LITRATURE REVIEW-

CHAPTER 2

2.1 Biopolymers and their applications

Recent discoveries in the field of biopolymers have opened up new avenues for medical applications of biopolymers due to their novel and unique properties like biodegradability, biocompatibility and renewability. Thus biopolymers provide immense scope for wide application in different fields. Animals, plants and microbes are the source of these biopolymers. Various biopolymers are now being used to construct diverse types of biomaterials. According to conference of the European Society, biomaterials are defined as 'material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body [O'Brien et al., 2011]. Biopolymers are classified in three main categories; *viz.* ceramics, synthetic polymers and natural polymers. In 1811 Henri Bracannot first discovered Chitosan, at that time he was the director of the botanical garden in Nancy, France, [Kavitha et al., 2011]. Then, dextran was discovered as a microbial product in wine by Louis Pasteur in 1861[Pasteur, 1861]. In 1886 Adrian J. Brown reported a cellulose producing bacteria and given it the name *Bacterium xylinum* [Brown, 1886]. In 1886 Borzi reported cyanophycin as first intracellular reserve in cyanobacteria [Borzi, 1887]**.** Near about after four decades in 1926 a polyester named polyhydroxybutyrate was found in *Bacillus megaterium* [Lemoigne et al., 1926]**.** Most other biopolymers of medical significance; such as xanthan [Leach, 1957], alginate [Linkeret al., 1966], poly-γ-glutamate [Ivanovics, 1937] were reported in the early to mid twentieth century.

Biopolymers may be of plant, animal or microbial origin. Plants and animals provide various types of polymers such as cellulose, rubber, starch, inulin, agar, glycogen, silk, collagen, gelatin etc. Microorganisms also synthesize various biopolymers through metabolic processes to fulfil their requirements such as reserve material or as part of a protective structure thus these biopolymers can provide some unique features to microorganisms which make them more

compatible for survival under certain environmental conditions. Biosynthesis of the biopolymers in the microbes is under control of complex regulatory pathways and sometimes external stimuli controls production and even their material properties. Given figure (Fig. 2.1) represents the major metabolic pathways and intermediates which are involved in the biosynthesis of these biopolymers [Verma and Banik 2013].

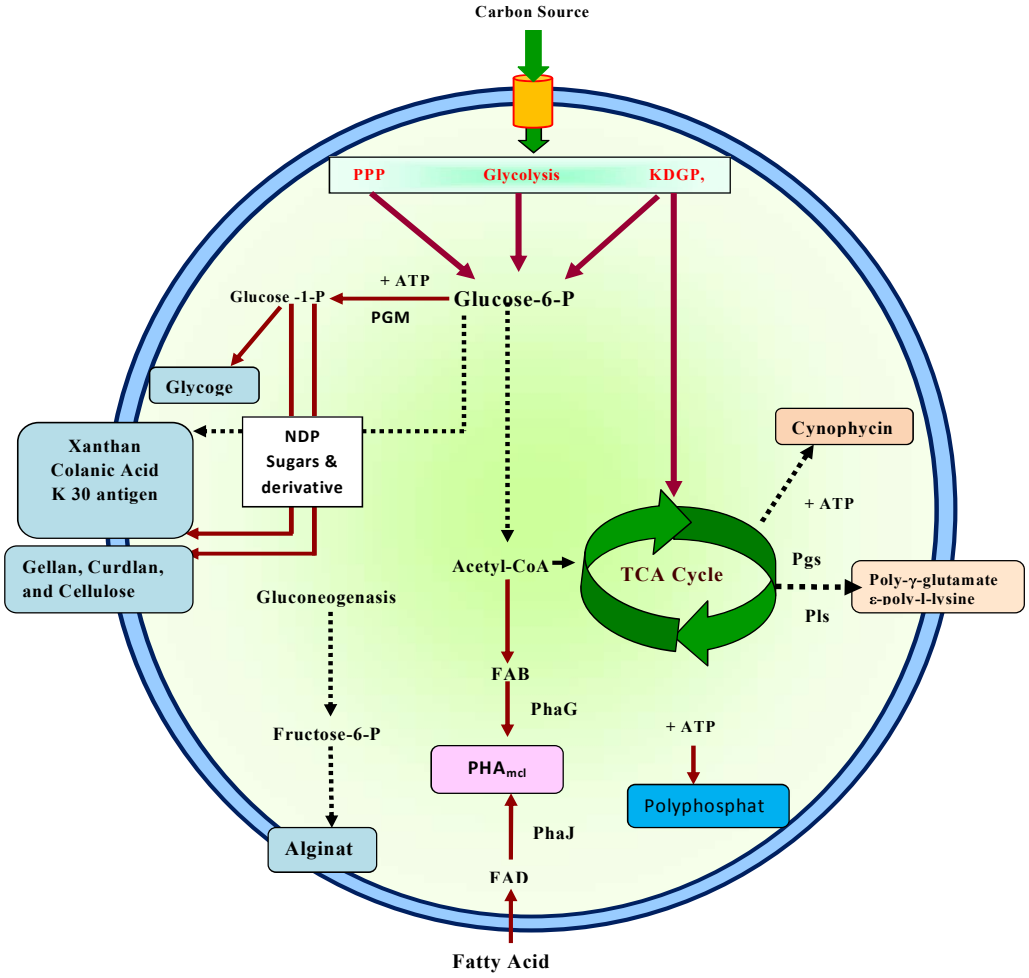

Figure 2.1: Summarized representation of major metabolic pathways for synthesis of the various biopolymers [Verma and Banik; 2013]**.** *Dashed lines represent multiple enzymatic steps. Solid lines indicate either direct enzyme-catalysed conversions or linking of primary metabolic pathways. Abbribiations; FAB, fatty acid de novo biosynthesis; FAD, fatty acid β-oxidation; KDPG, 2-keto-3-deoxy-6-phosphogluconate pathway; NDP, nucleoside 5′-diphosphate; P, phosphate; PGM, phosphoglucomutase; Pgs, Poly-γ-glutamate synthetase; Pls; poly-l-lysine synthetase, Pha, polyhydroxyalkanoate synthesis enzyme; PHAmcl, medium-chain-length PHAs; TCA cycle, tricarboxylic acid cycle.*

On the basis of chemical structure, polymers are classified into four main categories:

a) Polysaccharides
b) Polyamide and Protein:
c) Polyester
d) Polyanhydrides

a) Polysaccharides:

Polysaccharides are long chain complex carbohydrate [$(CH_2O)_n$] molecules composed of monosaccharide [simple sugar] monomer units joined together by glycosidic bonds. Microbial polysaccharides can be subdivided into exopolysaccharides, capsular polysaccharides and intracellular polysaccharide. Nucleoside diphosphate, their acids and derivatives are precursors for the synthesis of microbial polysaccharides.

Table 2.1. Some important polysaccharides and their versatile applications

Polymer	Remark
Alginate	Alginate is used extensively as a biomaterial in prosthetics (such as in dental impression-making material) and for making scaffolds for drug delivery and tissue culture [Lee et al., 2009]. Alginate also applied in food, textile, and cosmetics industries.
Chitosan	Chitosan is commonly used to construct scaffolds for tissue culture and drug delivery system [Muzzarelli, 2010]. Due to its antimicrobial and hemostatic activities it is also used in surgical treatment [Muzzarelli et al., 1999]. It is also used for the construction of contact lenses, antacids and wound dressings [Muzzarelli et al., 1999, Hisamatsu et al., 1982].

Curdlan This is mainly used as Food additive and acts as thickener or a gelling agent. It is also known to prevent various diseases such as diabetes, digestion troubles and bowel disease [Harada and Harada 1996].

Dextran It is used in microsurgery to reduce the risk of vascular thrombosis by altering the electronegativity of the cells and vascular endothelium. In the eye drops dextran is added as a lubricating agent [Angelopoulou et al., 2012].

Glycogen The most common disease due to abnormal glycogen metabolism is diabetes, in which, liver glycogen either accumulated or depleted.

Levan Many drug delivery formulations contain levan as a coating material. Levan also represents anti-tumor activity [Leibovici et al., 1985].

Hyaluronic Acid (HA) Hyaluronic acid is used for extra lubrication to the bone joints through the process of visco-supplementation and used to treat osteoarthritis [Uthman et al., 2003]. In veterinary science it is proved that HA is effective in the treatment of articular disorders in horses. In our body hyaluronic acid has a vital role in wound repair process [Dechert et al., 2006]. It has also been attempted to fabricate scaffolds for tissue culture [Garcia-Fuentes et al., 2009].

Cellulose Cellulose is used in wound dressings and in the preparation of scaffolds for tissue engineering [Czaja et al., 2006, Alvarez et al., 2004].

Pullulan Pullulan and its derivatives such as carboxymethyl pullulan serve as delivery stems for controlled release of drugs [Gheorghe et al., 2008]. Recently two new anti-cancer bioconjugates were synthesized by pullulan derivatisation [Scomparin et al., 2011].

Xanthan It is an efficient laxative and used in anti diarrheal & amebicidic suspensions. Xanthan gum has wide application in various industries *viz.* food industry, cosmetics & in the oil industry [Daly et al., 1993].

Gellan Gum Gellan Gum is an emerging polymer for drug delivery systems. In food industry it is used as gelling agent [Rinaudo et al., 2000], it also serves as a drug delivery vehicle [Miyazaki et al., 1999].

Emulsan It is used for bioemulsification and controlled release of drugs [Castro et al., 2008].

Kefiran It is a water-soluble branched glucogalactan having nearly equal proportions of galactose and glucose residues; isolated from kefir, Kefiran has antimicrobial and healing activity [Rodrigues et al., 2005].

Succinoglycan This exopolysaccharide helps the bacterium during invasion and nodule formation in host plant [Pellock et al., 2000].

Welan gum Welan gum have similar properties as gellan gum, it is used in cement industry as a rheology modifier [Allen et al., 1995].

b) Polyamide and Protein

In polyamides; the amides monomers are joined by amide bonds. In biological systems they are of two types; one is synthesized by ribosomal mediated synthesized proteins and second is non-ribosomally synthesized polyamides. Proteins are the polymers of amino acids and these amino acids are jointed together via peptide bonds. Some of the important proteins used for biomedical applications are silk, gelatin, collagen, keratin etc. There are only three members in the category of non-ribosomally synthesized polyamides; two of them are extracellular polyamides [poly-γ-glutamate [PGA] and ε-poly-l-lysine [Pl] and one of them is intracellular polyamide [cyanophycin granule peptide [CGP]. CGP also used as raw material to prepare some useful derivatives such as poly aspartic acid, Polyglutamic acid and pacitaxal which acts as an anticancer drug [Li et al., 1998].

c) Polyester

Microbial polyesters are produced by microorganisms as carbon and energy reserves. Polyesters and their copolymers produced by micro-organisms have variety of applications as biomaterial; these are used to make fixation devices such as sutures, surgical mesh and orthopaedic pins, designing scaffolds for tissue culture and wound dressings, drug carrier and in tendon repair devices [Chen et al., 2005]. Along with these medical applications these bio polyesters are also used to make bioplastic [Chen et al., 2005].

d) Polyanhydrides

Living cells contains only singe polyanhydrides that is “inorganic polyphosphate *viz.* Poly P”**.** Poly ‘P’ is a widely explored polyanhydride. Researches show that ‘poly P’ can serve as a source of phosphorus for the biosynthesis different biomolecules such as of nucleic acids and phospholipids under phosphorus

starvation conditions. 'Poly P' have important role in tissue remodeling [Kasuyama et al., 2011]. Practical interest in 'poly P' includes many industrial applications.

2.2 Scaffold

Scaffold is a 3D framework which provides the support to any system. In tissue engineering scaffolds are being constructed by various polymers and they are capable of providing the support for the cell growth. It is vital for the scaffold to mimic the in-vivo system for guiding the tissue formation process effectively. An ideal scaffold should have following properties:

- A highly porous matrix with interconnected pores which is capable to provide the support to growing cell and could also allow the division as well as the migration of cells deep inside the scaffold which ultimately lead to formation of new tissue.
- Scaffolds should be able to provide a biocompatible and nontoxic environment to growing tissue.
- Scaffolds should be capable to deliver desired bioactive molecules and drugs at targeted site.
- Scaffolds should have proper shape and size according to need.
- Scaffolds should meet appropriate mechanical and biodegradation properties as per need of application.

2.3 Biopolymers used in fabrication of scaffold

Many synthetic and natural biopolymers have been explored for the various biomedical applications. Several of them have been utilized for the construction of the scaffolds and used as a vehicle for drug delivery and tissue engineering and wound healing. Name of some of major examples of such biopolymers are ***Galactose*** [Hutmacher, 2001], ***Agar*** [Hollister et al., 2000; Chu et al., 2002; Mikos et al., 1994, Marra et al.,1999], **Heparin** [Chung et al., 2007], ***Carrageenans*** [Hutmacher, 2001;

Hollister et al., 2000; Yang et al., 2002; Sachlos and Czernuszka, 2003; Cheah et al., 2004, Hutmacher et al., 2004], ***Cellulose*** [Vimalraj et al., 2016; Lohani et al., 2016], ***Gellan gum*** [Chang et al., 2012; Pacelli et al., 2015; Wang et al., 2008; Shiva et al., 2012; Osmałek et al., 2014], ***Hyaluronic acid*** [Tripodo et al., 2015; Park et al., 2016; Lai et al., 2016; Fan et al., 2015], ***Chondroitin sulphate*** [Lim et al., 2011; Venkatesan et al., 2012; Yu et al., 2013; Ni et al., 2015], ***Collagen*** [Fitzgerald et al., 2015; Li et al., 2016; Joanne et al., 2016; Cao et al., 2015], ***Gelatin*** [Martínez-Vázquez et al., 2015; Kanungo et al., 2015; Aduba et al., 2013; Gentile et al., 2016; Sahoo et al., 2015], ***Silk fibroin*** [Farokhi et al., 2014; Shahverdi et al., 2014; Miroiu et al., 2015; Wang et al., 2016; Werner et al., 2015; Shahbazi et al., 2015; Bhardwaj et al., 2015], ***Chitosan*** [Nazemi et al., 2015; Wers et al., 2015; Yao et al., 2015; Gentile et al., 2016; Mahmoud and Salama 2015; Raftery et al., 2010; Kanimozhi et al., 2016], **Starch** [Balmayor et al., 2009; Subramanian et al., 2014; Duarte et al., 2009; Ghavimi et al., 2015] and **Alginate** [Quinlan et al., 2015; Lee et al., 2011; Wiltsey et al., 2015; Kirdponpattara et al., 2015; Liu et al., 2015; Quraishi et al., 2015; Pan et al., 2016].

But here only those polymers are being discussed in detail which has been utilized in the presented research *viz.* Silk Fibroin, Gellan Gum and Chitosan.

2.3.1. Silk Fibroin (SF)

Silk fibers are spun from various arthropods and insects such as spiders and silkworms. Cocoon silk of *Bombyx mori (B. mori*) is the most investigated among all type of silk categories. *B. mori* produces the silk by its major ampullate glands. In current few decades, silk-based composites have been extensively studied and it has emerged as a strong candidate for various applications, such as biomedical applications, tissue engineering, drug delivery systems, biosensing, textiles and structural applications [Li et al., 2013]. Silk fiber is composed of two proteins viz.

fibroin and sericin proteins. In a silk fiber, the fibroin protein is embedded inside the glue-like sericin protein (Fig 2.2).

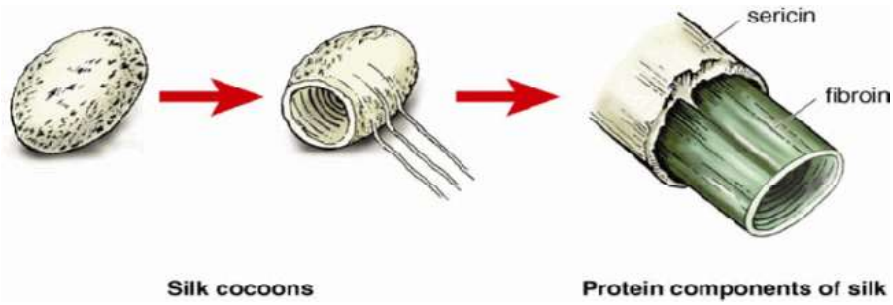

Fig. 2.2: **Two protein components of silk fiber i.e. fibroin and sericin proteins [Fig adapted from: Li et al., 2013].**

Silk fibroin (SF) is effectively being used for the construction of biomedical composite materials [Farokhi et al., 2014; Shahverdi et al., 2014]. SF also had proven to have promising properties for tissue engineering and drug delivery applications [Miroiu et al., 2015; Wang et al., 2016; Werner et al., 2015; Shahbazi et al., 2015; Bhardwaj et al., 2015]. SF is capable to provide the microenvironment for the growth of cell due to its similar structure with the native extracellular matrix (ECM), which is a crucial issue to design an appropriate scaffold for tissue engineering application [Li et al., 2013]. Silk also contains naturally occurring arginine-glycine-aspartic acid (RGD) tripeptide sequence, which have well reported role in cell attachment and in cell migration [Maghdouri-White et al., 2015]. SF contains eighteen amino-acids and repetitive Gly-Ala-Gly-Ala-Gly-Ser (GAGAGS) sequences which give rise to anti-parallel β-sheet structure of SF after self-assembly [Kratky et al., 1950; Heslot 1980]. SF also shows crystalline dimorphism which strongly influences its solubility in water [Carlisle and Bernal 1955]. Various studies has revealed that SF show four different structures viz. silk I, silk II, silk III and random coil [Valluzzi et al., 1996]. Silk I has good water solubility whereas Silks II is insoluble form of SF. Silk I is formed of alternate α-helix and β-sheet while Silk II is rich in β-sheet. Silk I is brittle whereas silk II form has strong mechanical

properties. All the silk forms can be interconverted to each other via regulating the processing environment thus tuning of physicochemical properties of SF is possible which makes it a suitable material for tissue engineering application. SF also proven to have good biocompatibility because, it is nontoxic for normal cells and its degradation products do not exhibit any adverse effects on the organisms [Li et al., 2013]. SF is a biodegradable biopolymer and its biodegradability is determined by its secondary structure which can be tuned during the preparation of regenerated silk materials [Horan et al., 2005]. SF had already been investigated for drug delivery vehicle and for the fabrication of scaffolds for various biomedical applications [Wang et al., 2016; Werner et al., 2015; Shahbazi et al., 2015; Bhardwaj et al., 2015]. From the above literature it is clear that SF can fulfill the different requirement of native living tissue therefore selected for the scaffold construction in present study.

2.3.2 Gellan Gum (GG)

Gellan gum (GG) is a linear anionic exopolysaccharide composed of repeating units of α-L-rhamnose, β-D-glucose and β-D-glucuronate, in 1:2:1 molar ratio (Fig. 1) [Milas et al., 1990].

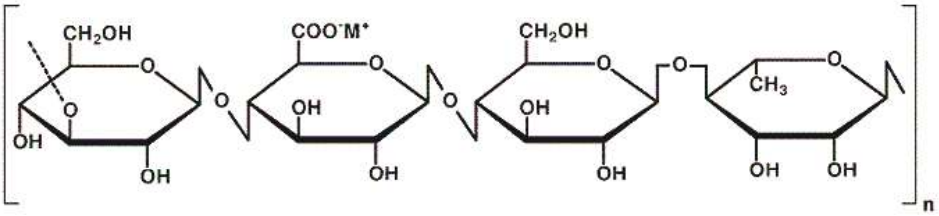

Fig. 2.3 Structure of gellan gum

It is produced by the bacteria *Sphingomonas elodea* (formerly known as *Pseudomonas elodea*) [Bajaj et al., 2007]. On the basis of presence and absence of acyl substituent, GG may be acetylated or deacetylated. GG also posses temperature dependent hydrogel forming characteristic in presence bivalent or trivalent cation.

On heating, GG forms a clear solution in water and on subsequent cooling the aqueous solution of GG undergoes conformation coil-to-helix transition and forms hydrogel. Freeze drying of GG hydrogel leads to formation of scaffolds [Osmałek et al., 2014]. GG has emerged as an excellent and effective carrier for delivery of different drugs [Vijan et al., 2012; Kesavan et al., 2010; Franklin-Ude et al., 2007]. GG has also been reported to be useful in versatile biomedical applications such as in the field of tissue culture, wound healing, surgery, angiogenesis and arthritis treatment [Miyamoto et al., 2010; Oliveira et al., 2010; Lee et al., 2010; Lee et al., 2012]. On the basis of these attractive characteristics GG was also shortlisted for the current study.

2.3.3 Chitosan (CS)

Chitosan (CS) is a natural linear exopolysaccharide mainly produced by deacetylation of chitin under alkaline conditions by enzymatic hydrolysis in presence of a chitin deacetylase enzyme. Microbial production of CS has been reported. *Cunninghamella elegans, Mucor racemosus* and*, Aspergillus Niger* are good producers of CS*.* CS is composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine subunits (Fig. 2.4).

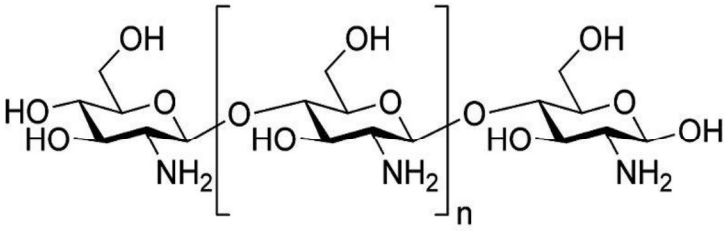

Fig. 2.4 Structure of chitosan

It is the second most abundant polymer after cellulose. Biocompatiblity, biodegradablity, non toxicity, film and scaffold forming ability, antimicrobial activity, haemocompatibility and renewability are some attractive properties of CS

which makes it a suitable biomaterial for various biomedical applications [Rinaudo et al., 2006]. CS has been widely explored as a suitable material for drug delivery, scaffold construction and tissue engineering [Nazemi et al., 2015; Yao et al., 2015; Subramanian et al., 2014; Barroso et al., 2014; Wang et al., 2015; Yao et al., 2013]. CS also used in surgical treatment, construction of contact lenses and also shows hypercholesterolemic, antacids and anticancer activities along with wound healing properties [Muzzarelli et al., 2014]. Beside pharmaceutical and medical applications CS also used as a water purifier, fungicide, insecticide, seed coating agent and for soil amendment [Kumar, 2000]. In the light of above mentioned properties CS was also selected for scaffold fabrication.

2.4 Techniques used for scaffold fabrication

Various methodologies has been developed for scaffold fabrication, some of them are [Subia et al., 2010]:

- Particulate Leaching Method
- Freeze-Drying Method
- Electrospinning Technology
- Gas Foaming Method
- Self assembly
- Phase Separation Technique
- Rapid Prototyping Technology
- Fiber Bonding Technology
- Fiber Mesh Technique
- Membrane Lamination
- Melt Molding Technique

All the above mentioned methods have their own merits and demerits; some of them are being enlisted here in Table 2.2.

Table 2.2: Summarized merits and demerits of some scaffold fabrication techniques

Technique	Merits	Demerits
Particulate Leaching Method [Xiang et al., 2006]	Control over Porosity, pore size and crystallinity	Poor mechanical property, residual solvents & porogen material
Freeze drying [Boland et al., 2004]	High temperature and separate leaching step not required	Long processing time
Electrospinning [Liang et al., 2007]	Control over porosity, pore size and fiber diameter	Limited mechanical property, pore size decrease with fiber thickness
Gas foaming [Ikada., 2006]	Free of harsh organic solvents, control over porosity and pore size	Limited mechanical property, inadequate pore interconnectivity
Self assembly [Zhang et al., 2006]	Control over porosity, pore size and fiber diameter	Expensive material, complex design parameters
Phase separation [Smith et al., 2006]	No decrease in the activity of the molecule	Difficult to precisely control the scaffold morphology
Rapid prototyping [Hutmacher et al., 2001]	Excellent control over geometry, porosity, no supporting material required	Limited polymer type, highly expensive equipment
Fiber mesh [Chen et al., 2002]	Large surface area for cell attachment, rapid	Lack the structural stability

	nutrient diffusion	
Fiber bonding [Mooney et al., 1996]	High surface to volume ratio, high porosity	Poor mechanical property, limited applications to other polymers
Membrane Lamination [Maquet & Jerome, 1997]	Provide 3D matrix	Lack required mechanical strength,
Melt molding [Thompson et al., 1995]	Independent control over porosity and pore size	Required high temperature for non amorphous polymer

Among all the techniques mentioned in the above table; three of them are most popular techniques in the area of tissue culture and biomedical engineering *viz.* particulate leaching methods, freeze-drying method and electrospinning technology.

2.4.1 Particulate Leaching Method

In particulate leaching method the scaffolds are made with the help of porogen. A porogen is any granulated inactive substrate which is used to create pores inside the scaffold. Particulate leaching methodology is of two types: (1) solvent casting–particulate leaching and (2) melt molding–particulate leaching method. In first approach, a polymer solution is mixed with porogen salt particles and casted in to moulds. After casting; the solvent is evaporated to make a polymer monolith having embedded salt particles. Embedded salt particles are then removed from the monolith by washing the monolith with water which results in to the formation of a porous scaffold. In second approach the polymer porogen mixture is cast into a mold and then the polymer is set down by applying heat and pressure. Settled porogen is leached away by washing with water to yield a porous scaffold. The solvent casting method is simple, reproducible and does not require

sophisticated apparatus. The pore size in the scaffold can be controlled by controlling the amount and the size of porogen added. The major drawback associated with this technique is that it needs long period of soaking in water to leach out all of the porogen particles. The long leaching period may lead to leaching of loaded drugs resulting in to payload very poor loading efficiency. Moreover certain critical characteristics of scaffold such as pore shape and inter-pore connectivity are not controlled and it can only produce thin wafers or membrane up to 3 mm thick [Moore et al., 2004]. To conquer these shortcomings new technologies have been developed.

2.4.2 Electrospinning technology

The electrospinning technique is a sophisticated technique based on utilization of the electrostatic force, generated through high intensity electric field between two electrodes, for the production of thin polymeric fibers. In this technique polymer solution is pumped through a syringe pump in a high electric field, which intends to overcome the surface tension of the solution. As a result, jet of polymer is ejected from the syringe pump and produces the fibers. In meanwhile, the solvent starts to evaporate and nanofibers are deposited to the collector. Overlapped deposition of these polymeric fibers leads to scaffold fabrication. More than 200 polymers have been used for electrospinning like collagen, silk fibroin, chitosan, gelatin etc [Mathews et al., 2002; Zarkoob et al., 2004; Jin et al., 2004; Ohkawa et al., 2004; Ma et al., 2005]. Although this technique offers many advantages over other techniques but sophistication, requirement of technical expertise and cell seeding problem are the some main problems associated with electrospining technology [Baker et al., 2008; Leong et al., 2008].

2.4.3 Freeze drying technique

Freeze drying technique is also very popular for the fabrication of porous scaffolds [Schoof et al., 2001]. This technique is based on the principle of sublimation. Polymer is first dissolved in a suitable solvent obtain a solution of desired concentration. Prepared solution is casted into the moulds and deep frozen at low temperature. After freezing, the solvent is removed by lyophilization under the high vacuum in order to fabricate the scaffold with high porosity [Mandal & Kundu, 2009]. This technique have been applied over a variety of polymers including silk proteins, gellan gum and chitosan [Vepari & Kaplan, 2007, Chang et al., 2011; Yao et al., 2013]. The tuning of pore size is possible by controlling the pH and freezing rate. Faster freezing rate produces smaller pores. Controlled solidification in a single direction can be used to create a homogenous 3D-pore structure [Schoof et al., 2001]. Main advantage of this technique is that it neither requires high temperature treatment nor it requires a separate leaching step like particulate leaching technique. Moreover this technique is simple and requires less technical expertise like electrospining technology. Due to these reasons this technique was chosen for the fabrication of scaffolds in the current study.

2.5 Wound and its treatment

2.5.1 Wound?

Wound is defined as an injury to the body that typically involves laceration or breaking of a membrane (as the skin) and usually damage to underlying tissues.

2.5.1 Wound healing

Wound healing is one of the most complex biological processes in which multiple biological pathways are synchronized to restore the tissue integrity and homeostasis of the injured tissue. During healing of the wound, many types of cells such as endothelial cells, keratinocytes and fibroblasts undergo marked changes in

gene expression and phenotype, leading to cell proliferation, differentiation and migration [Singer et al., 1999; Aarabi et al., 2007]. Normal wound healing without any medicinal supplement is also known as classic wound healing. There are three main stages of classic wound repair [Gurtner et al., 2008].

***Stage 1. Inflammation**:* this stage is characterized by following events:

- Inflammation starts from injury and persists until 48 h after injury in normal case.
- At the wound site; the surrounding native environment becomes hypoxic (ischaemic).
- A fibrin clot gets formed in this ischaemic environment at wound site.
- At this stage Bacterial load is very high at the wound site.

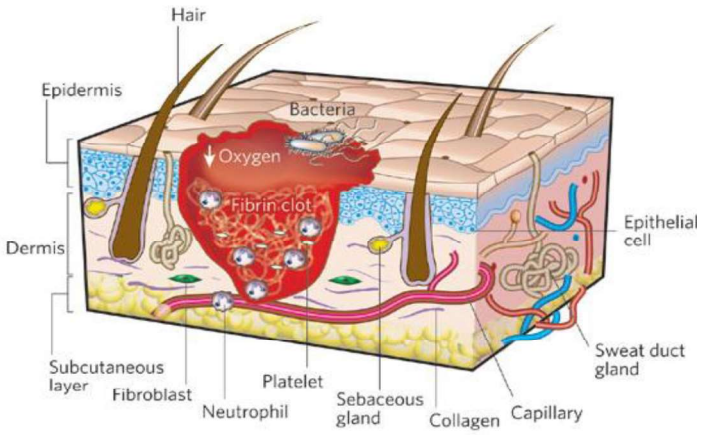

Fig. 2.5 Inflammation; the Ist stage of wound healing
[Fig. adapted from Gurtner et al., 2008]

Stage 2. New tissue formation: This stage starts about 2–10 days after injury. This stage is characterized proliferation and migration of different types of cells

- The first event is the migration of keratinocytes at the site of injury.
- New blood vessels are then formed inside injured tissue.

- Fibroblasts and macrophages comes out from growing capillaries and replace the fibrin matrix with granulation tissue at wound site and generate a new substrate for keratinocyte migration.
- The keratinocytes which are behind the leading edge of developing capillaries proliferate and mature to restore the barrier function of the injured epithelium.
- Finally in the later part of this stage, fibroblasts are stimulated by macrophages, and some of them get differentiated into myofibroblasts. Fibroblasts and myofibroblasts together produce extracellular matrix (ECF), mainly in the form of collagen, which ultimately forms the scar on the surface of the wound.

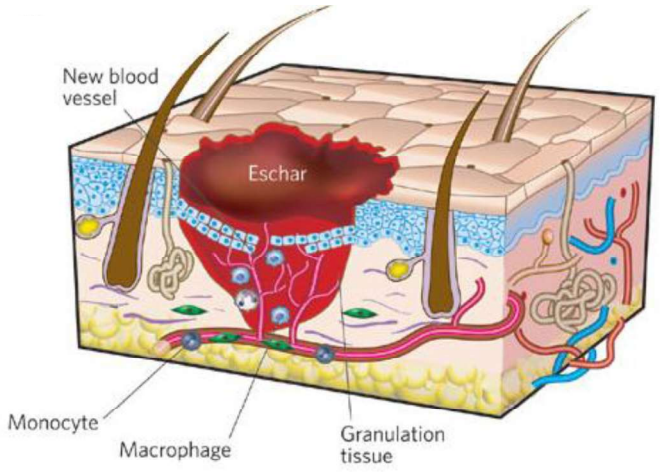

Fig. 2.6 New tissue formation; the IInd stage of wound healing
[Fig. adapted from Gurtner et al., 2008]

***Stage 3. Remodelling*:** This is the longest stage lasts for a year or longer. During this prolonged stage the disorganized collagen fibbers has been laid down by fibroblasts and ECM regains its original native architecture but healed region does not contain normal skin appendages.

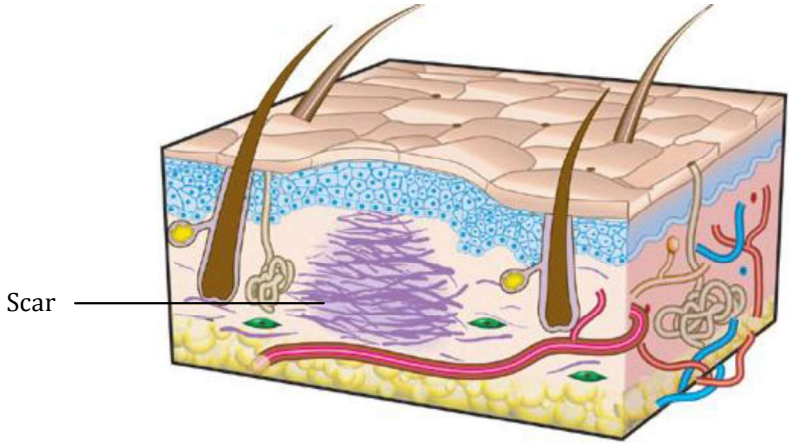

Fig. 2.7 Remodelling; the IIIrd stage of wound healing
[Fig. adapted from Gurtner et al., 2008]

2.5.3 Factors affecting wound healing

Wound healing is the result of complex cascade of reactions and interactions among blood, cytokines, growth factors and ECM. The cytokines controls various healing pathways such as increasing inflammation, stimulating the production of new ECM, preventing dehydration and the formation of granulation tissue. Two types of factors are mainly responsible for controlling these pathways *viz*. local and systemic factors [Finn et al., 2006]. Local factors includes pain, infection, hypothermia, radiation and tissue oxygen tension whereas systemic factors are the overall health of the individual that affect individual's ability to heal [Guo and Dipietro 2010]. In addition to these factors, poor nutrition, protein, vitamins and mineral deficiency as well as age of an individual also prolongs the healing period. For wound healing various types of dressings are being used from very past of time some of them are being discussed here.

2.5.4 Conventional wound dressings

Various types of synthetic wound dressings are available in the market. Practically, no single dressing is perfect for the treatment of all types of wounds because of variable requirements for wound healing environment. Many types of

dressings are being used during the treatment of a single wound. An ideal wound dressing should contain the following properties:

- Able to provide bacterial protection
- Able to maintain the moist environment at the wound interface
- Provide mechanical protection and thermal insulation
- Allow gaseous and fluid exchange
- Capable to absorb excess exudates from wound
- Should be non-adherent to the wound and easily removable without trauma
- Should be non-toxic, non-allergenic and non-sensitising

Wound dressings can be classified in to three main categories as enlisted in *Table 2.3*; whereas *Fig 2.8* shows some commercialized wound dressings available in the market for the treatment of the wounds.

Table 2.3. Classification of wound dressings [Zahedi et al., 2010].

Dressing	Description	Product Names
Passive products	These are traditional dressings that provide cover over the wound surface.	*Gauze & Tulle dressings*
Interactive products	These are polymeric films; mostly transparent, permeable to water vapour and oxygen. The films are non-permeable to bacteria	*Semipermiable films* *Hydrogels &* *Foam dressings*
Bioactive products	These dressings are able to deliver therapeutic substances at the site of wound and promote the active wound healing	*Hydrocolloids, Alginate, Collagens & Chitosan patches, Hydrofibers*

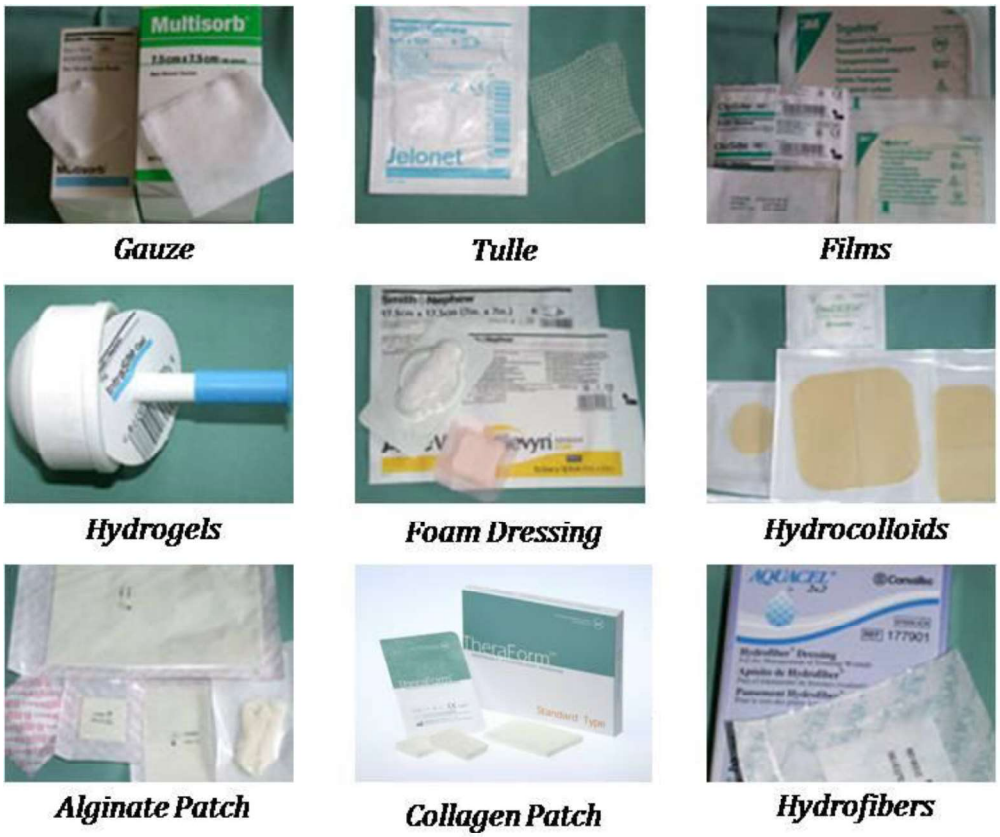

Fig. 2.8 Some commercial wound dressing products

2.5.5 Regenerative medicinal therapy: a modern approach for wound healing

In current scenario, regenerative medicinal therapy is in lime light of research interest. It includes the altered wound repair mechanism to achieve faster and better wound healing without scar formation. For this purpose; scientists have followed many innovative approaches including the use of biomimetic scaffolds, tissue grafting, manipulation of the mechanical and electrical environment of native tissue, use of gene-therapy approaches and the use of stem cell-based strategies (Fig. 2.9) [Gurtner et al., 2008, Lutolf et al., 2005]. Advances in material science have made all these approaches feasible. During vast research, drug loaded scaffolds have been proven as an invincible tool in the sector of regenerative medicinal therapy.

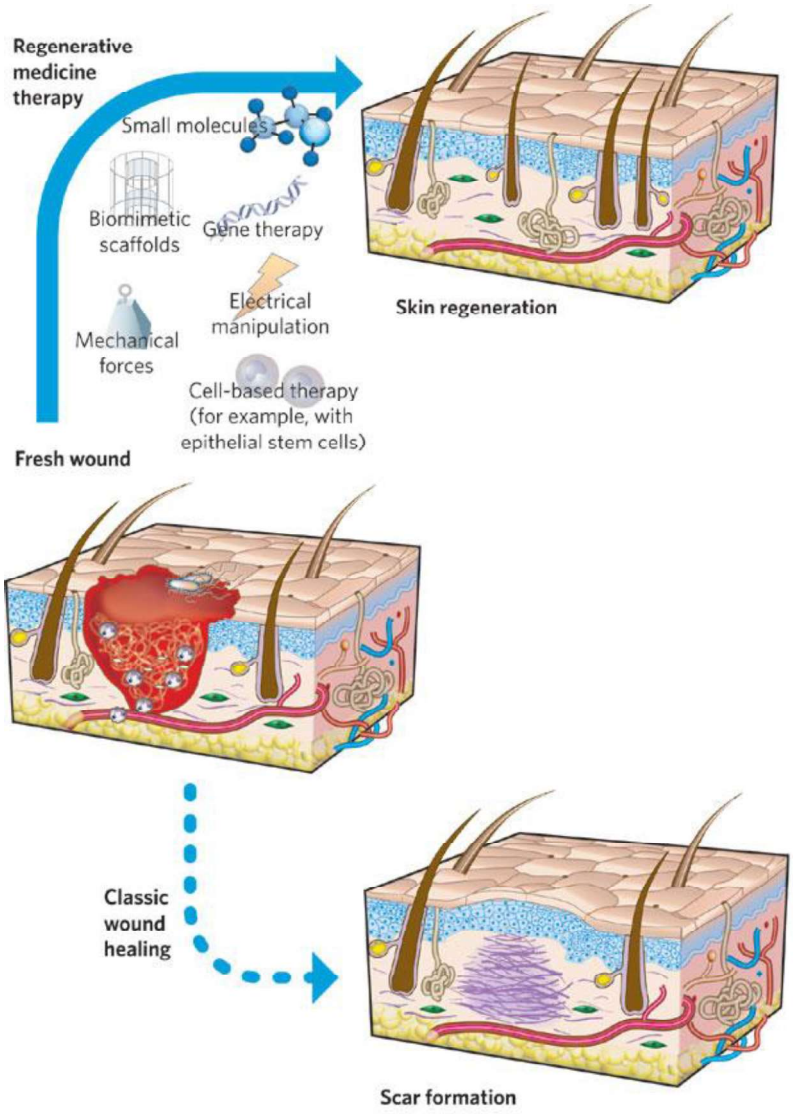

Fig. 2.9 Regenerative medicinal therapy for advanced wound healing without scar formation. Figure also shows the comparative difference of healed tissue in case of regenerative medicinal therapy and through classical wound healing ***[Fig. adapted from Gurtner et al., 2008]***

2.5.6 Drug (antibiotic) loaded scaffolds as a tool of regenerative medicinal therapy for wound healing

As previously described; that scaffolds are capable of providing the support to the growing cells at the wound site. Moreover, it has also been proven from various studies that loading of antimicrobial drugs, growth factors or cells enhances the wound healing capability of scaffolds [Mahmoud et al., 2016; Gainza et al., 2015; Ahman et al., 2009]. Among these; the antimicrobial drug loaded scaffolds have

gained special attention of researchers because bacterial infection at the wound site is a major problem for the wound healing cascade [Howell-Jones et al., 2005]. *S. aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa, Enterobacter cloacae, Klebsiella species, Streptococcus species* are some common bacteria responsible for wound infection [Howell-Jones et al., 2005]. Bacterial infection delays wound healing and make the wound chronic. Thus it is very critical to eliminate the bacterial load from wound vicinity. Thus, the early use of antibiotics for the treatment of wound is generally advocated (Jeffcoate 1999). Some of suggested antibiotic for this purpose includes ampicillin, co-amoxiclav, second or third generation cephalosporin and a quinolone, and metronidazole with a quinolone [Howell-Jones et al., 2005]. Utilization of topical antibacterials (e.g. silver sulfadiazine, metronidazole and fusidic acid) and topical antiseptics (such as chlorhexidine, sodium chloride and povidoneiodine) is in common practice [Dow et al., 1999; O'Meara et al., 2000]. Till the date many polymeric scaffolds loaded with various types of bioactive drugs have been investigated for their potency to heal the wounds [Zilberman et al., 2015; Whelan et al., 2014; Laçin et al., 2014; Lan et al., 2014; Losi et al., 2013; Catanzano et al., 2015; Ninan et al., 2015; Egozi et al., 2015; Phaechamud et al., 2016; Namazi et al., 2016]. On the basis of literature we have shortlisted two antimicrobial agents for loading into the scaffolds in current study *viz.* Ciprofloxacin hydrochloride (*cpr*) and Silver nanoparticles (AgNPs). Here some details regarding these two antimicrobials are also been given for better clarity and understating about these two drugs.

a) Ciprofloxacin hydrochloride (cpr)

Ciprofloxacin hydrochloride *cpr* is one of the most commonly used second-generation fluroquinolone broad spectrum antibiotic. Chemically *cpr* is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic

acid having empirical formula $C_{17}H_{18}FN_3O_3$ with molecular weight of 331.4 g/mol. Structural formula of cpr is given in Fig 2.9. *cpr* is a faintly yellowish to light yellow crystalline substance which is soluble in water. It is effective against variety of Gram positive and Gram negative bacterial pathogens. *Cpr* is being used to treat a number of bacterial infections. This includes skin infections, bone and joint infections, intra abdominal infections, diarrhea, respiratory tract and urinary tract infections [Appelbaum and Hunter 2000]. It kills the bacteria by inhibiting DNA gyrase and topoisomerase IV enzymes which are necessary for DNA replication thereby inhibiting the bacterial cell division [Drlica et al., 1997]. It is available as a very less expensive generic medication with wholesale cost between 0.03 to 0.13 USD per dose.

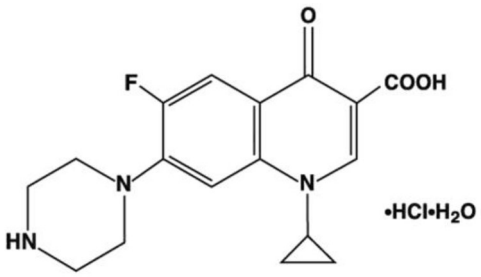

Fig. 2.10 Chemical structure of Ciprofloxacin hydrochloride (*cpr*)

b) *Silver nanoparticles* (AgNPs)

AgNPs are nanoparticles of silver with size between 1 nm to 100 nm. AgNPs are composed of zerovalent silver Ag^0 and generated through the reduction of Ag^+ ions. AgNPs are being used in various fields such as; wound dressings, antibacterial formulations, surgical purpose, bond prostheses, biosensing and water treatment [Dubas et al., 2008; Filippo et al., 2010; Raju et al., 2012; Xu et al., 2008]. AgNPs have unique characteristics like antimicrobial activity, electrical conductivity, catalytic power, chemical stability and sensing ability [Ahmad et al., 2010; Huang et al., 2011; Sharma et al., 2009]. Although AgNPs have greatly been investigated for their

potential in wound healing but very few reports are there for AgNPs containing scaffolds for wound healing [Wen et al., 2015].

2.6 Plan of work

From discussed literature it is clear that there is a need to explore as well as to design the new and better systems for enhanced wound healing. Thus the aim was set to develop a 3D biopolymeric wound dressing (scaffold) with impregnated antimicrobial agent/s, which could be able to deliver these loaded antimicrobials at the wound site in sustained manner up to desirable period of time. Moreover, it was also targeted to ensure the better porosity, swelling, biodegradability, surface roughness, haematocompatibility and biocompatibility of the scaffold so that it could be able to provide the support to the new growing tissue at the wound site. Furthermore during designing of scaffold this fact was kept in mind that blending of two polymers together, specially one protein and one carbohydrate, better bio-mimics the extracellular matrix [Alberts et al., 1994; Bhardwaj et al., 2011; Teimouria et al., 2015; Wenk et al., 2011].

To achieve targets the work was planed as follows:

1. Selection of suitable polymers on the basis of literature and to utilize them for the fabrication of drug loaded 3D scaffolds.
2. Characterization of prepared scaffold
    i. Measurement of scaffold porosity
    ii. Determination of in-vitro enzymatic degradation
    iii. Swelling behavior study
    iv. Measurement of mechanical properties
    v. Study of ultra-structure through Scanning Electron Microscopic (SEM) analysis
    vi. Energy Dispersive X-Ray Spectroscopy (EDX) analysis
    vii. Fourier Transform Infrared Spectroscopic (FTIR) analysis

viii. X- Ray Diffraction (XRD) analysis

ix. Atomic Force Microscopy (AFM) analysis etc.

3. Evaluation of prepared scaffold for its drug loading and release properties.
4. Drug release kinetic studies and prediction of drug release mechanism through following mathematical models:
   i. Zero order mathematical model,
   ii. First order mathematical model,
   iii. Higuchi models
   iv. Korsmeyer-Peppas models
5. Evaluation of antimicrobial potency of scaffold by cell viability test
6. Modifications (*if required*) in the scaffold architecture, polymeric composition and loaded antimicrobial agents for better results.
7. Re-characterization of modified scaffold/s through above mentioned techniques and studies.
8. Preparation of an adhesive wound dressing using the finally developed suitable scaffold.
9. Evaluation of prepared wound dressing for its effect on wound healing in *invivo* system.