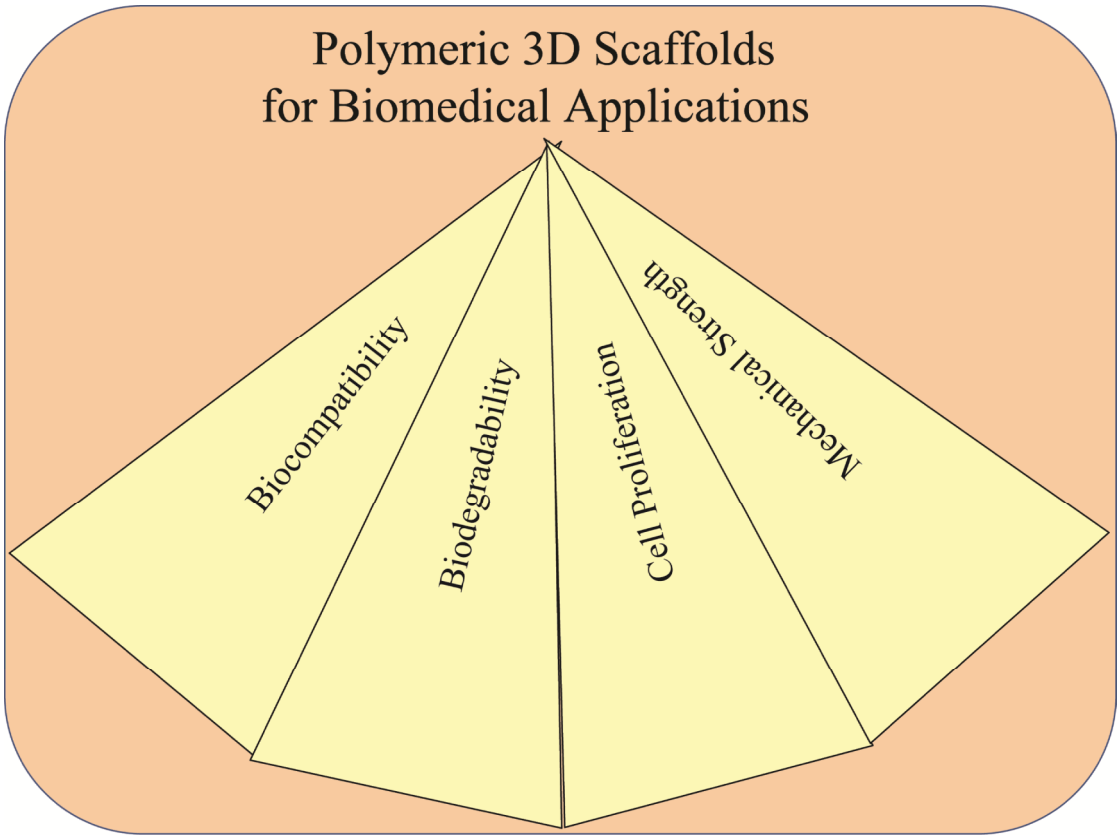
1. INTRODUCTION



This chapter briefly explains about the various parameters required for a three dimensional (3D) scaffold to be used for biomedical application. Further it also exemplifies the characteristics of synthetic polymer poly(Lactic acid) (PLA) and natural polymer gelatin and emphasizes the current challenges of 3D scaffolds in tissue engineering applications.

1.1. Biomaterials

The definition of biomaterials has been radically changed in the last five decades as the discovery in the field has been continuously evolving. In general, a biomaterial can be defined as any material used to make devices to replace a part or a function of the body in a safe, reliable, economic, and physiologically acceptable manner[1]. The rising interest on biomaterials research allowed multiple biomaterials related products entry to the market and indeed that have improved the quality of life for uncountable number of people. A variety of biomaterials based devices are used in the treatment of disease or injury such as sutures, needles, bone plates, hip prosthesis, contact lenses, heart valve, arterial prosthesis, dental implant, skin etc.

1.1.1. Biomaterials for Tissue Engineering

In recent days, there is a large demand for tissue engineered products which is to replace the damaged part of tissue occurred by traumatic injuries or diseases. Various surgical processes have been developed to replace the damaged tissue using xenografts, allografts or autografts. Xenografts are highly available but there is a risk of infection and immunological responses that could lead to the rejection of the graft from human body; the lack of human donors and the risk of immune response limits the allografts. On the other hand, autografts from the patients other part of tissue avoids all the immunological based rejection but it requires long period for the explanted tissue site to heal and to come to the normal stage.

Biomaterials based cell therapy could be an alternative to all the above strategies because the limitations can be avoided in this new strategy. They play central roles in modern tissue engineering and regenerative medicine as the biomaterial could be

designed to direct cellular behavior and function in a controllable manner. Biomaterials have been used as carriers to deliver transplanted cells or matrices to induce morphogenesis in bioengineered tissues constructed *ex vivo* in cell therapy approaches; and in acellular therapies, materials induce ingrowth and differentiation of cells from healthy residual tissues *in situ*. Hence, the direction provided by biomaterials may help for restoration of structure and function of damaged or dysfunctional tissues. One of the critical parameter includes three dimensional support to interact with cells to control their function, guiding the complex multicellular processes of tissue formation and regeneration spatially and temporally. Therefore, biomaterial based tissue regeneration requires three components. They are scaffolds, cells and biochemical signals. Scaffolds should exhibit the interconnective pores and required physicochemical and mechanical properties in order to trigger a cascade of cellular events leading to the regeneration of damaged tissue. Cells are the source for the targeted tissue to be regenerated and the biomolecules are grow factors, hormones, and small molecules required to do the proper cellular function [2]. The scaffold design and development is a major challenge in biomaterial based cell therapy approach.

1.1.2. Three dimensional (3D) scaffolds for tissue engineering

The extracellular matrix (ECM) of a natural tissue is 3D structured, therefore the material to be used as a scaffold should mimic the 3D structure. 3D porous scaffolds can be fabricated by the following techniques.

1.1.2.1. Solvent casting and particulate leaching

Solvent casting is one of the simple and largely used conventional techniques to produce 3D scaffolds. It involves the dissolution of the polymer along with a porogen

agent (salt, sugar or nano/micro particle) in an organic solvent and casting into a predesigned mold. After the evaporation of the solvent, the porogen is leached out and leaving the pores behind. The profound drawback of this technique is formation of interconnectivity between adjacent pores is very low [3].

1.1.2.2. Freeze Drying

Freeze drying process involves the drying of polymeric solution prepared in a mold at a frozen condition. Thermally-induced phase separation (TIPS) is one of the principle technique involving freeze drying, which is a five step process involving polymer dissolution, phase separation and gelation, solvent extraction, freezing, and freeze drying. TIPS takes advantage of the thermodynamic instability of polymer solutions at certain temperatures [4].

1.1.2.3. Electrospinning

Electrospinning was first developed in the early 1900s for textile applications. Later it has been used for tissue engineering applications since Doshi et al. explored it [5]. It is a versatile technique to fabricate fibrous mats of biodegradable polymers. The process uses electric field to overcome the surface tension of a polymer solution and to shoot a jet of liquid out from the needle towards a conducting collector [6]. Under the right conditions polymer fiber is formed with a diameter that can range from tens of nanometers to microns. The critical parameters affecting this process include polymer properties, solvent properties, solution flow rate, voltage, distance from needle to collector, and polymer concentration [7].

1.1.3. Materials for Biomedical applications

A great number of polymers, ceramics and metal composites have been used for biomedical applications such as drug delivery and tissue engineering. Polymers are widely used since we can tailor the physicochemical properties easily.

1.1.4. Biodegradable polymer

There are two kinds of biodegradable polymers,

1. Naturally derived polymers that include polysaccharides (chitosan, chondroitin sulfate, starch, alginate, hyaluronic acid, cellulose) and proteins (collagen, gelatin, xanthan gum, soy, fibrin gels, silk).

2. Synthetic polymers. Synthetic polymers are the largest family of biodegradable polymers. They offer various advantages such as controlled conditions during the synthesis which leads to produce reproducible physicochemical and mechanical properties of polymers.

Polyesters, Polyamides, Polycarbonates and polyurathene are the widely used synthetic polymers for the biomedical applications. Among them, hydrophobic aliphatic poly(lactide) (PLA), poly(caprolactone) (PCL), and poly(glycolide) (PGA) are important polymers. These are biocompatible; biodegradable by the various enzymes in the biological system under physiological conditions; low immunogenicity and good mechanical properties. Importantly, they are FDA-approved for their clinical use. These unique properties have facilitated their use in biomedical applications as sutures, implants, drug delivery carriers and tissue engineering scaffolds [8, 9]. In case of PLA, lactic acid is constituting the chains of PLA which is a byproduct of glycolysis pathway

in humans and mammals [10]. Therefore, PLA is a promising material and we are more interested in PLA based synthetic polymers than other polyesters.

1.1.4.1. Poly(lactic acid) (PLA)

The chemistry of PLA is based on processing and polymerization of lactic acid monomer. Lactic acid (2-hydroxypropionic acid) is a chiral molecule that exists as two enantiomers, L- and D-lactic acid (**Figure 1.1**). The L isomer rotates clockwise and the D isomer rotates anticlockwise on the plane of polarized light. The L or meso form is an equimolar (racemic) mixture of D(−) and L(+) isomers and they are optically inactive [11].

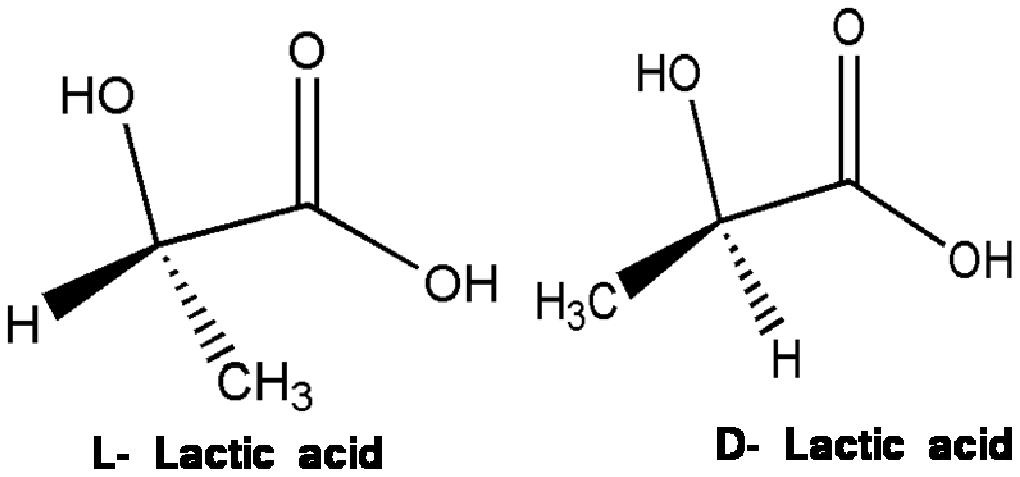


Figure 1.1. Isomers of Lactide

High molecular weight of PLA can be synthesized in two ways (**Figure 1.2**)

1. Direct condensation—which involves solvents under high vacuum.
2. Formation of the cyclic dimer intermediate (lactide)—which is solvent free

Direct condensation method was initially proposed by Carothers, where the reaction was conducted at high vacuum and high temperatures for the removal of water produced during the condensation reaction [12, 13]. The resultant polymer molecular weight was observed as low to intermediate molecular weight [14] due to some common side-reactions (such as cyclization, incomplete reaction, the presence of water etc.) and the polymer was tend to be brittle, which limited their usage in industry. Further, it was found that a high conversion is required to obtain a high degree of polymerization to obtain higher molecular weight PLA.

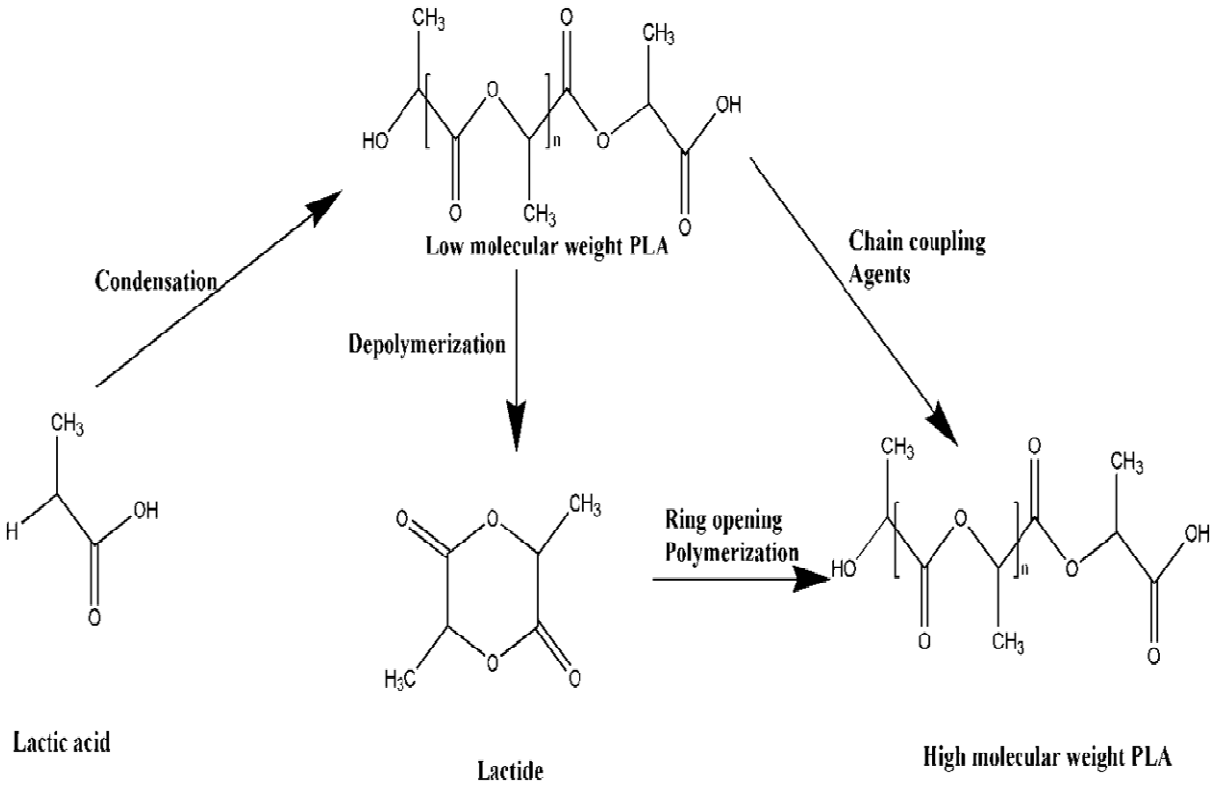


Figure 1.2. Common routes to synthesis high molecular weight PLA

The PLA with controlled molecular weight can be achieved by ring-opening polymerization (ROP), which is a solvent-free reaction. In addition, it is also possible to control the ratio and sequence of D- and L-lactic acid units by controlling residence

time and temperature in combination with catalyst type and concentration [14-16]. ROP of lactides was first explained by Carothers in 1932, but he only obtained low molecular weight PLA due to impurities [17] and in 1954, Dupont improved the lactide purification techniques to obtain high molecular weight PLA [18]. Thus, ROP proceeding via a coordination– insertion pathway is efficient in terms of producing high molecular weight PLA [19, 20] (**Figure 1.3**). Another advantage of the coordination–

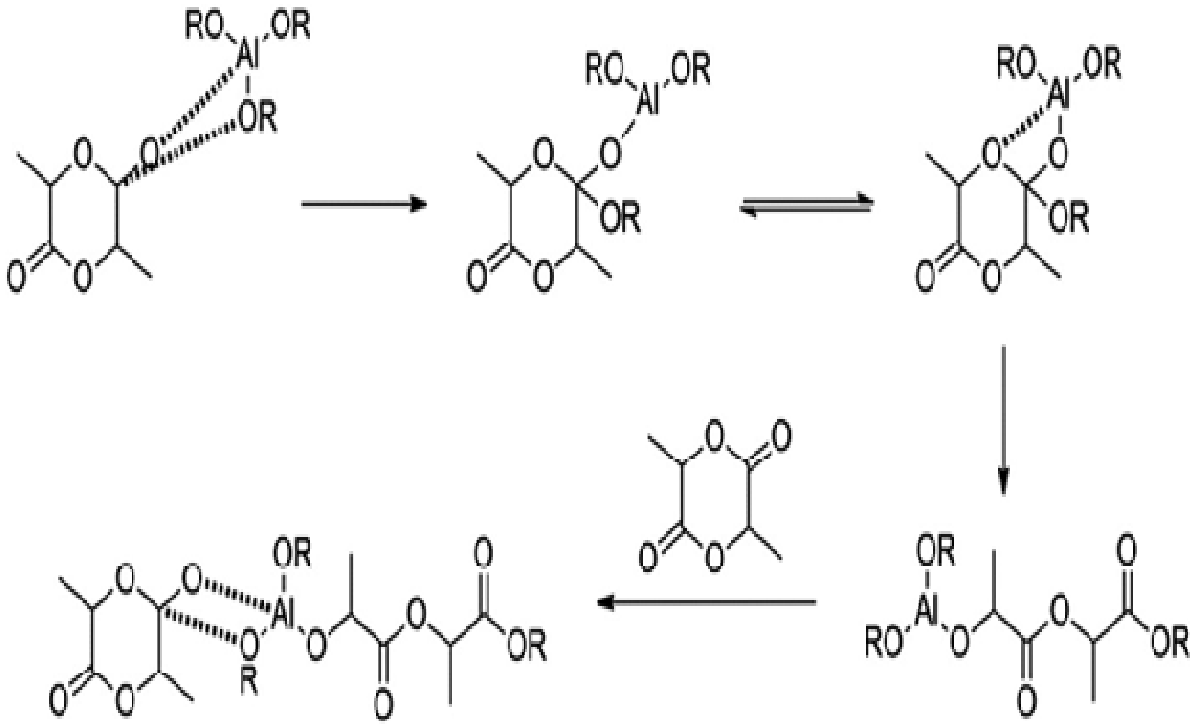


Figure 1.3. Coordination-Insertion mechanism using Aluminium oxide [10])

insertion pathway is that it can be performed in bulk (whilst anionic and cationic ROP often require solvents). Many catalysts have been screened and heavy metal based catalysts such as Tin, zinc, aluminium were found to be best to produce high molecular weight PLA, [19] which showed more than 90% conversion and less than 1%

racemisation. Tin octoate (tin(II) 2-ethylhexanoate) is the catalyst which is mostly used in industry because of its greater stability and lower toxicity [21].

1.1.4.2. Star poly(lactic acid)

Star polymers are the branched, multi-armed polymeric materials where the branches extend from its core. Inter and multiple disciplinary scientists have been showing their constant interest on star polymers because to obtain interesting rheological and mechanical properties, such as increase of the polymer strength. Star PLA can be categorized into discrete, polymeric and dendritic/hyperbranched cores. These star

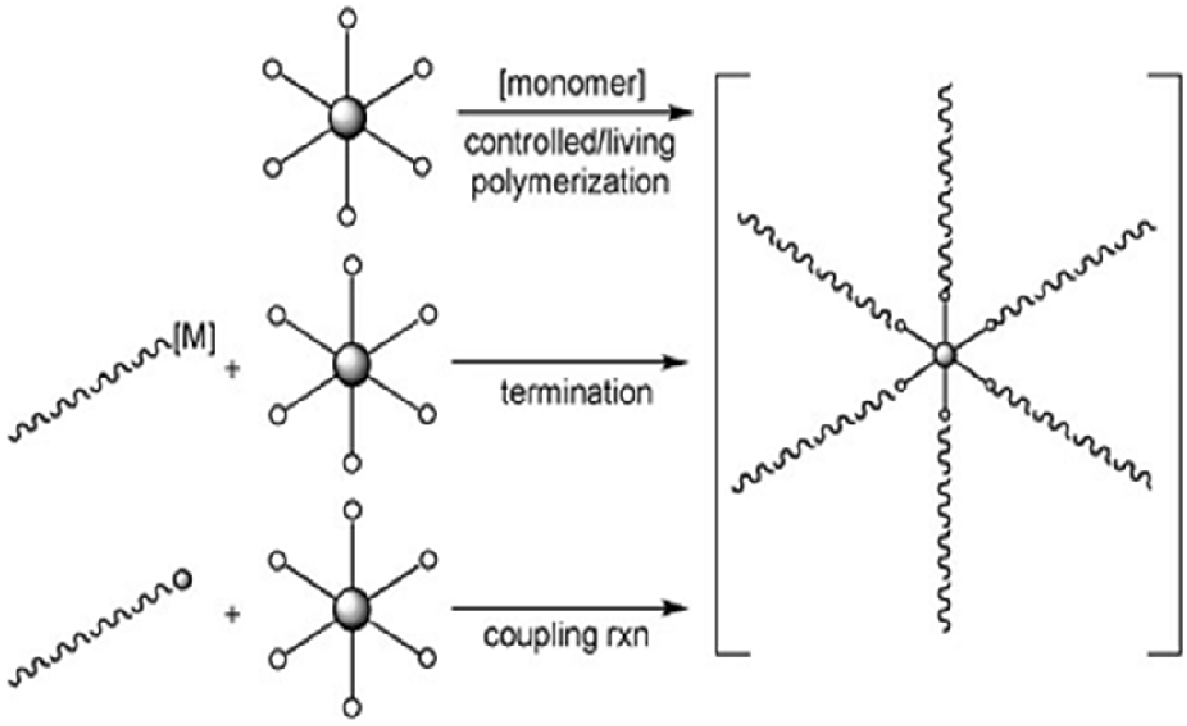


Figure 1.4 Synthesis methodologies of star polymers ([22])

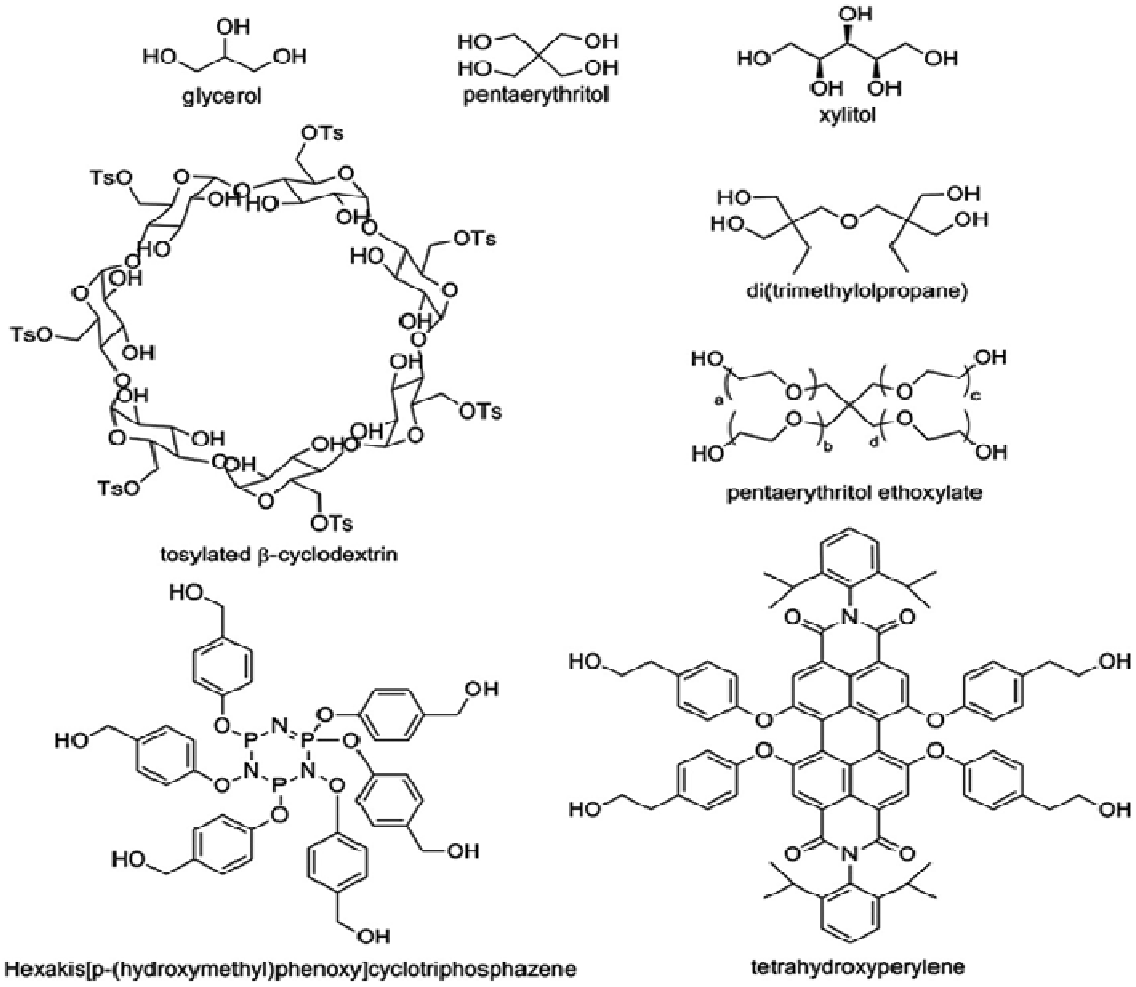


Figure 1.5. Multifunctional alcohols used to synthesize multiarm PLA ([10]

polymers were first described by Schaefgen and Flory (1948), who synthesized four- and eight-arm star polymers of ε-caprolactam, eventually made a efficient path in this area. Although star polymers were first reported in 1948, the first star PLA macromolecule was introduced in 1989 [23] extensive research in this area was started in the last two decades due to their unique properties when compared to linear PLA..

In general, three methods have been used for star polymer synthesis, as shown in **Figure 1.4** [22, 24]. The core-first method involves, ring opening of monomer by multifunctional initiator and further the living polymerization of a reactive monomer. In

this method the polymer chains are grown directly on the core. The arm-first method involve coupling linear polymer chains with a reactive core molecule. Multi-functional reagents will be used in this method to terminate linear living polymers. The other method can exploit the latent reactivity of telechelic linear polymers to chemically attach the polymer arms to the core.Aliphatic polyesters are the important classes of star polymer because the reactions are relatively easy and readily available multi-functional alcohols to initiate the ROP of cyclic esters through a core-first approach polymer synthesis. The ROP reactions are same as of linear polymers, whereas multifunctional alcohol will be used instead of monofunctional alcohol and catalyzed by a metal complex to form an active metal alkoxide that further follows a coordination–insertion mechanism to produce the star polymer. Most commonly used multifunctional alcohols for ring opening is listed in **Figure 1.5**.

1.1.5. Properties of poly(lactic acid)

1.1.5.1. Crystallinty and Thermal behavior of poly(lactic acid)

PLLA and PDLA are crystalline due to enantiomeric purity and stereoregularity of the PLLA/PDLA chain. Conversely, PDLLA (equimolar of L- and D-lactic acid) is fully amorphous because of its irregular structure. In general, crystallization, and thermal behavior of polymers depend on the polymer molecular weight, polymerization conditions and purity of the polymer.

Pure PLLA and PDLA have almost same properties, where the glass transition temperature (T_g) is around 50-70 °C, the melting temperature is (T_m) between 170 to 190 °C, and the crystallinity is approximately 35% [25-28]. In case of syndiotactic

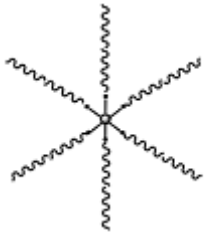
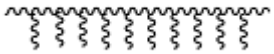
polylactide, lower glass transition temperature and melting temperature were observed as of 34 and 155 °C, respectively due to the different stereotactic configuration [29]. It was further observed that increase in molecular weight leads to significant increase in T_g/T_m and crystallinity for PLLA and PDLLA, respectively [30].

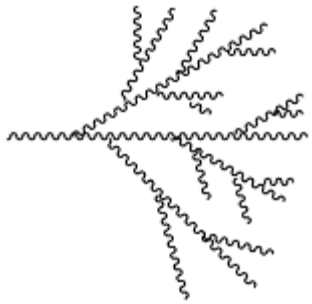
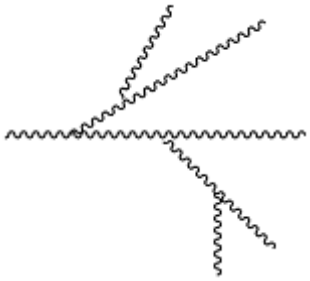
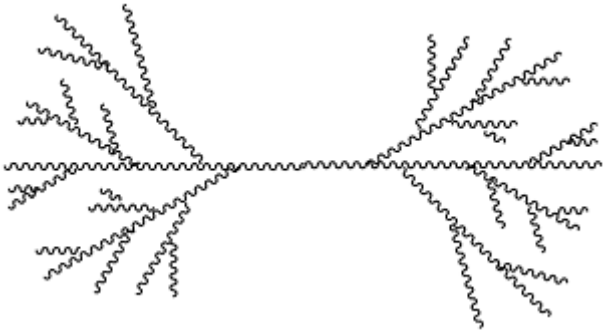
Star PLA have shown lower T_m, T_g, crystallization temperature (T_c) and hydrodynamic volumes compared to linear PLA [31, 32] but higher viscosity than linear PLA [31]. In addition, star-shaped PLA exhibit coiling nature [33]. S.H Lee, et al [34] studied the thermal characterization of star PLA with different multifunctional alcohol. The T_g was closely dependent on molecular weight than the number of arms present in the star PLA. The T_g ranged from 49.1 °C (M_n = 12 700) to 58.1 °C (M_n= 31 700) for the star PLA [29]. In addition, improper control in ROP resulted in lower T_g indicating that polymer star uniformity playing an important role in controlling the thermal properties. Further, the crystallization behavior revealed the choice of initiator has a larger effect on T_c, where pentaerythritol-based PLLAs have lower T_c values compared to dipentaerythritol based PLLAs [34]. Recent reports also complies with reported results [35, 36]

1.1.5.2. Rheology and Mechanical Property

In general, increase in molecular weight usually lead into increase in strength but contradictory results were observed by Perego et al, (1996). PLLA showed better behavior when compared with PDLLA where tensile strength was 59 MPa (M_n = 67,000) and 44 MPa (M_n= 114,000) for PLLA and PDLLA, respectively. This can be due to the stereoregularity of the polymer chain. In case of viscosity, star-shaped

Table 1.1 Properties of Branched PLA ([10])

Structure	**Properties**	**References**
Star Shaped 	Higher solution viscosity More prominent shear-thinning behaviour Higher storage and loss moduli Lower T_g, Tm and crystallinity	[31] [31] [31] [31]
Comb Shaped 	Higher degradability Lower T_g, Tm and crystallinity Increased hydrophilicity Lower intrinsic viscosity	[37-39] [38] [40, 41] [39]
Hyper branched	Lower T_g, Tm and melting enthalpy ΔH_m Higher thermal	[42-45] [45]

	stability	
Long chain Branched 	Lower crystallinity Lower contraction factor Lower intrinsic viscosity	[42] [43] [43]
Dumbell shaped 	Lower T_g, T_m, T_c Lower crystallinity Decreased hydrophobicity	[46] [46, 47] [46]

PLLA showed higher viscosity and greater dependence of viscosity on temperature. It was observed that branched polymers have well entangled arms, which results in higher viscosities due to the suppression of longitudinal motions [31, 33, 48].

1.1.6. Need for the modification of PLA

Challenge of PLA based biomaterials for the tissue engineering applications is, it doesn't have any integrin binding moieties to support the cell growth though it has goog mechanical strength to mimick the natural Extracellular Matrix (ECM) [49]. This can be achieved by attaching some hydrophilic polymer into PLA so that protein adsorption will be increased, this increased protein adsorption property will help for the cells to adhere into the matrix [50, 51]. Researchers have studied the pendant –COOH functionalized PLLA and found that the functionalization increased the hydrophilicity of the surface thus the cells seeded on -COOH modified PLLA scaffolds had higher adhesion and proliferation rates and spread well over a larger area compared to unmodified scaffolds [52]. Functionalizing scaffolds with the integrin binding moieties will increase the cell-material interactions since these bioactive molecules are the constituents of natural ECM proteins. Often gelatin which is a denatured form of collagen has been used to alter the surfaces of 3D PLLA scaffolds [49, 53, 54]. The gelatin modified scaffolds greatly increased the adhesion, proliferation, spreading of cells and collagen deposition than on the control scaffolds [53].

1.1.6.1. Gelatin

Gelatin is a soluble protein extracted from collagen by partial hydrolysis. Collagen is the main protein constituent present in connective tissues of bones, cartilages and skins [55]. Insoluble native collagen must be pre-treated to make it suitable for extraction,

which involves heating in water at temperatures higher than 45 ˚C. This pre-treatment disorganize the protein structure and helps solubilization of collagen [56]. The consequent heat treatment destabilize the triple-helix by breaking the hydrogen and covalent bonds, resulting helix-to-coil transition and conversion into soluble gelatin [57, 58]. The degree of conversion to gelatin is dependent on pretreatment and the warm-water extraction process, which are critically controlled by pH, temperature, and extraction time [55]. Based on pre-treatment two types of gelatin can be obtained, which are commonly known as type-A gelatin (isoelectric point at pH ~ 8-9) and type-B gelatin (isoelectric point at pH ~ 4-5) obtained under acid and alkaline pre-treatment conditions respectively. The source, age of the animal, and type of collagen, are all intrinsic factors influencing the properties of the gelatin. The most abundant sources of gelatin are pig skin (46%), bovine hides (29.4%) and pig and cattle bones (23.1%) [59, 60]. Fish gelatin represented less than 1.5% of total gelatin production in 2007 [60, 61]

1.2. Scope of the Thesis

The failure of an organ or tissue is one of the most frequent, and devastating problems in human health care. The materials based tissue Engineering has been a major clinical breakthrough in the 20th century, despite of the clinical problems associated with xenografts, alografs and autografts. PLA is a biodegradable polymer which is widely used to fabricate 3D scaffolds and further to create cell construct. However, the lack of cell adhering moiety in the PLA limits the successful application in the field of tissue engineering. The conventional modification involves immobilization of biomolecules or surface treatment of PLA surface to make the PLA favourable for the cell adhesion followed by other cellular function. Yet, uniform cell proliferation was not able to

achieve. The lacuna in the design of tissue engineering scaffold motivated us to design, develop and validate gelatin grafted PLA for biomedical applications.

- Tailoring the cell adhesion property of PLA by gelatin has not been reported yet.
- The degree of gelatin grafting to PLA lead to difference in hydrophilicity, mechanical property and the rate of various cell proliferation has not been reported yet.
- Therapeutic efficiency of gelatin grafted PLA as anti-amyloidogenic property has not been studied yet.
- The *in vivo* biocompatibility of silane crosslinked gelatin grafted PLA has not been investigated in detail.

1.3. Objective

The main objectives of the present thesis are,

- Synthesis of gelatin grafted linear and star shaped PDLLA (l-pLG and ss-pLG) and their physicochemical characterization.
- Fabrication of 3D scaffolds using freeze drying technique and their characterization.
- Studying degradation kinetics of 3D scaffolds in enzyme containing media and their characterization.
- Evaluation of rate of cell proliferation within 3D scaffolds *in vitro*.
- Evaluation of hemocompatibility of the 3D scaffolds.

- Increasing the stability of 3D scaffolds using crosslinker silane.

- Studying the *in vivo* compatibility of 3D scaffolds.