked in a 12-well cell culture plate containing DMEM culture medium for a couple of days before cell seeding. Approximately 1 million hUMSC in 1 ml Dulbecco's Modified Eagle Medium (DMEM) were seeded on each scaffold disc. The DMEM medium contained 10% fetal bovine serum (FBS), 50IU ml^{-1} penicillin and 50µg ml^{-1} streptomycin. The scaffolds were transferred to another cell culture plate after 3 hours of cell seeding. The culture medium in the plate was changed in 24 hours for the first time and every three days thereafter. A hemocytometer was used to determine the concentration of cells in cell suspension. The morphology of cells cultured on scaffolds was examined scanning electron microscopy.

7.2.2 Cell proliferation

Cell proliferation was assessed using the Almar Blue Assay. Scaffolds were seeded with cells in DMEM cell culture medium. After 24 hours of cell seeding,

the scaffolds were washed with PBS and placed into 12-well culture plates. 2 ml of 10% v/v Almar Blue in DMEM medium was added to each well containing scaffolds and the plates were incubated for 4 hours at 37^{o}C. Absorbance of the solution in each plate, which is proportional to the number of cells attached to the sample, was measured with a micro plate reader (Biorad, imark, USA) at wavelengths of 570 and 600nm. A calibration curve generated from a known number of huMSC reacting with the Almar Blue was used to calculate the number of cells. Samples were assayed at 3,7,14 and 21 days, respectively after cell culture.

7.2.3 Cell viability

The MTT assay is used to quantify spectrophotometrically the amount of living cells, due to the reduction of MTT to purple formazan by the mitochondria of the living cell. For the MTT test, the mesenchymal stem cells were seeded at a concentration of 2×10^5 cells/mL in respective medium for 24 h in a 96well microtiter plate. After 24 h of incubation, the old media were replaced with media containing different concentrations of MNPs and the cells were exposed for an incubation time of 48 h. Blue formazan crystals, from the metabolism of MTT in the mitochondria of viable cells, were washed with PBS and were dissolved in 50 mL of dimethylsulfoxide and measured at 550 nm by the plate reader. The experiments were repeated three times and the data was graphically presented as the mean. The percentage of cell viability (%) compared with the control well containing cells without scaffold is calculated by the equation,

$$Cell\ Viability\ (\%) = \frac{[A\ absorbance]tested}{[A\ absorbance]control} \times 100 \qquad (7.1)$$

7.2.4 Histology

Scaffolds seeded with huMSc were washed twice with PBS and fixed with 4% neutral buffered formalin for histological assay. The scaffolds were embedded in paraffin and cut into 5mm thick sections using a microtome. The sections were stained with hematoxylin-eosin. The satined sections were then mounted on glass slides and examined with a Nikon e200 microscope.

7.2.5 BET Surface Area Analysis of Chitosan-Alginate Scaffolds

BrunauerEmmett-Teller (BET) surface area analysis was performed using a Quantachrome NOVA1000e, USA surface area and porosity instrument to determine the surface area and average pore diameter of the scaffold. Four scaffolds were sliced from a cast sheet, to give an approximate weight of around 0.2 g. The samples (scaffolds) were then re-suspended and washed in distilled water before re-freeze drying under the previously described protocol to give an undeformed clean sample. The sample was then placed into a long neck, round bottom sample tube and loaded into sample ports and evacuated to a high vacuum (2 lmHg). Nitrogen adsorption measurements were taken at 77 K, up to a pressure of 230 mmHg (pressure of 0.3bar).

7.3 Results and discussion

7.3.1 Seeding of stem cells on scaffolds

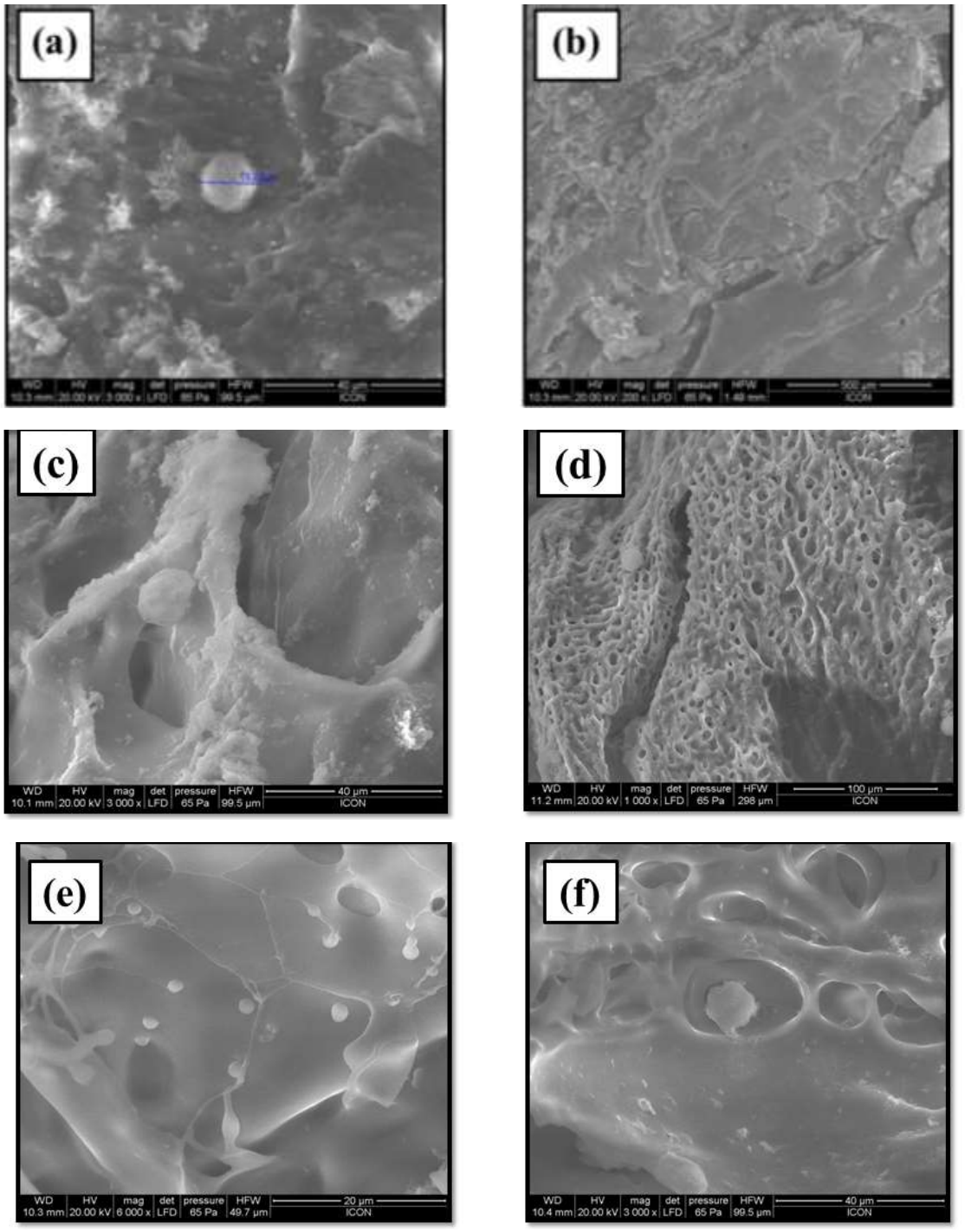


Figure 7.4 E- SEM microphotographs of mesenchymal stem cells grown on (a,b) chitosan-alginate and (c,d) chitosan-alginate scaffolds after 7 days cell culture and on (e,f) chitosan-alginate scaffolds after 14 days cell culture with 1000X,3000X & 6000X magnification respectively

To observe the cell morphology inside the micro and the constructs were cross-sectioned in thick slices and observed by SEM Figure 7.4. (CA1-b, CA4-d,CA8-f). Figure 7.4 shows SEM images of huMSC grown on chitosan-alginate scaffolds after 14 and 21 days of cell culture. Figures (7.4 e & f) show the images taken at day 21 at a higher magnification in order to detail the cell morphology. At day 14, huMSC on scaffolds proliferate well and exhibited a spherical morphology. At day 21, however, the cells on the chitosan-alginate scaffolds became more flattened and started to assume a fibroblast- like morphology. As can be seen, cells spread on the surface of the scaffold and after 21 days culture the whole surface was covered. The observed results suggest that the material system promoted intercellular contacts and the spatial arrangement of the cells.

7.3.2 Cell proliferation

Mesenchymal stem cells isolated from human umbilical cord were seeded onto chitosan-alginate scaffolds. Cell proliferation on both chitosan and chitosan-alginate scaffolds as a function of time was assessed using the Almar Blue assay and the results are shown in Figure 7.5.

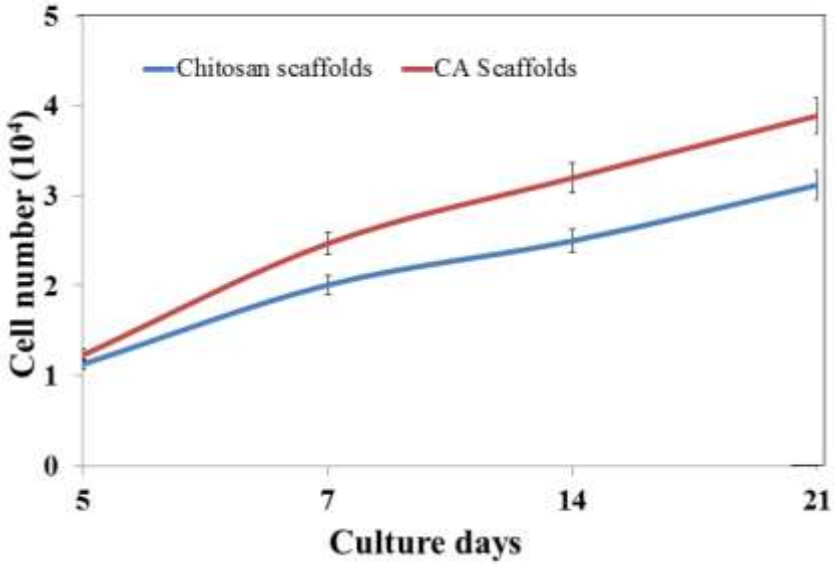


Figure 7.5 Cell proliferation as measured by the Almar Blue assay. Data show the average and its level of statistical significance ($p< 0.05$)

The cells on both scaffolds were doubled within the first week of cell culture and continued to increase over time. Three samples in triplet were tested and the results were analyzed. No significant difference in cell number was observed between the chitosan and chitosan-alginate scaffolds at day 3 and 7 after cell culture. However, the cell number on the chitosan-alginate scaffolds became notably higher than that on the chitosan scaffold at day 14 and day 21.

7.3.3 Cell viability

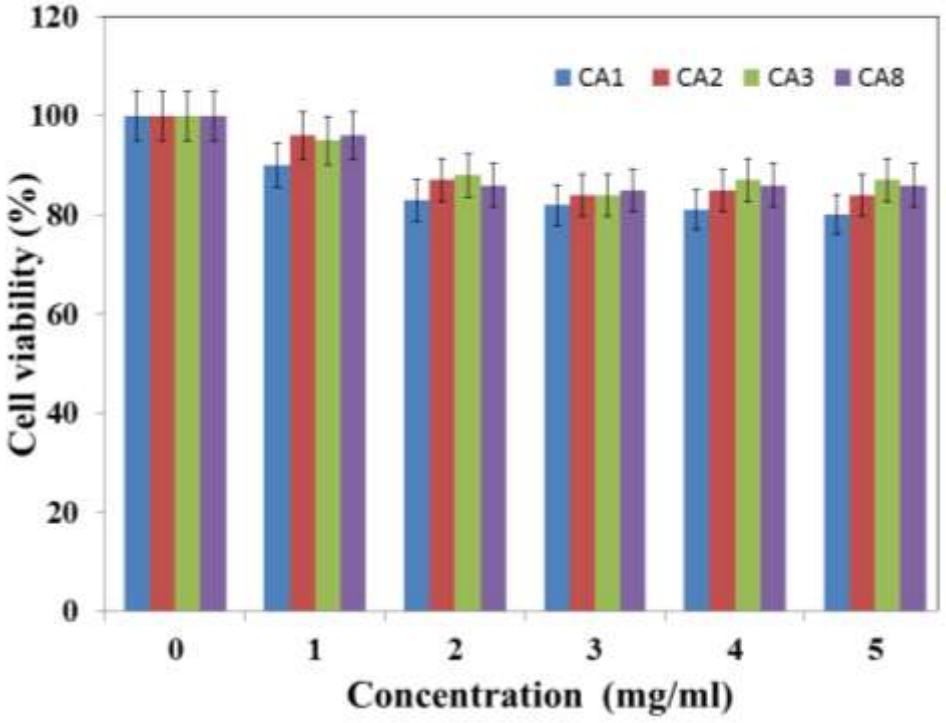


Figure 7.6 Cell viability versus concentration of CA Scaffold for CA1, CA2, CA3 and CA8 samples on huMSC after 48 h

Despite having antibacterial activity, the suitability of material for tissue engineering applications depends that scaffolds must not have intolerable toxic effects in human body. The effect of scaffolds on the viability of huMSC was studied as the reflection of their suitability and the values obtained were shown in Figure 7.6. After 48 hours of incubation, sample CA 3 shows slightly higher absorbance as compare to other samples at every concentration (approx > 80%)

indicated the presence of more number of cells on sample. Therefore, these scaffolds can be considered as biocompatible product having nontoxic effects. The cyto-compatibility of chitosan and chitosan-alginate were already been proved [28-30]. Therefore, considering good biocompatibility, chitosan-alginate scaffolds could be used for tissue engineering materials.

7.3.4 Histology

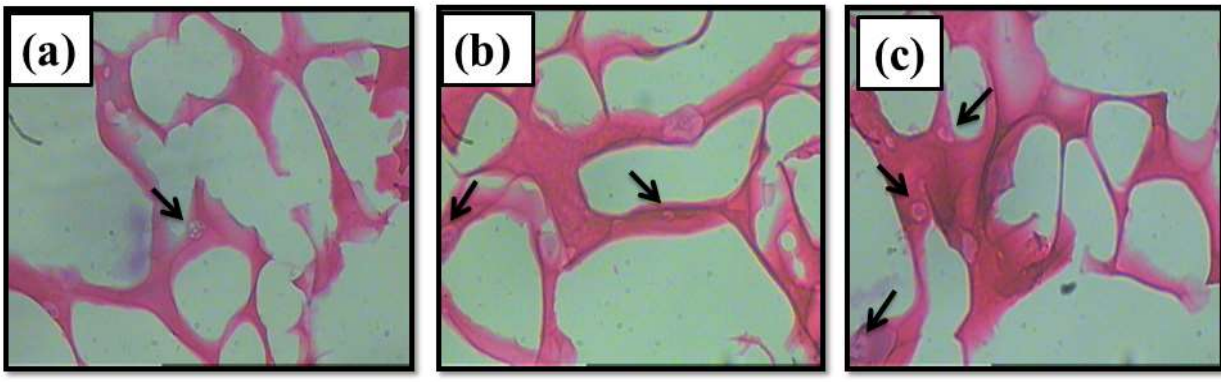


Figure 7.7 Microscopic images of huMSC's on (a) pure chitosan and (b & c) chitosan-alginate scaffolds after 21days of cell culture

Hematoxylin & Eosin was used to stain the harvested scaffolds in order to study the interaction of cells with the materials. Fig 7.7 shows the microscopic images of huMSC's stained with H & E and grown on chitosan-alginate scaffolds after 21 days of cell culture with the cell nuclei appearing in violet and cytoplasm and materials in pink. The cells on the chitosan-alginate scaffolds had smaller nuclei and larger cytoplasm. Furthermore, the cells on the chitosan-alginate scaffolds tended to form flattened fibroblast-like morphology.

7.3.5 BET Surface Area Analysis of chitosan-alginate Scaffolds

The pore structure of hybrid scaffolds is not uniform; they are typically birds nest like morphology with many shattered fragments as shown in Figure 7.8 (a&b). Numerous small pores interconnect the large pores. Comparatively, the

pore structure of pure chitosan looks more like birds nest; there are fewer fragments and fewer small interconnecting pores shown in Figure 7.8 (c).

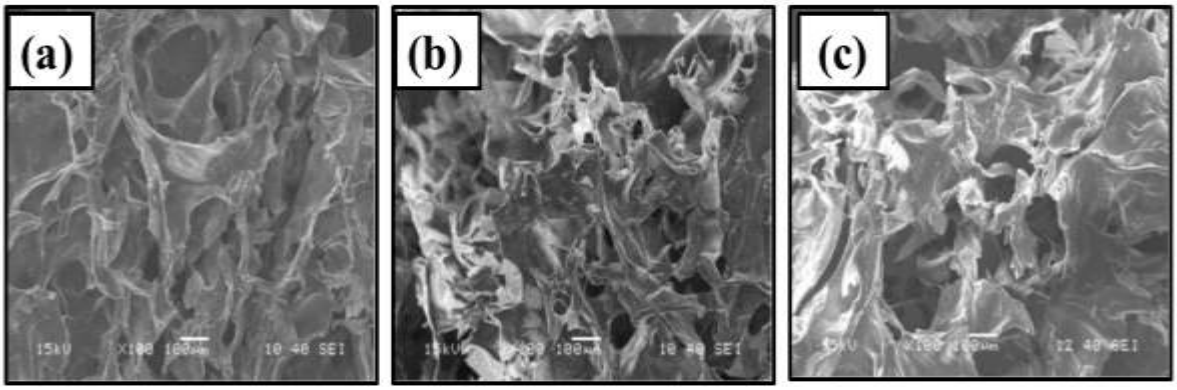


Figure 7.8 comparesion the pore structure of the hybrid chitosan- alginate scaffolds (a & b) and the pure chitosan scaffolds (c)

Average pore diameter, total pore volume and surface area of hybrid chitosan-alginate scaffolds was measured by BET analysis method.

Table 7.1 BET experimental results of chitosan-alginate scaffolds when seeded 10^3 cells/ml

Scaffolds	Surface area	Average pore diameter	Total pore volume (cc/g)
CA6-1	29.305 m²/g	3.13586e+01 nm	0.1584
CA6-2 (1 day)	26.028 m²/g	3.38870e+01 nm	0.1532
CA6-3 (5day)	17.770 m²/g	3.54754e+01 nm	0.1250
CA6-4 (10 day)	4.252 m²/g	3.62400e+01 nm	0.0519

Table 7.2 BET experimental results of chitosan-alginate scaffolds when seeded 10^5 cells/ml

Scaffolds	Surface area	Average pore diameter	Total pore volume (cc/g)
CA6-1	29.305 m^2/g	3.13586e+01 nm	0.1584
CA6-2 (1 day)	24.028 m^2/g	3.48760e+01 nm	0.1476
CA6-3 (5day)	16.770 m^2/g	3.67844e+01 nm	0.1135
CA6-4 (10 day)	3.252 m^2/g	3.738640e+01 nm	0.0316

CA6-1 is having surface area of about 29.305 m^2/g. when 10^3 cells/ml are loaded onto scaffolds, cells will uniformly distributed in and on the scaffolds, reducing surface area with increase in incubation period. After 5 days of incubation surface area is reduced from 26.028 m^2/g to 17.770 m^2/g. while after 10 incubation surface area is found to be 4.252 m^2/g only.

Vice versa, average pore diameter of scaffolds increases with time. It is observed that average pore diameter of scaffolds is increasing from 3.13586e+01 nm to 3.62400e+01 nm within 10 days of incubation period. Similarly, total pore volume of scaffolds is decreases with time which is found to be 0.1584cc/g - 0.0519cc/g. similar kind of results obtained when 10^5 cells/ml are seeded on the chitosan-alginate scaffolds. Surface area and pore volume decreased to 3.252 m^2/g, and 0.0316cc/g whereas average pore diameter is increased from 3.13586e+01 nm to 3.738640e+01 nm. In summary, with increasing concentration of cells, surface area will reduce, average pore size will increase, and the pore volume tends to decrease but not much.

7.4 Conclusions

Cell attachments and proliferation on a material are the indications of the cellular compatibility of the material and the suitability of the material for tissue engineering applications. Our experimental results show that huMSC's attached well on both chitosan and chitosan-alginate scaffolds without involvement of special cell adhesion techniques such as dynamic seeding and incorporation of adhesion peptides. The cell adhesion property of chitosan is believed to be related to its polycationic nature, as it has been shown that cell adhesion is inhibited with increasing degree of deacetylation and thus decrease in positive charge. Chitosan-alginate hybrid material appeared to inherit such cellular adhesive property. The cells increased consistently on both chitosan and chitosan-alginate scaffolds over cell culture time and proliferate faster on the chitosan-alginate scaffolds than on the chitosan scaffolds. Furthermore, the cell viability assay revealed more live cells on CA3 scaffolds as compare to other samples. And histological studies proves the morphology of cultured mesenchymal stem cells. BET analysis confirms the effiecent binding of isolated stem cells on chitosan-alginate scaffolds. Overall, as a scaffolding material, chitosan-alginate exhibits combined superior biological and mechanical properties over its chitosan counterpart and shows great potential to accelerate tissue growth and retained the phenotype of mesenchymal stem cells. Hence, its use can surely be expanded to other tissue engineering applications in view of its superior mechanical, microstructural and biological properties.

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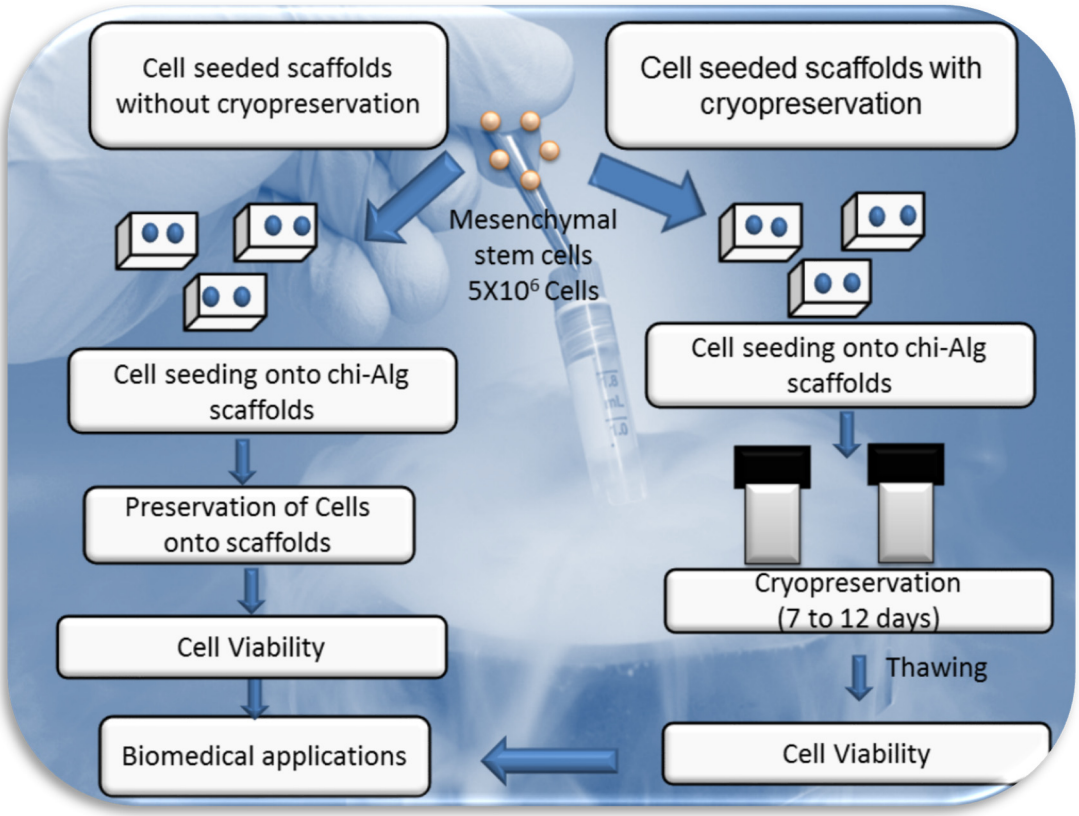
Chapter 8

Application of Scaffolds for Storage of Stem Cells



8.1 Introduction

Tissue engineering is emerging with the time while cell-scaffold constructs are likely to find an increasing number of applications in the regeneration of human tissues [1]. The engineered tissue substitutes are enduring several clinical trials and expecting a rising demand of cultured cells and tissues. However, tissue engineering community is becoming more concerned. It is facing the challenge of providing sufficient amounts of such products to the market [1, 2]. A major such difficulty can be overwhelmed by preserving and storing living biomaterials. The simple preservation techniques such as freezing and tissue culture have disadvantages like risk of contamination, high cost, limited shelf-life and genetic drift. Developing tissue substitute's process may take long time spans up to several weeks. It starts from the autogenous cells isolation to the in vitro expansion followed by seeding of cells on a scaffold and finally implantation bring about in extremely long incapacitation of patients [3-6]. To overcome this drawback an alternative approach is the cryopreservation of cell-scaffold constructs. This approach is based on the principle that chemical, biological, and physical processes are effectively suspended at cryogenic temperatures [8].

Scaffolds are proposed to replace tissues possess highly and fully interconnected open-pore geometry. It provides large surface area to volume ratios and allows enough diffusion of nutrient and gases. It also enables the tissue and vascularity ingrowth upon implantation [8, 9]. Cryopreservation of Cell-scaffold constructs with sustained cell viability and functionality is a desirable approach to overcome shortage in supply and would allow immediate application of the constructs by their ready-to-use character. Even differentiation in various tissue types *in situ* is possible [10-14]. However, very few studies are reported on cryopreservation of cell-scaffold construct. *Miyoshi H. et al* [8] studied effect of cryopreservation on fibroblast cells and 3D polyvinyl formal (PVF) resin scaffolds with collagen coating. *Yin H. et al* [9] showed effect of cryopreservation on bone marrow mesenchymal stem cells with demineralized bone matrix scaffolds.

Similarly, *Rupf T et al* [12] observed effect of cryopreservation on fibroblasts and keratinocytes seeded on collagen/elastin scaffolds. In comparison with, the current study is intended at evaluating the effect of cryopreservation on seeded as well as nonseeded cells and the effect of cryopreservation on the morphological and mechanical properties of the scaffold itself those are critical for the engineering of various tissues.

Amongst the numerous classes of materials used as scaffolds, alginate shows variety of advantages for biomedical applications. It is the natural polysaccharide forms hydrogel when crosslinked with multivalent cations. It is biocompatible and biodegradable [15, 16]. Furthermore, pore size in alginate scaffolds can be adjusted and allows immuno-isolation of encapsulated grafts along with nutrient supply. [17-22]. After fabrication such as cryogenic methods, like freeze-drying and cryostructuring, that produced a macro-porous cryogel scaffolds by large ice crystals at low cooling rates and temperature up to -20^{o}[23-25]. However, untreated alginate is not famous for cell adhesion, especially human umbilical cord derived mesenchymal stem cells (hUMSCs) remain round-shaped with little attachment sites [26]. To increase cell adhesion, alginate scaffolds are coupled with different polymer like chitosan, gelatin and elastin [27-29]. For effective cryopreservation, the strained cytoskeleton of adherent cells has to be protected against injury caused by freezing and thawing procedures. Since it is well known that water molecules are the main reason for cryoinjury [30], scaffolds like alginate seem to have beneficial effects for cryopreservation procedures.

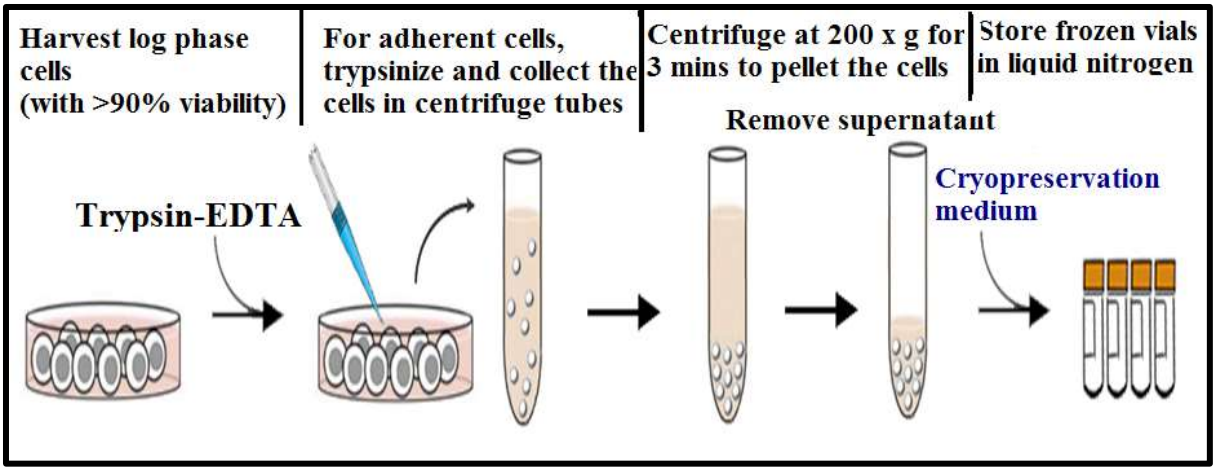


Figure 8.1 General procedure of cryopreservation of mesenchyml stem cells

Mechanism of cryopreservation

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to solid state.Due to the presence of salts and organic molecules in the cells,the cell water requires much more lower temperature to freeze(even up to -68°C) compared to the freezing point of pure water(around 0°C).When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to standstill.

Generally, two methods are used to cryopreserve almost cell lines. They are slow freezing–rapid thawing method and vitrification method [31]. The slow freezing–rapid thawing method using dimethylsulfoxide (DMSO) as a cryoprotectant can be easily applied to the cryopreservation of MSC lines such as BMMSCs [32], human dental pulp – derived mesenchymal stem cells [33], and umbilical cord blood derived mesenchymal stem cells [34]. In most research, cells are suspended in freezing medium containing 5-10% DMSO, transferred into cryovials and then frozen by steps with slowly decreasing temperatures: 4°C for 10 min, -20°C for 1 hour, -80°C for 1-2 days and -196^{o}C for 3-6 months. Using this method can obtain some advantages, like a large cell volume which might be frozen in one vial are required for applications such as drug screening or clinical applications. In addition to keeping balance to protect cryopreservated cells, it also

prevent the damage caused by various factors, including crystal formation in the cytoplasm of cells, fracture, toxic and osmotic damage by decreasing temperature slowly with low concentration of cryoprotectants [35]. However, it is difficult to eliminate injuries by intracellular ice formation which is the main source of fracture and damage to the cytoplasm [36]. It is also a time – consuming procedure and requires a programmable freezer. Alternately, vitrification, a rapid freezing method using a high concentration of cryoprotectants, could also be used. Vitrification can totally eliminate damages caused by intracellular crystal formation of cells [37]. It is also advantageous because of controlling penetration of cryoprotectants and dehydration rate, preventing prolonged temperature shock and damage from intracellular ice formation. Besides, it is a relatively short time and nonprogrammable temperature decreasing procedure. However, the vitrification method requires a high concentration of permeable or nonpermeable cryoprotectants exposed to cells before plunging into liquid nitrogen. It results in extreme osmotic stresses and chemical toxicity for cryopreservated cells [38]. The vitrification method has been commonly used to cryopreserve many different cell types such as embryonic stem cells (ESCs) [37] and umbilical cord derived mesenchymal stem cells (UCMSCs) [39].

Many studies on the cryopreservation of MSCs were carried out using slow-rate cooling methods [40, 41] which is often considered a superior method of preservation [42, 43]. However, limited evidence exists whether the freezing rate in fact affects stem cell growth and differentiation potential. Both ‘slow’ [44-48] and ‘fast’ [49,50] freezing protocols have reported ‘success’ as far as maintaining similar phenotypes, cell surface markers and growth rates in comparison with unfrozen MSC.

Ironically, the same cryopreservation process aimed at preserving living cells could also cause damage and compromise their survival. An understanding of the responses of cells and tissues to the physical changes that occur during the

freezing process is essential in the design of protocols that will minimize cryoinjury and ensure maximum recovery of viable and functional cells.

Role of cooling rates

The purpose of cryopreservation is to maintain life by slowing down metabolic activity of cells at temperatures as low as that of liquid nitrogen (-196^{o}C). Several mechanisms have been proposed to explain cell behavior during cryopreservation. In the course of freezing cells in suspension, water crystallizes in pure form in the extracellular space, resulting in the concentration of dissolved solutes in the remaining liquid. A concentration gradient is created across the cell membrane and, if cooling takes place slowly enough, water moves out of the cells in response to the resultant osmotic stress. As cooling continues and more ice is formed, salts concentrate to high levels as the cells become dehydrated and shrink. Damage caused by extended exposure to increased solute concentration at intermediate temperatures is referred to as slow-cooling injury. On the other hand, if the cooling rate is too fast for the cells to maintain equilibrium by loss of intracellular water, the unfrozen solution within the cells becomes increasingly super-cooled resulting in intracellular ice formation. Damage caused by intracellular ice is referred to as rapid-cooling injury. This two-factor hypothesis of freezing injury (i.e., solute effects and intracellular ice formation) as proposed by Mazur [51] has been elaborated upon by others [52, 53]. Successful cryopreservation of cells in suspension requires sufficiently high cooling rates to reach low temperatures quickly, minimize solution effects and avoid slow-cooling injury, but low enough cooling rates to decrease the formation of intracellular ice and avoid rapid-cooling injury.

Cryoprotectants

Cryoprotectants are classified as either permeating or non-permeating depending on their ability to traverse the cell membrane [54]. Permeating cryoprotectants, such as Me2SO, glycerol, ethylene glycol, propylene glycol (1,2-

propanediol), methanol, ethanol, propanol and formamide, protect against slow cooling injury by reducing extracellular ice formation, preventing excessive concentration of solutes and minimizing cell dehydration to a tolerable degree [55]. Non-permeating cryoprotectants can protect cells at lower molar concentrations, but they generally require more rapid rates of freezing to confer protection [54]. Examples include polyvinylpyrrolidone, sugars such as trehalose, sucrose, lactose and glucose, sugar alcohols such as mannitol and sorbitol, and the polymer hydroxyethyl starch (HES).

Table 8.1 Commonly used cryoprotectants, advantages and disadvantages

Cryoprotectant	Advantages	Disadvantages
Polyvinylpyrrolidone (PVP)	• Maintains the ability of some types of stem cells to differentiate post-freezing	• Inferior cell viability compared to intracellular cryoprotectants • Can affect nucleic acid integrity
Dimethyl Sulfoxide (DMSO)	• Provides superior protection to osmotic lysis	• Can be directly absorbed through the skin • Cytotoxic in some cell types
Glycerol	• Much less cytotoxic compared to other intracellular cryoprotectants • Increased operator protection compared to DMSO	• Greater osmotic lysis compared to DMSO • Must be removed prior to plating cells

Although intended to confer protection as their name implies, cryoprotectants can also cause injury to cells and several investigations have focused on determining, minimizing or eliminating their toxicity [56-62]. The solution for this is to use scaffolds for seeding of mesenchymal stem cells. Scaffolds can provide a protective cage for mesenchymal stem cells which will minimize the direct effect of cryoprotectants on the cells. Cryoprotectants in this way uniformly distributed throughout the scaffolds and slowly reach towards the cell in scaffolds. Scaffolds properties like high surface to volume ratio, porosity, surface architecture, and absorption properties which influence flow of cryoprotectants within a scaffolds and cell viability.

In the present study, cell seeded and nonseeded constructs subjected to a standard cryopreservation procedure for 1, 3 and 7 days. These constructs were characterized by cell viability assay and surface, mechanical properties of constructs also were studied. It is proposed that chitosan-alginate scaffolds (CA) are suitable for 3D culture and cryopreservation of MSCs which serve as ready-to-use scaffold constructs for regenerative medicine.

8.2 Experimental

Chitosan (from shrimp shells) DD (degree of deacetylation) was > 75%, calcium alginate (alginic acid calcium salt) were also obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Acetic acid, Sodium hydroxide (NaOH) pellets, Calcium chloride ($CaCl_2$) was purchased from Merck Ltd., Mumbai. A proliferation medium Dulbecco's modified Eagle's medium-low glucose (DMEM-LG), 20% fetal bovine serum (FBS), 100 U/mL penicillin and 100µg/mL streptomycin, Dimethyl Sulfoxide (Me2SO), Trypsin, Trypan blue and Phosphate Buffered Saline (PBS) were purchased from Sigma Aldrich Pvt. Ltd. All chemicals were of AR grade and used without further purification. All aqueous solutions were prepared in deionized water throughout the experiment.

8.2.1 Cell cultivation

hUMSCs were harvested from the umbilical cord obtained after normal full-term delivery of patient and isolated as reported elsewhere [52]. It was then expanded in low-glucose DMEM supplemented with 1% antibiotic and 10% FBS. When confluence reached, cells were trypsinized and ressuspended at passage 4. From the obtained cell suspension, and surface area available for cell attachment, cellular concentration was prepared such as 5 x 10^5 cells in 300 µL volumes and seeded onto the surface of CA scaffolds. All the scaffolds were placed in 24-well nonadherent plates to perform seeding. After seeding, these samples were transferred into the incubator for 3h, before adding 5mL of DMEM basal medium. The samples were cultured for 7 days and the medium changed after 2 days. Scaffolds without cells were kept in the same conditions to be used as experimental controls.

8.2.2 Cryopreservation and cell recovery

After 7 days of culture, half of the previously seeded constructs and unseeded scaffolds, were collected for physiochemical characterization viz. environmental scanning electron microscopy (E-SEM), Fourier Transform Infrared Spectroscopy (FT-IR), and MTT assay while the other half of the constructs were cryopreserved along with some more unseeded scaffolds. For the cryopreservation step, a cryopreservative solution composed of Me2SO and FBS was used for suspending the seeded and unseeded scaffold. The concentration of Me2SO to use in the cryoprotective solution was determined by estimating the amount of cells in the scaffolds after 7 days of culture as reported [7]. A period of 7 days of cryopreservation was chosen taking into consideration previous studies performed in the field, which states that the duration of the storage in liquid nitrogen (-196^{o}C) has a negligible impact on constructs [64-67]. Thus, for each millions of cells, a 10% concentration of Me2SO was added to the cryopreservative solution. All seeded and unseeded scaffolds were suspended in

cryoprotective solution inside standard cryovials and placed inside a cryogenic tank. The samples were thawed at 37°C in water bath and placed into non-adherent well plates containing 0.5 ml of PBS. The PBS was replaced with culture medium. Immediately after thawing some samples were stained for viability, the others were placed into incubator for recovery. Recovery time points were 1, 3 and 7 days respectively after thawing. Figure 8.2 shows schematic representation of the cryopreservation experiments. Scaffolds were used and the experiment was repeated three times. All the remaining samples were used for further analysis.

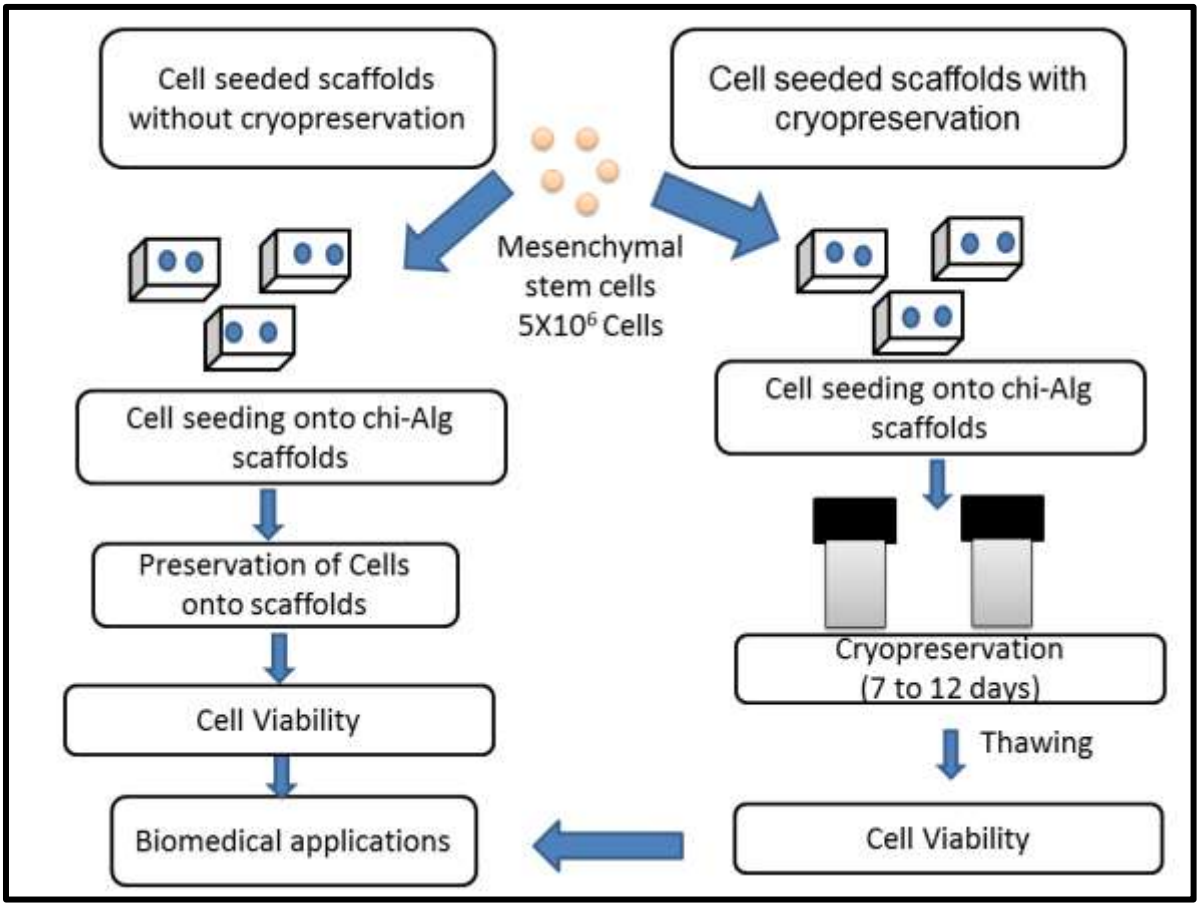


Figure 8.2 Procedures of cryopreservation experiments of cell seeded chitosan-alginate scaffolds

8.2.3 Physicochemical properties

Surface morphology was studied by an environmental scanning electron microscope (E-SEM) JEOL Model JSM-6360LV at an accelerating voltge10kV to evaluate cell morphology and cell attachment within the scaffolds. The chemical structure and possible interaction between CA scaffold and the cell-scaffold construct was analyzed by FT-IR, Bruker, ALPHA 100508, USA spectrophotometer in the range 4000 to 500 cm^1.

The rheological study was performed by using Rheometer (AR2000, TA Instrument Ltd.). The sample was tested for compressive modulus before and after cryopreservation.

8.2.4 MTT Assay for cell viability

MTT assay for Cell viability was performed onto cell seeded scaffolds with and without cryopreservation. Similarly, on mesenchymal stem cells with and without scaffolds as showed in Figure 8.3. This assay is used to quantify spectrophotometrically the amount of living cells, due to the reduction of MTT to purple formazan by the mitochondria of the living cell. For the MTT assay, the hUMSCs were seeded on CA scaffolds at a concentration of 5×10^5 cells/mL in respective medium for 24 h in a 96 well microtiter plate. After 24 h of incubation, the media were replaced with fresh media. Then cells were kept for an incubation time of 48 h. Blue formazan crystals forms from the metabolism of MTT in the mitochondria of viable cells, were washed with PBS and were dissolved in 50 mL of Me2SO and measured at 550 nm by the plate reader. The experiments were repeated three times and the data was graphically presented as the mean. The percentage of cell viability (%) compared with the control well containing cells without scaffold is calculated by the equation,

$$Cell\ Viability\ (\%) = \frac{[A\ absorbance]tested}{[A\ absorbance]control} \times 100 \qquad (8.1)$$

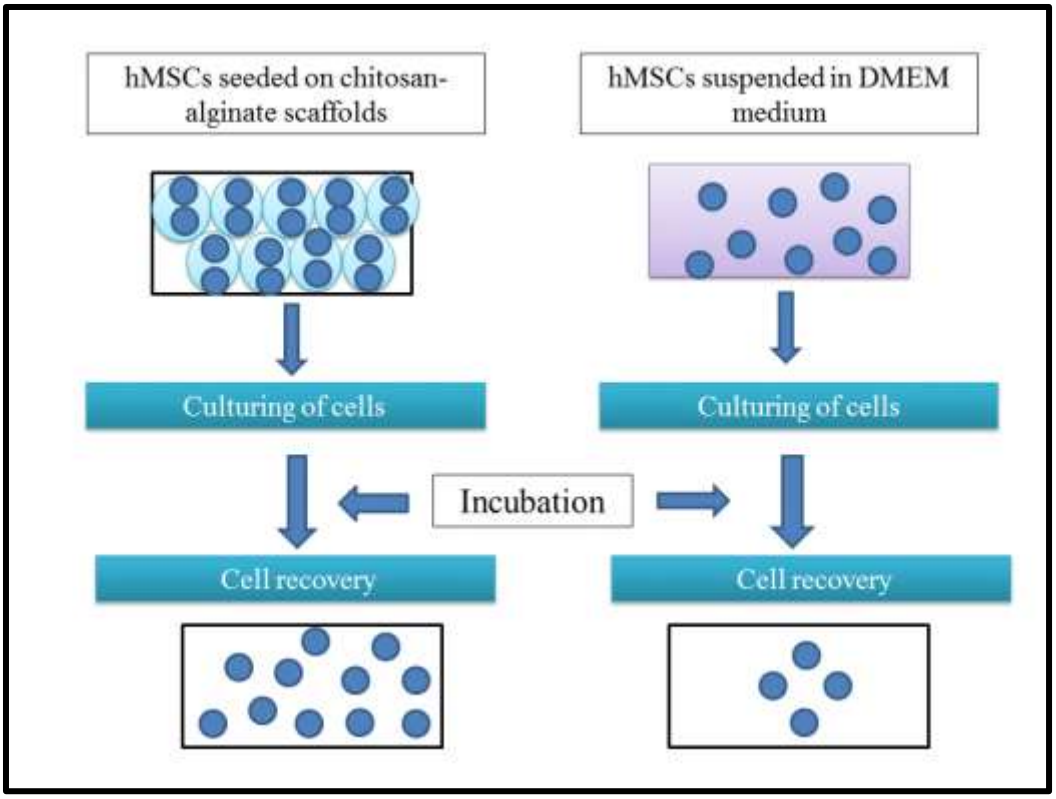


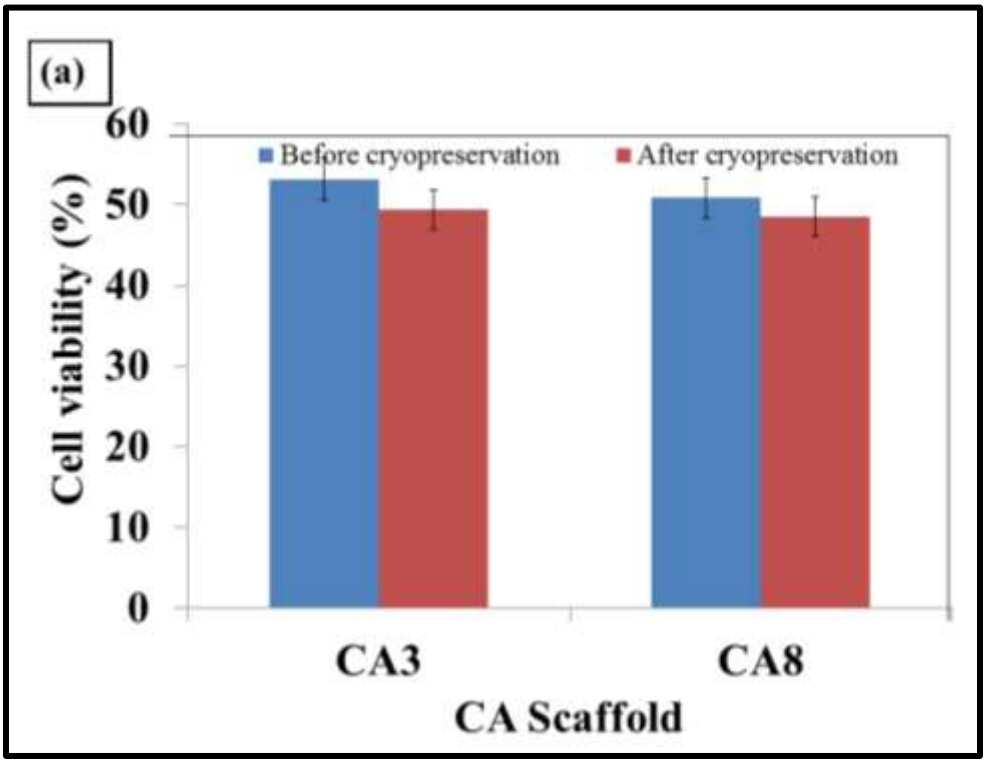
Figure 8.3 Schematic representation of cell recovery with and without scaffolds

8.3 Results and discussion

8.3.1 Viability of hUMSCs on porous scaffolds before and after cryopreservation

The hUMSC were successfully seeded and cultured under basal culture conditions on the porous scaffolds for 7 days. Constructs were cryopreserved under standard conditions for 3 days, thawed and then cultured for a further period of 1, 3 and 7 days respectively to allow for full cellular recovery and efficient leaching of Me_2SO residues. Cells and tissues that are submitted to cryopreservation always require time to recover. Studies found in the literature have shown that, after thawing, cellular viability tends to decrease for a period of at least 7 days before stabilizing [66]. This recovery culture period also allowed for a more prolonged and more efficient leaching of toxic Me_2SOs residues from

the porous constructs. The recovery culture period was not prolonged for 9 days since the strategy involved in a medically oriented usage of these constructs consist of applying these constructs into tissue defects as quickly as possible after thawing, to reduce patient's immobilization time. By examining the results obtained from the MTT assay (Figure 8.4) performed on seeded scaffolds before and after cryopreservation, constructs were able to maintain cellular viability.



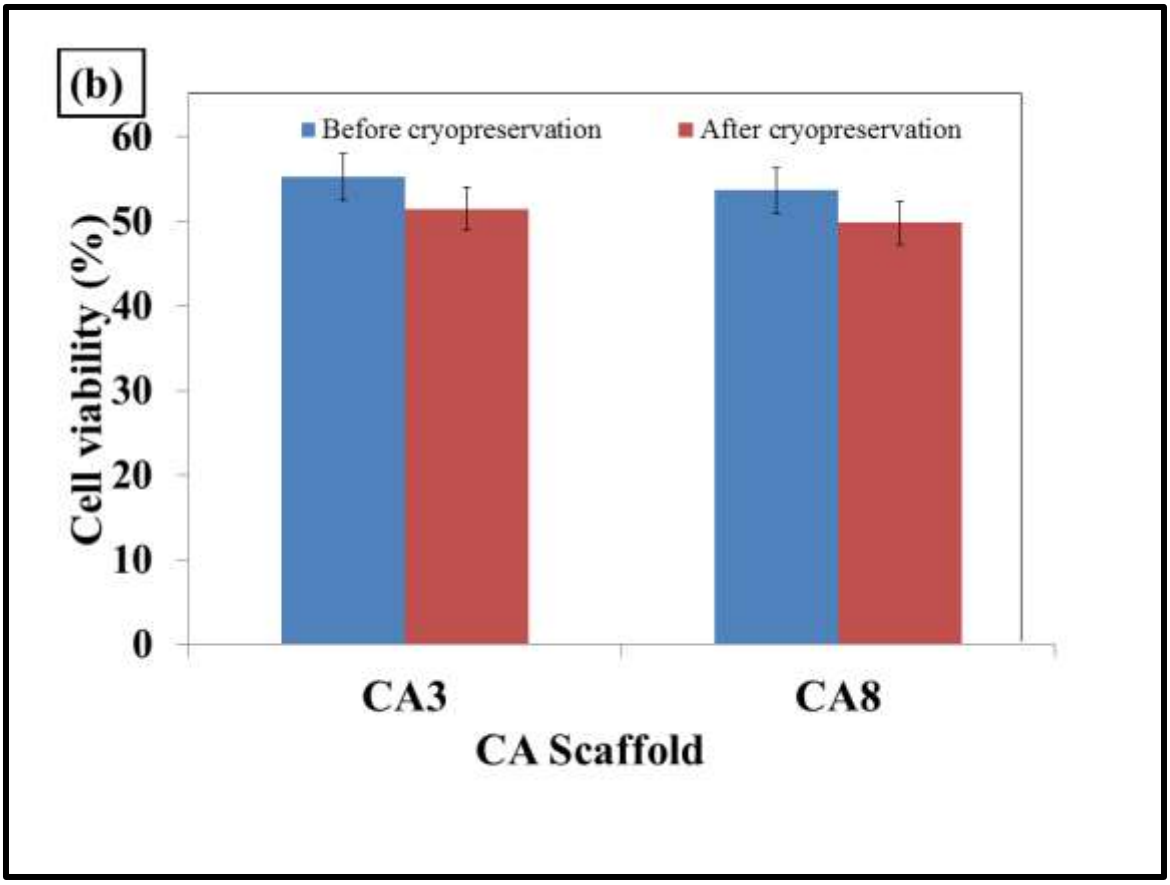


Figure 8.4 Cell viability versus concentration of CA Scaffold samples seeded with huMSC after (a) 3 days of incubation and (b) 7 days of incubation before and after cryopreservation

It was also possible to notice that cellular viability was efficiently maintained in porous scaffolds after cryopreservation. Table 1 showed cellular viability of cell seeded on scaffolds at 3 and 7 days of recovery period was observed. However, no significant differences in viability between them. The results shows that cells seeded in porous scaffolds would be better cryopreserved as pores in the scaffolds would retain the cryoprotectant more efficiently. Along with a prolonged recovery time for cellular viability stabilization, an effective removal of the cryoprotectant from the porous scaffold after cryopreservation is possible. Cryoprotectants like Me_2SO, are known to be highly toxic for cells therefore it is crucial not only to maintain the cryoprotectant inside the scaffold

during cryopreservation but also to make sure it would be efficiently removed after cryopreservation. The efficiency of the cryoprotectant's removal contributed to the scaffold's specific porosity and architecture [64].

Table 8.2 Cellular viability of CA scaffolds before and after cryopreservation at 3 and 7 days of recovery period respectively

Cellular Viability (%)	3Days		7Days	
	Before cryopreservation	After Cryopreservation	Before cryopreservation	After Cryopreservation
	53.12±3%	49.41±2%	55.35±2%	51.47±2%

8.3.2 Physicochemical properties

8.3.2.1 E-SEM Morphology

In tissue engineering, scaffold should have high porosity and inter connected pore structure to enhance a compatible biological condition for cell attachment, proliferation and differentiation. E-SEM investigation of hMSCs in scaffolds before and after cryopreservation indicated good attachment and spreading of hMSCs on CA scaffolds. The scaffolds were smooth and showed no ruptures or damages after cryopreservation. By comparing the E-SEM micrographs (Figure 8.5) before and after cryopreservation it was possible to observe that the cryopreservation process did not change the material's surface morphology. After cryopreservation, the surface of porous scaffolds showed the same smooth morphology as before cryopreservation. This was more accurately confirmed by performing E-SEM which revealed no significant difference between surfaces of CA scaffolds before and after cryopreservation.

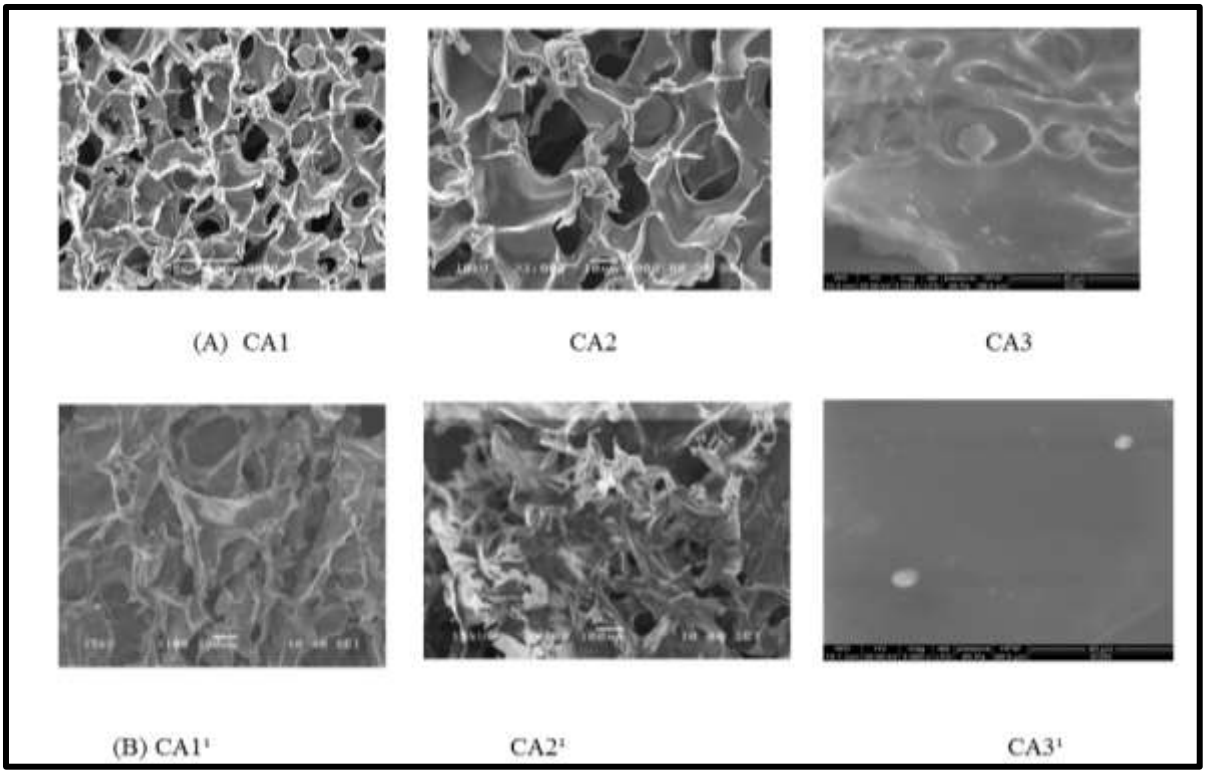


Figure 8.5 E-SEM pictures of chitosan-alginate (CA1, CA2, CA3), scaffolds with X100, X500, x1000 and x3000 resolution before and after cryopreservation after (A) 3 days of recovery and (B) after 7 days of recovery

Immediately, after thawing for 1hr recovery of the scaffolds, cultivated for 7 days before cryopreservation, round shaped cells were observed. As shown in figure 8.5, E-SEM analysis depicts the cellular morphology which was not affected by the cryopreservation process and that cell remained well attached to the surface of the material after thawing. In the same way, stress-strain experiment was performed on the porous scaffolds showing that cryopreservation process did not alter the general architecture of scaffolds and porosity as shown in Table 8.3.

Table 8.3 Quantitative analysis performed to compare the total porosity of scaffolds before and after cryopreservation. The values of average porosity before and after cryopreservation were not significantly different

	Before Cryopreservation	**After Cryopreservation**
Average Porosity (%)	40.76 ± 3.76	40.08 ± 3.71

8.3.2.2 FTIR Analysis

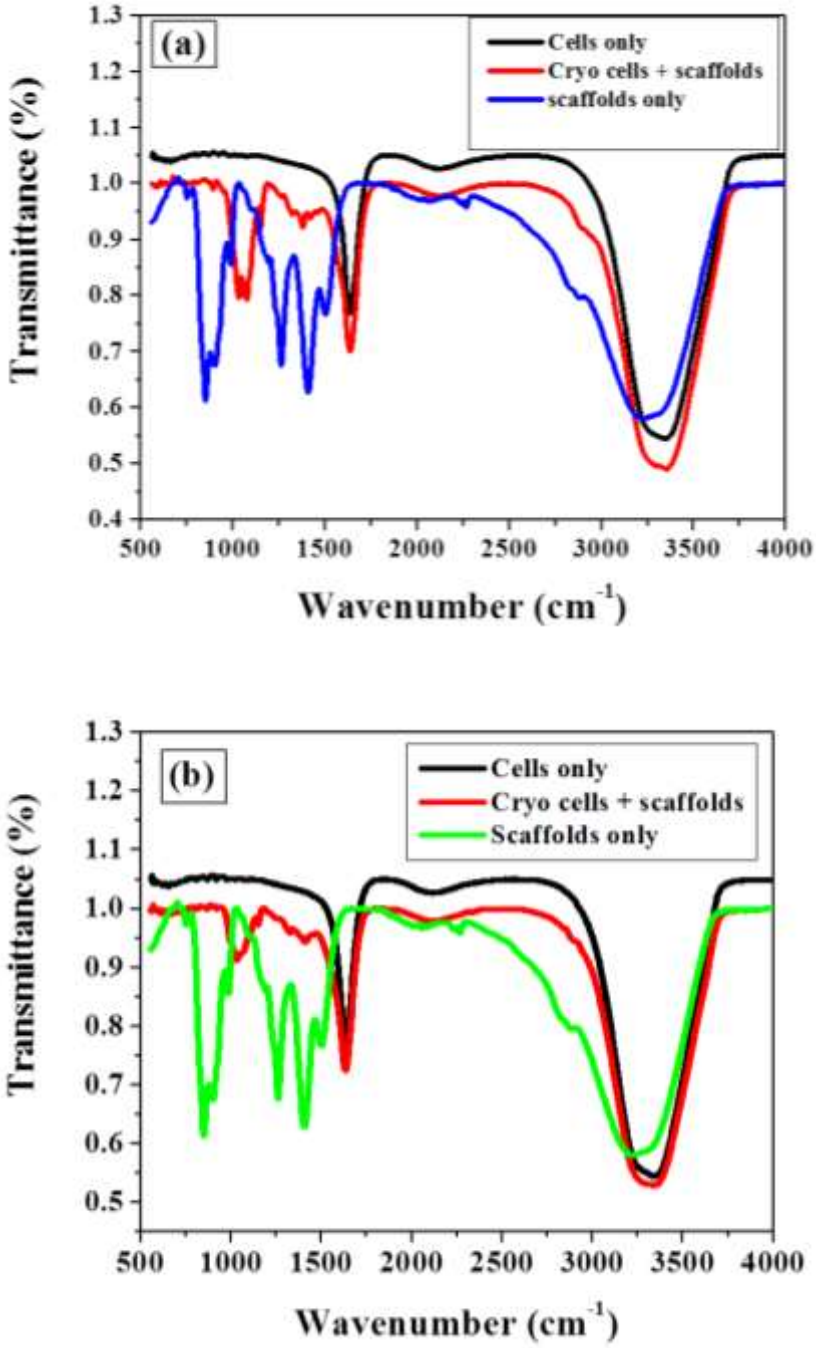


Figure 8.6 FTIR spectrum of mesenchymal stem cells, seeded scaffolds and cryopreserved seeded scaffolds (a) after 3 days of incubation and (b) after 7 days of incubation

FTIR spectra of MSCs, seeded CA scaffolds and cryopreserved seeded CA scaffolds are illustrated in Figure 8.6a and 8.6b, respectively. The spectrum of MSCs showed presence of intense peaks at 3337 cm^{-1} and 1635 cm^{-1} which was due to N-H and O-H stretching vibrations of polysaccharides, C=O stretching vibrations of proteins (Amide I) respectively as shown in figure 5a. The absorption bands was observed at 3222 cm^{-1}, 1507cm^{-1}, 1411cm^{-1} and 1277cm^{-1} significantly which was due to CO-O-C asymmetric strectching vibraitions, skeletal vibration involving the COO stretching respectively and they were characteristic of its saccharine structure present in scaffolds [65]. After cryopreservation of the seeded scaffolds, 1411cm^{-1} and 1277cm^{-1} peak were disappeared. Similarly, 845cm^{-1} and 908cm^{-1} peaks were shifted to 1036cm^{-1} and 1069cm^{-1} respectively. Similarly, after 7 days of incubation (Figure 5b) the spectra of MSCs showed presence of intense peaks at 3337 cm^{-1} and 1635 cm^{-1} as shown above [65]. In cryopreserved seeded scaffolds 1036cm^{-1} peak was shifted to 1031cm^{-1} and 1069cm^{-1} peak was absent. The change was due to lowering the temperature up to -196^{0}C. Remaining peaks did not show any significant change with respect to control sample.

8.3.2.3 Mechanical properties

Compression mechanical tests were performed on cryopreserved and non-cryopreserved scaffolds. The results showed that the cryopreservation process did not alter the mechanical compressive properties since there was no significant difference found when comparing the values for Young's modulus before and after cryopreservation as shown in Table 8.4.

Table 8.4 Comparative Compression Mechanical Analysis on Scaffolds before and After Cryopreservation

Average young's modulus (Mpa)		
Sample	Before Cryopreservation	After Cryopreservation
Scaffolds	33.46 ± 4.20	32.07± 3.56

8.3.2.4 Cell recovery with and without scaffolds

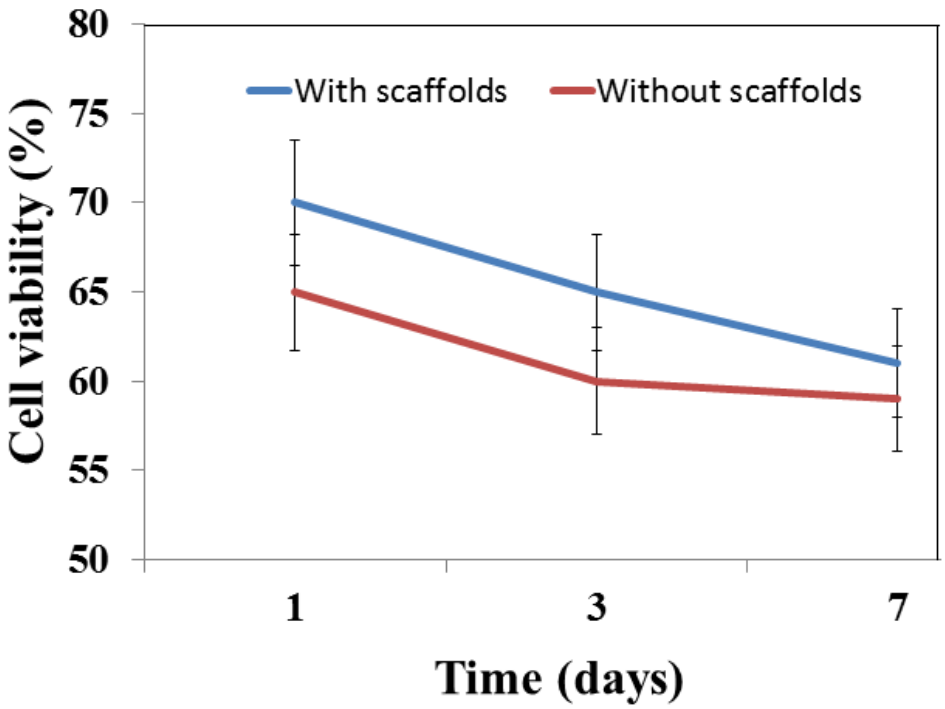


Figure 8.7 calculated viability of hMSCs seeded on (a) chitosan-alginate scaffolds and (b) without scaffolds at recovery period of 1, 3 and 7 days

The CA scaffold acts as an extracellular matrix for cell adhesion and regeneration. Here, hMSCs are seeded onto CA scaffolds and examined for cell recovery in presence and absence of scaffolds. Figure 8.7 showed higher cell recovery in presence of CA scaffolds as compared to cells without scaffolds. Cell viability rate is higher after 7 day recovery of cells. It is shown that in presence of scaffolds cell recovery is maximum i. e. 61% , 65% and 70% at recovery period of 1, 3 and 7 days respectively. When compared to cell recovery without scaffolds it is found to be 59%, 62% and 65% at same recovery period. Hence, scaffolds provide a microenvironment for cells to grow, attach and differentiate.

8.4 Conclusions

From present study, it is observed, successful cryopreservation of adherent hMSCs within CA scaffolds possess the potential to serve as a ready to use tissue engineered product. In present work, it is proposed that, cell seeded constructs maintain cellular content and cellular viability in standard cryopreservation conditions as shown by the MTT assay.

In comparison with the work of *Xu et al.* [27], viability of cryopreserved hMSCs adherent to scaffolds could be enhanced after 7days. The CA scaffolds synthesized here are porous and spongy. Mechanical strains between shrinking cells and stiff scaffolds during cooling and ice crystallization occur in lesser amount. As both have similar thermal contractions, so that cells do not detach [29, 64]. Further, alginate is a potent hydrogel, absorbs water and thus can avoid harmful formation of large ice crystals. The scaffolds remain soft and elastic after cryopreservation. Literature reveals that, in cryopreservation of adherent cells use recovery times of 24 h up to 7 days prior to freezing [68-70]. However, present result shows that the longest recovery time (7 days) yielded highest viability of hMSCs after thawing (Figure. 8.7). Also, the porosity found in scaffolds influence the viability and retention of cells in constructs exposed to cryopreservation processes. This observation may be explained by a possible retaining and protective effect exerted by the scaffolds over the cells contained inside the scaffold's pore against the intrinsic damaging effects of cryopreservation [70].

Tripathi A et al.[71] showed after 24h cultivation hMSCs were more spread than the cells in other groups and formed closed cell–cell and cell-substrate contacts mediated by integrins and cytoskeletal structures such as actin stress fibers [72,73]. Former studies already showed that rigidity of plasma membrane and cytoskeleton play key roles in freezing and thawing of adherent cells [61]. Disruption of the cytoskeleton by ice crystal formation during cryopreservation can lead to cell detachment with subsequent cell death [72]. Present results of hMSCs cultivated for 7 days before cryopreservation confirmed this thesis.

cytoskeletal proteins are not studied as did others [73], but investigated cell state after cryopreservation by high-resolution SEM and checked cell shape. It is found that attachment with CA scaffolds allows an indirect proof of cytoskeleton breakdown of cells with maintained cell-substrate focal contacts.

As for the morphology of scaffolds, it was not damaged or altered by the cryopreservation process at a macroarchitectural level as shown by E-SEM studies. This is an important observation since the scaffold's architecture and surface morphology are known to greatly influence the efficiency of cell adhesion and subsequent proliferation over material's surfaces.

It enables that the cryopreservation process did not alter the mechanical compressive properties of CA scaffolds since there was no significant difference found when comparing the values for Young's modulus before and after cryopreservation. It means that it would be possible to accurately define the construct's mechanical properties prior to cryopreservation aiming at specific tissue without having any detrimental effect caused by the cryopreservation process. The same principle applies to the architecture and surface morphology of scaffolds, as show E-SEM. It is also not affected by the cryopreservation process. Similarly FT-IR spectra shows slight changes in peaks of cell seeded construct before and after cryopreservation. Also, we showed that cell recovery rate is increased when cells are seeded onto scaffolds at time interval of 1, 3 and 7 days. This is may be due to role of scaffold in attachment and proliferation of hMSCs which improves result.

Present work showed the influence of cryopreservation on adherent hMSCs within CA scaffolds. It showed that in an optimum period of 7days cells recovered most rapidly and shows higher percentage of viable cells as compared to the short recovery time of 2days. Also, FT-IR and mechanical properties confirm negligible effect of cryopreservation on cells and scaffold material. Overall, the obtained result shows that it is possible to maintain viable cells and scaffolds properties upon cryopreservation of tissue-engineered constructs based on CA

scaffolds and hMSCs using standard cryopreservation methods. Also, present study suggests that the architecture found in porous scaffolds favors the retention and viability of the construct's cellular content during cryopreservation processes. In particular, major finding is that use of CA scaffold for effective preservation of mesenchymal stem cells. Scaffolds mimic the extracellular matrix of the native tissue, recapitulating the *in vivo* milieu and allowing cells to influence their own microenvironments.

Finally, these findings indicate that it may be possible to prepare off-the-shelf engineered tissue substitutes and preserve them to be immediately available upon request for patients.

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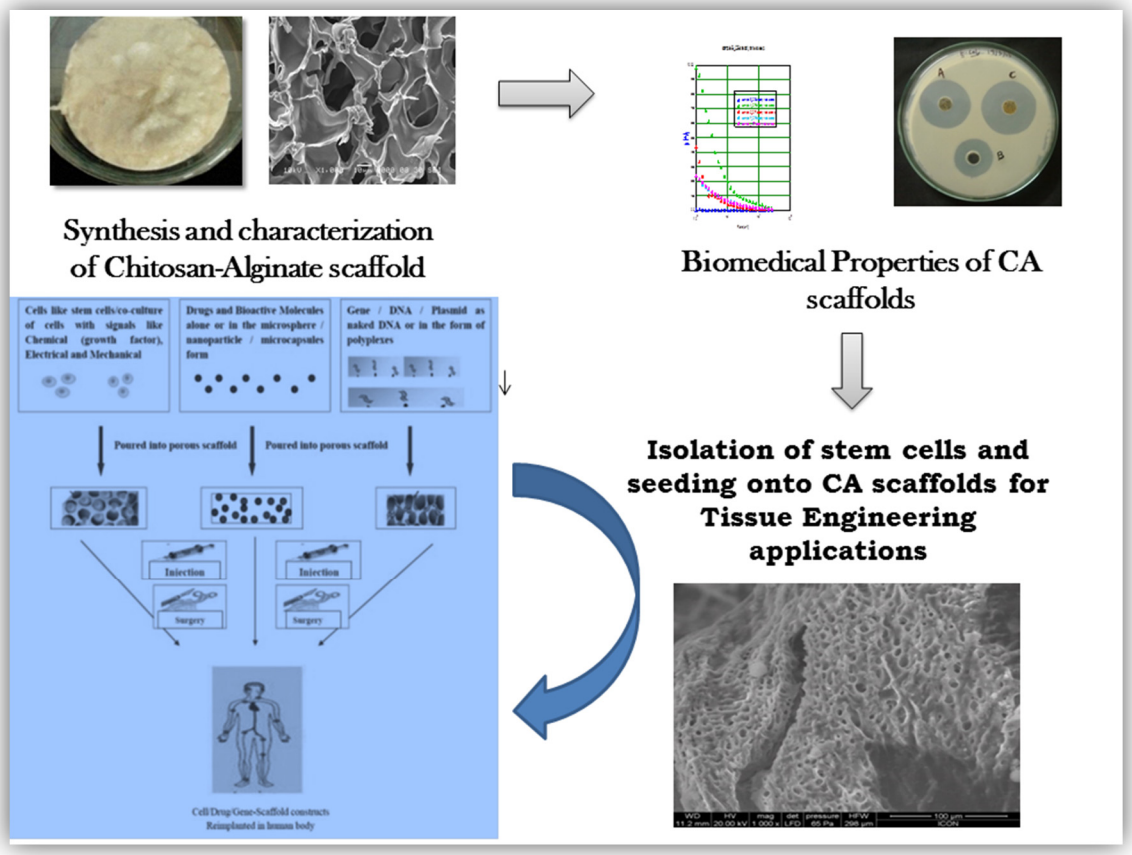
Chapter 9

Summary and Conclusions



9.1 Introduction

A wide range of scaffolding materials has been reported in literatures that have found application in tissue repair and tissue regeneration. Scaffolds with incorporated biological cues mediate tissue formation by guiding the adhesion, proliferation and differentiation of the transplanted cells or the native infiltrating cells at the site of tissue regeneration. In recent years many different types of scaffolds using varied biomaterials have been developed, specifically hydrogels; custom scaffolds; fibrous scaffolds; porous scaffolds; microspheres; native tissue scaffolds. An important characteristics of scaffolds includes proper mechanical properties, tunable degradation rates, cyto-compatibility, and the ability to direct cell growth and differentiation.

3-D substrates have great impact in the development of scaffolds for tissue engineering in regenerative medicine, especially in combination with multipotent stem cells, like human mesenchymal stem cells (hMSCs),that are capable to differentiate in various cell types of mesoderm germ layer. These scaffolds provide native environments required for cell proliferation and differentiation at an optimal growth-area-to-volume-ratio. Among the numerous kinds of materials serving as scaffolds, chitosan-alginate composite convinces by following advantages for medical applications: the natural polysaccharides forms a hydrogel when crosslinked with multivalent ions, is cyto and biocompatible, as well as biodegradable. Furthermore, pore sizes in chitosan-alginate scaffolds can be adjusted and allows immuno-isolation of encapsulated grafts along with nutrient supply and metabolic factor diffusion as well as cell motility and contact in scaffolds with large, open pores.

Recently, lot of attention has been focused for development of new combinations of polysaccharides as scaffolds; hence in the present thesis an attempt has been made to synthesize chitosan-alginate scaffolds by freeze drying methods with efficient mechanical and antibacterial properties which may be helpful for biomedical applications.

9.2 Competent Components of Thesis

The principle aim of the thesis is focused on synthesis and characterization of chitosan-alginate scaffolds for seeding mesenchymal stem cells isolated form umbilical cord tissue for various biomedical applications. Natural polymers can be considered as the first biodegradable biomaterials used clinically as scaffolds. Natural materials owing to the bioactive properties have better interactions with the cells which allow them to enhance the cells performance in biological system. After reviewing extensively chitosan and alginate are selected as a potential material for the present work due to the physical, chemical, biological, rheological and degradation properties.

In the present study, chitosan, alginate and chitosan-alginate composite scaffolds were successfully fabricated by freeze drying method. These materials were interrogated via physiochemical characterization method, which provides a new route as an injectable material. This finding provides the basic information about the particle size, molecular structure and surface morphology after the formulation of scaffolds. The synthesized scaffolds were characterized by applying orthogonal and physical approaches viz; EDS, XRD, E-SEM and FTIR. The SEM micrographs showed porous smooth surface having highly interconnected pores which is needed for cell attachment, adhesion and proliferation. The molecular structure of chitosan-alginate scaffolds were determined by FTIR spectroscopy while amorphous nature of synthesized scaffolds confirmed by X-ray diffraction technique. This work provides a simple method which will be helpful in order to fabricate chitosan-alginate scaffolds which will be later useful as a potential material for tissue engineering applications. Hence, could also be utilized as a carrier for delivery of bioactive compounds like growth factors, hormones and biomolecules like DNA, RNA and cells.

The use of biomaterials as scaffolds in tissue engineering is growing with continuous development. The scaffolds fulfill a serial of requirements when used

for clinical applications like biocompatibility, anti and biodegradability. From the observed data it is clear that chitosan-alginate scaffolds are showing antibacterial activity. Herein, the mechanism of antibacterial activity of chitosan-alginate scaffolds is discussed in detail. **Additionally, the antibacterial activity results revealed that chitosan-alginate scaffolds have excellent antibacterial property against *E.coli* and *Staphylococcus epidermidis* showing maximum zone of inhibition.** Similarly, Degradation of CA scaffolds can be studied by using lysozyme enzyme. The results shows that CA6 scaffold which containing lower chitosan concentration and moderate alginate concentration degrades faster as compare to other combinations of chitosan and alginate.

The most of biomaterials used in biomedical applications are showing little toxicity, which is not favorable for there *in vivo* administration as it is. In the present case, chitosan-alginate scaffold found to be biocompatible after extensive literature review. The biocompatibility of the CA3 composite shows higher biocompatibility than other composites due to higher concentration of chitosan. The most common method, which is used to find out the cytotoxicity of CA scaffolds, is MTT assay. **The CA3 composite is showing about 90% cell viability up to 48h.** Hence, chitosan-alginate scaffolds serve as a potential material for invitro applications.

On the other hand, Rheological and Hemocompatibility studies are the two major properties of scaffolds which have taken into consideration. From the observed data it is clear that chitosan –alginate scaffolds showing stable 3D porous network because of having oppositely charged interparticle interactions. Also, these scaffolds are highly hemocompatible in nature. **Both results indicate potential of chitosan-alginate scaffolds in invivo applications.** Once the scaffolds get synthesized, it is necessary to check its suitability for various biomedical applications. In this research work, we have studied thermal properties of scaffolds. As well as swelling behavior and porosity is also determined. The observed data shows chitosan-alginate scaffolds possess excellent thermal stability

as compare to chitosan scaffolds. Porosity or pore size of synthesized scaffolds compares with the chitosan scaffolds. It seems porosity of CA scaffolds quite lesser than chitosan scaffolds due to interference of sodium alginate. Also, **the scaffolds containing10%chitosan, 90% alginate, showed a higher degree of swelling than the other chitosan– alginate scaffolds,** likely due to the higher amount of hydrophilic alginate present in these scaffolds.

In the next part of the thesis we have focused on isolation of mesenchymal stem cells from umbilical cord tissue which is a biological waste. Culture is maintained for its characterization and growth studies. Later, isolated cells were seeded on chitosan-alginate scaffolds to study the interaction of cells and scaffolds. Such cell seeded scaffolds has greater advantage in the tissue engineering field. Result shows that, Mesenchymal stem cells were successfully isolated from cord tissue (Wharton's jelly) and confirmed by flow cytometry analysis. **A flow cytometry study confirms that isolated cells are nothing but MSCs.** Further, for successful seeding of cells on the scaffolds, the molecular characteristics should be well understood. In order to know in detail about the structure of chitosan-alginate scaffolds the studies have been carried out which provides the basic information about the conformational transitions occurring during the seeding of cells onto the material. The work presented here provides proof of concept and demonstrates the efficacy of the complementary characterization approach for the investigation of cell seeded scaffolds. This study aids to explore the conformational changes of cell seeded scaffolds as shown in FT-IR spectroscopy which signify major changes occur after cell seeding. SEM analysis shows the effective binding of the cells on scaffolds which is supported by histochemical analysis. Similarly, the observed results suggest that the material system promoted intercellular contacts and the spatial arrangement of the cells.

Further, cell seeded scaffolds undergo cryopreservation process serves as storage system which can be utilized immediately whenever needed. This approach opens up new dimension which will be helpful in the clinical

applications. The obtained result shows **the percentage of cellular viability was 55.35±2% before cryopreservation and 51.47±2% after cryopreservation at 7 days incubation. Similarly, cellular viability obtained was 53.12±3% before cryopreservation and 49.41±2% after cryopreservation at 14 days of incubation.** Also**,** the results suggested that the cryopreservation process did not alter the mechanical compressive properties of chitosan-alginate scaffolds when comparing the values for Young's modulus before and after cryopreservation. The same principle applies to the architecture and surface morphology of scaffolds that, as shown by the performed SEM, are also not affected by the cryopreservation process. Finally, these findings indicate that it may be possible to prepare off-the-shelf engineered tissue substitutes and preserve them to be immediately available upon request for patients.

9.3 Major Conclusions

- Chitosan-Alginate scaffolds were synthesized by simple freeze drying method. The changes in the structural and morphological characteristics have been studied extensively by using different spectroscopies techniques.

- Chitosan-Alginate scaffolds containing more positively-charged particles (CA6) exhibited higher viscosity, suggested that the scaffolds were desirable for injectable applications. Also, such scaffolds are highly hemocompatible and biocompatible in nature.

- Chitosan-Alginate scaffolds possess excellent thermal stability as compare to chitosan scaffolds. Similarly, other properties like surface chemistry and swelling behavior studies reveals the use of chitosan-alginate scaffolds in tissue engineering field.

- The antibacterial activity results revealed that chi-alg (CA6) Scaffolds have excellent antibacterial property against *E. coli* and *S. epidermidis* showing maximum zone of inhibition.

- Mesenchymal stem cells were successfully isolated from umbilical cord tissue and seeded on chitosan-alginate scaffolds to study cell-scaffold interaction. Interaction study proves the efficient binding and proliferation of cells onto scaffolds.

- It is possible to maintain viable cells and scaffolds properties upon cryopreservation of tissue-engineered constructs.

- Finally, these findings indicate that it may be possible to prepare off-the-shelf engineered tissue substitutes which is immediately available for clinical applications.

9.4 Future Scope of Thesis

It is an exhilarating challenge for future research on cell seeded chitosan-alginate scaffolds for carrying out *in vivo* experiments on the animal model for various biomedical applications. Currently, we are planning to grow human embryo on chitosan-alginate scaffolds and study its interaction pattern which further will be useful in IVF trials.

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Date of registration: 1st August, 2011 at D. Y. Patil University, Kolhapur.

EXPERIMENTAL KNOWLEDGE

- Expertise in synthesis of chitosan nanoparticles, chitosan- alginate scaffolds and its composites. I have used different methods for preparation viz; freeze drying for scaffolds preparation and solvent casting/particulate leaching for synthesis of chitosan nanoparticles.

- ❖ Expertise in isolation of mesenchymal stem cells from umbilical cord tissue and cord blood; it's culturing and passaging, cryopreservation of stem cells, cytotoxicity assays, along with some basic molecular biology techniques including electrophoresis, SDS-PAGE, PCR.

- ❖ Characterization instruments handled: X-ray diffractometer, Particle size analyzer, Scanning/Transmission electron microscope (SEM), Fourier transform infrared (FTIR), Rheometer, FE-SEM, Energy Dispersive X-ray Spectroscopy (EDS), Particle size analyzer, TGA/DSC etc.

- ♣ **Some part of the research work is applied for the patent entitled "Scaffolds of umbilical cord for tissue engineering and preparation method thereof."(Application number 3247/MUM/2015)**

RESEARCH EXPERIENCE

1. Ph. D. Thesis entitled **"Synthesis and Characterization of Chitosan-Alginate Scaffolds and Seeding Mesenchymal Stem Cells for Biomedical Applications"** under the supervision of **Prof. (Dr.) S. H. Pawar**, Center for Interdisciplinary Research, D. Y. Patil University, Kolhapur-416006, India **(2011-2016)**.

2. Successfully guided the project entitled "Chitosan scaffolds synthesis and its antibacterial activity", "Activity of medicinal plant extracts on HeLa cell line", "Green synthesis of silver nanoparticles from neem plant and its antibacterial activity" of for the partial fulfillment of B.Sc degree in Biotechnology, Department of Biotechnology, University of Pune, Pune-411001**(2012-2015).**

3. Successfully completed the project entitled "**Chitinase production using penicillium ochrochloron MTCC 5175 strain"** for the partial fulfillment of M.Sc. degree in Biotechnology under the supervision of **DR. J.P.Jadhav,** Department of Biotechnology, Shivaji University, Kolhapur-411004 **(2009-2010)**.

4. Worked as Research Associate at Patki Research Foundation & Hospital, Kolhapur, Maharashtra (Aug 2010-May 2011)

TEACHING EXPERIENCE

1. Worked as Assistant Professor at Nowrosjee Wadia Collage, Department of Biotechnology, University of Pune, Pune-411001 **(August 2012 to May 2015).**

2. Worked as a Teaching assistant for research for M. Sc. Stem cells and Regenerative Medicine, Centre for Interdisciplinary Research, D. Y. Patil University, Kolhapur-416006, India **(Aug 2011-March 2012)**.

LIST OF PUBLICATIONS

1. **S.G.Kumbhar** and S.H.Pawar, "Facile synthesis, characterization and antimicrobial activity of chitosan-Alginate scaffolds, **Indian journal of applied research**, 5, 787-791 (2012). **(I. F. = 3.624)**

2. **S.G. Kumbhar,** S.H. Pawar, "Facile Synthesis, Characterization and Antimicrobial Mechanism of Chitosan Scaffolds", Trends Biomater. Artif. Organs, 30(1), 26-31 (2016) **(I.F. = 0.699)**

3. **S.G. Kumbhar,** S.H. Pawar, "Synthesis and characterization of chitosan-alginate scaffolds for seeding human umbilical cord derived mesenchymal stem cells", **Bio-Medical Materials and Engineering**, 27 (6), 551-560 (2016) **(I.F. = 0.988)**

4. **Kumbhar S.G** and Pawar SH, "Self-Functionalized, Oppositely Charged Chitosan-Alginate Scaffolds for Biomedical Applications", **Biotechnology: An Indian Journal**, 13(2), 1-15 (2017) **(I.F. = 0.03)**

5. S. S. Rohiwal, R. K. Satvekar, A. P. Tiwari, A. V. Raut, **S. G. Kumbhar** and S. H. Pawar, "Investigating The Influence of Effective Parameters on Molecular Characteristics of Bovine Serum Albumin Nanoparticles", **Applied Surface Science,** 334, 157–164, (2015). **(I. F. = 2.711)**

6. **S.G.Kumbhar**, R.A.Bohra and S.H.Pawar, "Nanomaterials for stem cell technology development", Medical Journal of D.Y.Patil University, Kolhapur (MJDYPU),Vol. IX, 4-16 (2015) ISSN0974-2743.

7. **S.G.Kumbhar** and S.H.Pawar, "Biomaterial scaffolds for medical applications, Annual journal of university research", 6, 221-226, (2016).

COMMUNICATED

1. S.G.Kumbhar, J.V.Meshram, S.M.Patki and S.H.Pawar, "Effective cryopreservation of mesenchymal stem cells using Chitosan-Alginate scaffolds" **Cryobiology**, **(I.F. = 1.799)**

PRESENTED INTERNATIONAL / NATIONAL CONFERENCES / WORKSHOP/ SYMPOSIUM

1. **S.G.Kumbhar** and S.H.Pawar, "Chitosan-Alginate scaffolds for seeding mesenchymal stem cells" International conference on Nanomaterials and Nanotechnology, (NANO2015), K.S.Rangasamy College of Technology, Tiruchengode, Tamil Nadu, India, 07-10 December,2015.

2. S.G.Kumbhar, and N.S.Patil, "Gender equity for prosperity and piece", National science day celebrations-2010, Shivaji University, Kolhapur, 24-28 February 2010.

3. **S.G.Kumbhar** and S.H.Pawar, "Designing and fabrication of chitosan-alginate scaffolds for stem cell technology" A Workshop on Nuclear Energy & Healthcare (NEHCA-2017), Sinhgad Institutes, Solapur, 18-20 February, 2017.

CONFERENCES/WORKSHOPS/ SEMINARS

1. International Conference on Nanomaterials and Nanotechnology (NANO-2015), at Centre for Nanoscience and Technology K.S.Rangasamy College of Technology, Tiruchengode, Tamil Nadu, India.

2. 1st national workshop on nanoparticles and their applications in medicine" (NAM-2012) at IIT Kharagpur, Kolkatta.

3. National Workshop on "Global Trends in Medical Education Technology GLOBMET -2012" at D.Y.Patil Medical College, Kolhapur.

4. National conference on Nuclear Energy and Health Care Applications (NEHCA-2011), at D. Y. Patil University, Kolhapur, Maharashtra.

5. National Conference on "Bioadvantage 2007" at Pune on 19th May2007.

6. National workshop on “Stem cell, Molecular biology and Bioinformatics, (SMB-2016) at D. Y. Patil University, Kolhapur.

7. ‘‘National science day 2010” University level poster competition at Shivaji University Kolhapur.

8. ‘‘National science day 2009” University level essay competition at Shivaji University Kolhapur

Date: 18/05/2017

Place: Kolhapur

Hereby I declare that the information given above is true to the best of my knowledge.

Ms. Sneha Kumbhar