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Synthesis and Characterization of Novel Polyester Scaffolds from Sugarcane Industry By-products for Use in Skin and Bone Tissue Engineering

Akanksha Kanitkar
Louisiana State University and Agricultural and Mechanical College, akanit1@lsu.edu

SYNTHESIS AND CHARACTERIZATION
OF NOVEL POLYESTER SCAFFOLDS
FROM SUGARCANE INDUSTRY BY-PRODUCTS
FOR USE IN SKIN AND BONE TISSUE ENGINEERING

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Dedicated to my parents, Mr. Vishwas G. Kanitkar and Mrs. Prerna V. Kanitkar,

and to Kartik Ramasubramanian

for their endless love, support and encouragement through all these years.

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ABSTRACT

The aim of this work was to synthesize non-toxic, biodegradable polyesters of aconitic acid, cinnamic acid and glycerol from by-products of the sugarcane industry as scaffolds for skin and bone tissue engineering. Utilizing the by-products, molasses and sugarcane bagasse, not only add value to the cane industry, but also paves a path for synthesizing novel bio-based materials from the isolated specialty chemicals.

Molasses contain an economically recoverable quantity of aconitic acid and its extraction was studied in detail as a part of this work. The yields of recovered aconitic acid varied from 25–69% depending on the extraction conditions. Under all the conditions, the purity values of extracted aconitic acid were higher than 99%. Subsequently, polyesters of aconitic acid, glycerol and cinnamic acid were synthesized. Different compositions of polyesters were characterized to determine their mechanical properties, porosity, mass loss in stromal medium, ability to support growth and proliferation of human adipose derived mesenchymal stem cells (hASC). Several biocompatibility tests such as mass loss over a period of time, alamarBlue® to analyze growth and viability of hASC on polyester scaffolds, picogreen for total DNA content synthesized indicated that these polyesters hold promising potential as tissue engineering scaffolds. The final step of this dissertation involved evaluating these polyesters as skin and bone tissue engineering scaffolds. For skin tissue engineering, especially for wound repair, thin film polyester scaffolds laden with hASC were grown in stromal medium supplemented with basic fibroblast growth factor (bFGF). Based on the amount of collagen synthesized and DNA quantification data, it was concluded that the polyesters can be used as scaffolds for wound repair by the addition of bFGF. Finally these materials were utilized as bone scaffolds where hASC were induced to undergo osteogenesis, and analyzed for mineralization and osteogenic target gene expression over a 21 days period. Based

on calcium deposition results, and alkaline phosphatase (ALP) and osteocalcin (OCN) expression

data, it was concluded that these scaffolds hold great potential for bone tissue engineering.

CHAPTER 1. INTRODUCTION

Sugarcane is a highly valued crop in Louisiana grown in about 22 parishes on over 400,000 acres of land and accounts for about 20% of the total sugar grown in the United States (Salassi, 2009; Zhong, 2003). There are a total of 11 operational mills in Louisiana and in the harvesting year of 2012-2013, they produced 1.7 million short tons of sugar with an average yield of cane produced per total acre of 37.2 tons (AMSCL, 2013). Louisiana is one of the oldest and historic sugar producing areas in the country, employing about 16,400 industrial workers in the sugarcane industry (AMSCL, 2013). Sugarcane production has an economic impact of $2.7 billion on the state's economy, making it an important agricultural commodity in the state (AMSCL, 2013; Salassi, 2009).

Sugarcane is planted vegetatively during the fall of each year. In the summer, after the stalks mature, cane is harvested the following fall. The stalks are cut into billets (250–300 mm) and transported to the mills, where they are crushed, and the juice is boiled down to a thick syrup from which raw sugar is crystallized (AMSCL, 2013). The raw sugar production process, as depicted in Figure 1.1, generates two by-products: sugarcane bagasse and molasses.

The major challenge currently being faced by the Louisiana sugar industry is the fact that the harvesting season lasts approximately three months of the year (September to December) and hence the industry must be very efficient in order to remain competitive (Grimaldo, 2013). Another challenge faced by the sugar industry is the fluctuation in the market price of sugar, making the industry vulnerable to price volatility (Zapata, 2007). Current research efforts are directed toward optimizing sugar processing operations and reducing the cost of production so that the industry can remain economically viable. One of the possible solutions to increase profitability is to utilize

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sugarcane industry by-products (sugarcane bagasse and molasses) to generate a variety of value added chemicals and products.
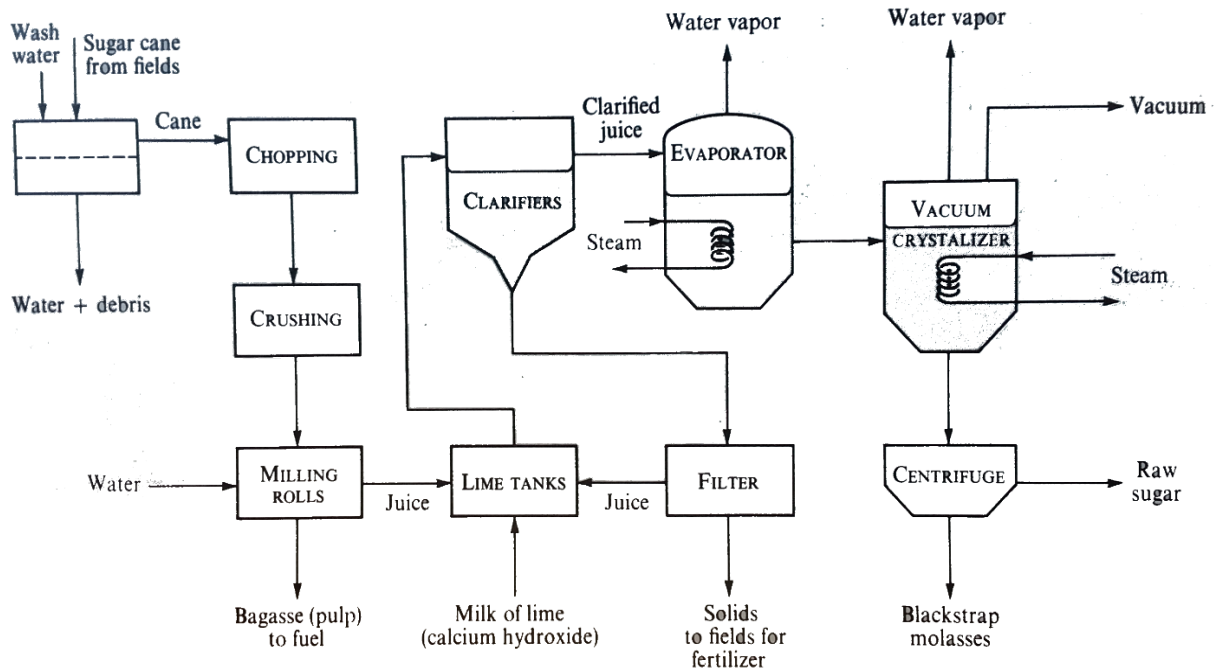
Figure 1.1. Steps for raw sugar production from sugarcane (Courtesy: King, 1980)

1.1. Sugarcane Bagasse (SB)

After the extraction of juice from sugarcane, the fibrous residue left behind is called sugarcane bagasse which is used as a source of fuel to power sugar mills. About 50% of the bagasse residue is burnt to provide energy to the mill, while the rest is stored in a stockpile and remains unused (Chandel *et al.*, 2012). Sugarcane bagasse is a lignocellulosic material that can be processed in a number of ways to yield value added products and has been at the forefront of sustainable engineering research. Approximately, 90% of dry weight of SB is primarily composed of (% w/w dry basis) cellulose (35–45%), hemicellulose (26.2–35.8%), lignin (11.4–25.2%), and other components (Canilha *et al.*, 2012; Zhao *et al.*, 2009). The conversion of SB into value added products such as xylitol, organic acids and ethanol using biotechnological routes can be complimentary to the cane industry by generating sustainable economic value (Canilha *et al.*,

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2012). Extensive research has been done on conversion of sugarcane bagasse to ethanol, referred to as second generation biofuel (Aita and Kim, 2010; Sun and Cheng, 2002). For the biochemical conversion of SB to ethanol it must be first broken down into simple sugars by a process termed as pretreatment. An effective pretreatment involves depolymerization of cellulose and hemicellulose into their corresponding monomeric sugars and their subsequent microbial fermentation to ethanol (Aita and Kim, 2010; Hari Krishna *et al.*, 2001; Saha, 2003). The biochemical conversion of SB to ethanol can be summarized in six steps: pretreatment, enzyme hydrolysis, detoxification, fermentation, ethanol recovery, and effluent treatment (Figure 1.2) (Aita and Kim, 2010).
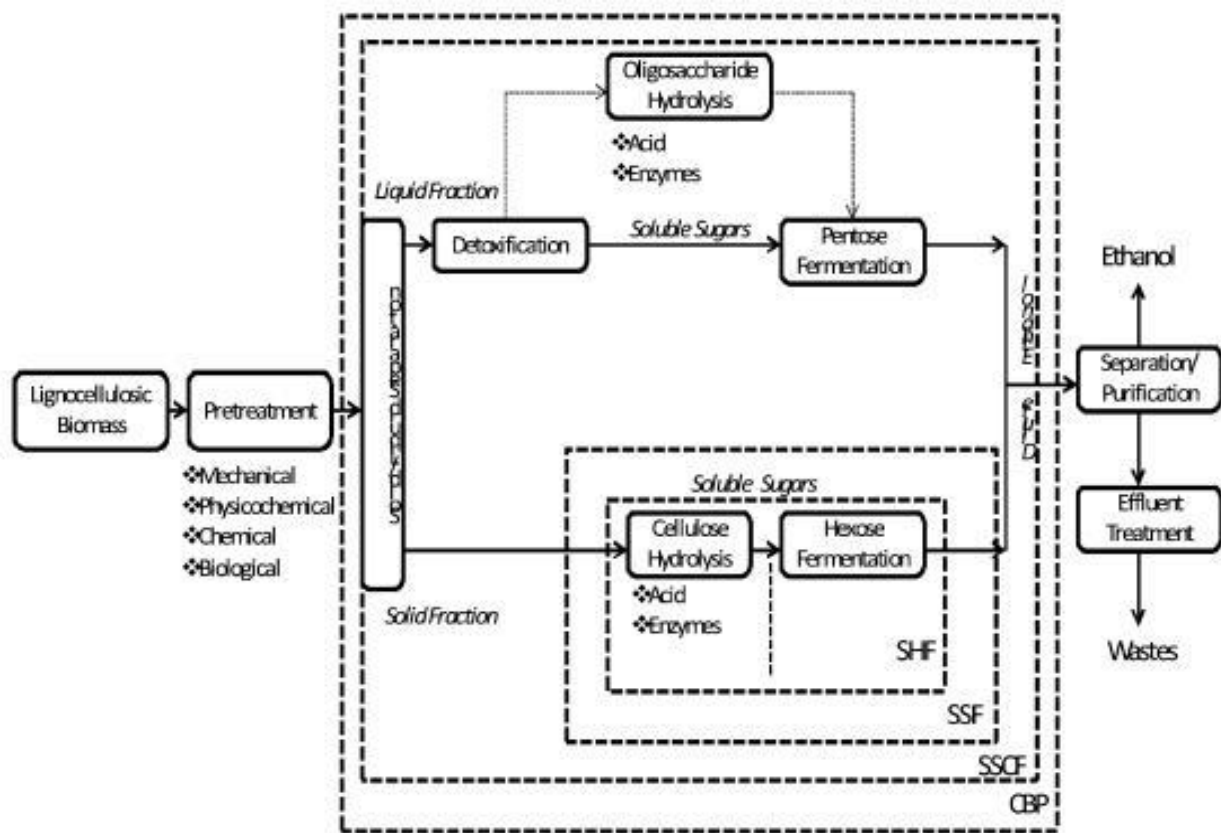
Figure 1.2. Flow sheet for ethanol production from lignocellulosic biomass.
SHF: Separate Hydrolysis and Fermentation; SSF: Simultaneous Saccharification and Fermentation; SSCF: Simultaneous Saccharification and Co-Fermentation; CBP: Consolidated Bioprocessing, where enzyme production, hydrolysis and fermentation of all sugars are performed in one step (Courtesy: Aita and Kim, 2010)

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Pretreatment of bagasse generates significant amounts of secondary compounds derived from sugars (furfural, 5-hydroxymethyfurfural, etc.) and from lignin (phenolics such as p-hydroxybenzaldehyde, ferulic acid, p-coumaric acid, hydroxyl cinnamic acid, etc.) (Aita and Kim, 2010). Weak acids (acetic, formic, levulinic, etc.) are also formed in the process (Peng *et al.*, 2009). All these chemicals can be used as building blocks to derive bio-based polymers from biomass (FitzPatrick *et al.*, 2010). Cinnamic acid and/or its derivatives can be isolated from the lignin in the pretreatment waste generated during bioethanol production from sugarcane bagasse. In this research project, cinnamic acid (Figure 1.3) was used as a monomer in polyester synthesis. The polyesters containing cinnamic acid can be further cross-linked via [2+2] photocycloaddition taking advantage of the double bond present in the side chain (Maekawa *et al.*, 2002).

In addition, sugarcane bagasse can be used for paper and pulp production, and for production of protein-enriched cattle feed and enzymes (Pandey *et al.*, 2000). In addition, other value added products such as 5-hydroxymethylfurfural (HMF), xylitol, butanediol, butanol, and various bio-polymers, like polyhydroxyalkanoates (PHA) and polylactates (PLA) can also be derived from hemicellulose (Canilha *et al.*, 2012). Thus, SB can be employed as a sustainable alternative for the production of various by-products that can add significant value to the sugarcane industry.
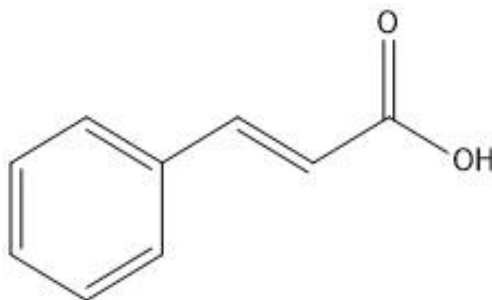
Figure 1.3. Structure of cinnamic acid

1.2. Sugarcane Molasses (SM)

In the process of raw sugar manufacture, the extracted juice from sugarcane is boiled down to a thick syrup. Raw sugar is crystallized from the syrup leaving behind molasses which is primarily used as a livestock feed (AMSCL, 2013). Molasses is primarily composed of water (20%), sucrose (35%), fructose (9%), glucose (7%), nitrogenous compounds (4.5%), and organic acids (5%) (Paturau, 1986). The primary use of molasses is as a cattle feed due to the presence of carbohydrates, proteins and trace elements such as cobalt, boron, molybdenum, zinc, etc. Molasses can be chemically and/or biochemically converted to a number of products ranging from alcohol and glycerin to inositol, lactic acid, single-cell proteins, etc. (Paturau, 1986). Molasses can be fermented to ethanol, glycerin, citric acid, itaconic acid, butanol, acetone, and also used as a carbon source for yeast (Olbrich, 1963). The non-sugar components in molasses, namely the nitrogenous substances can also be utilized to produce glutamic acid and betaines (Olbrich, 1963). These products can further be used as building blocks for a variety of specialty value added chemicals. Aconitic acid (prop-1-ene-1,2,3-tricarboxylic acid) (trans- isomer) is the major organic acid found in sugarcane molasses (Figure 1.4). The presence of three carboxylic acid groups is ideal for synthesis of polyesters of high molecular weight, and it was used as one of the monomers in the synthesis of biodegradable polymer synthesis in this project.
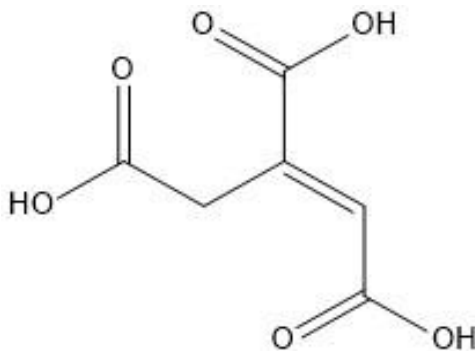
Figure 1.4. Structure of trans-aconitic acid

By utilizing the by-products of the cane industry, molasses and sugarcane bagasse, not only adds value to the cane industry, but also paves a path for synthesizing novel bio-based materials and specialty chemicals from the isolated molecules.

1.3. Sugarcane Industry as a Biorefinery

The concept of a bio-refinery which integrates the production of bio-based fuels and chemicals is becoming a viable option in midst of an energy crisis (FitzPatrick *et al.*, 2010). Biorefinery, like a petroleum refinery, refers to fractionation of input feedstock in this case biomass, for the co-production of fuels, chemicals, power, and multiple value added products (Edye *et al.*, 2006; Jong, 2010). In Louisiana, sugarcane harvesting, and hence the sugar mills operation occurs only for about 3 months a year that allows ample time for production of other co-products from leftover bagasse without impacting the raw sugar production during the rest of the year. Figure 1.5 below shows few of the various products that can be generated from the raw sugar biorefinery.
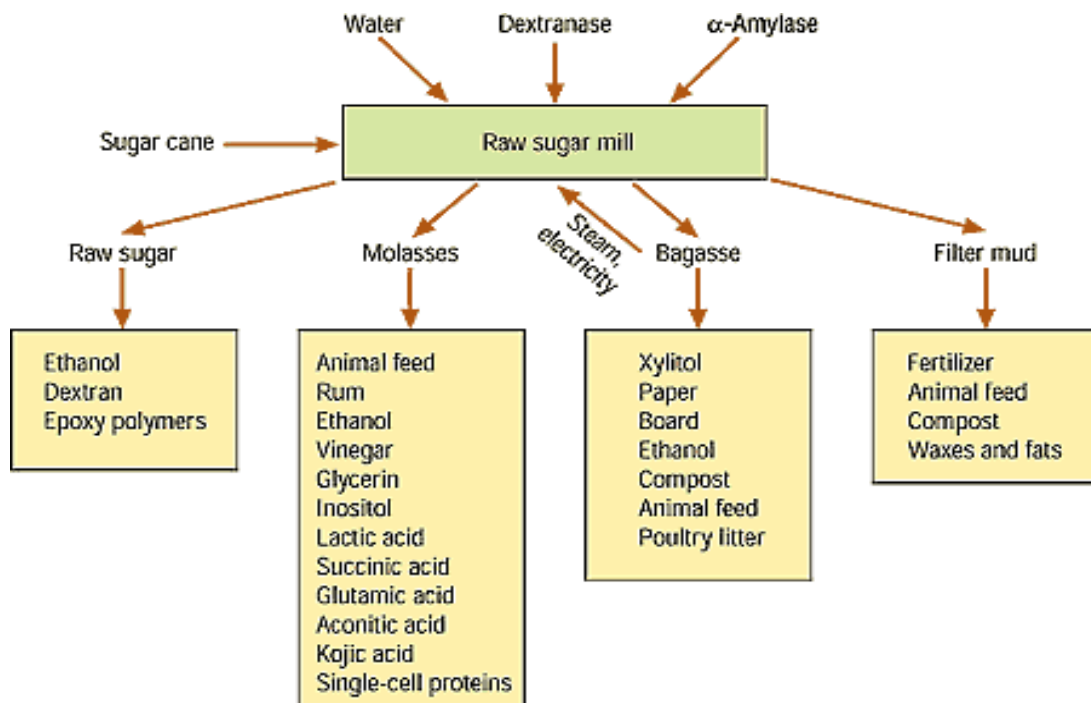
Figure 1.5. Raw sugar mill showing inputs, products, and chemicals that can be manufactured using sugarcane as a feed (Courtesy: Taylor, 2000)

Biorefineries today are based on utilization of the whole plant and/or complex feedstock, with integration of traditional and modern processes for utilizing these biological feedstocks. The range of products manufactured in a biorefinery should be economically sustainable and market competitive. The products should add economic value to the biorefinery operation and generate revenue (Jong, 2010; Kamm *et al.*, 2000). The concept proposed at LSU Agcenter's Audubon Sugar Institute utilizes sugarcane not only for the production of sucrose, molasses and bagasse, but also ethanol and bio-polymers. The research work presented here focuses on one aspect of the biorefinery, synthesis of bio-polymers from aconitic acid, glycerol and cinnamic acid. Aconitic acid can be recovered from molasses after sugar has been crystallized out of the juice. Glycerol can be recovered from the fermentation broth from pretreated sugarcane bagasse used for bio-ethanol production or from the fermentation of molasses. Cinnamic acid can be derived from the different phenolic compounds recovered from the lignin waste stream generated during the production of bio-ethanol from sugarcane bagasse.

1.4.   Biodegradable Polymers for Tissue Engineering

Traditional treatments to repair damaged and degenerated tissues involved autografting (transplanting tissue from one site to another of the same patient) or allografting (transplanting tissue from one individual to another). Autografting is expensive, painful and has anatomical constraints, while allografting suffers from the disadvantage of risk of rejection by the patient's immune system, and increased chances of infections and disease transmission from the donor to the patient (O'Brien, 2011). Tissue engineering is an alternative approach to surgical procedures for the replacement or restoration of damaged or traumatized tissue (Enrione *et al.*, 2010; Shaker *et al.*, 2012; Zhu *et al.*, 2008). The term tissue engineering was officially coined at a National Science Foundation workshop in 1988 to imply 'the application of principles and methods of

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engineering and life sciences toward the fundamental understanding of structure-function relationships in a normal and pathological mammalian tissues and development of biological substitutes to restore, or maintain tissue function' (O'Brien, 2011). Tissue engineering is a highly multidisciplinary field where the fields of cell and molecular biology converge with material sciences and biomedical engineering. Scaffolds are an indispensable aspect of tissue engineering that interact with cells and act as a template for tissue formation, and provide an appropriate environment for the regeneration of tissues and organs (Saltzman, 2013). Scaffolds are seeded with cells and are either cultured *in vitro* to synthesize the target tissue by providing appropriate growth factors, and later implanted to an injured site, or directly implanted *in vivo* where the regeneration of tissues or organs is achieved inside the body (Rouwkema *et al.*, 2011). Figure 1.6 depicts the tissue engineering triad at the heart of which lies the biomaterial scaffold.
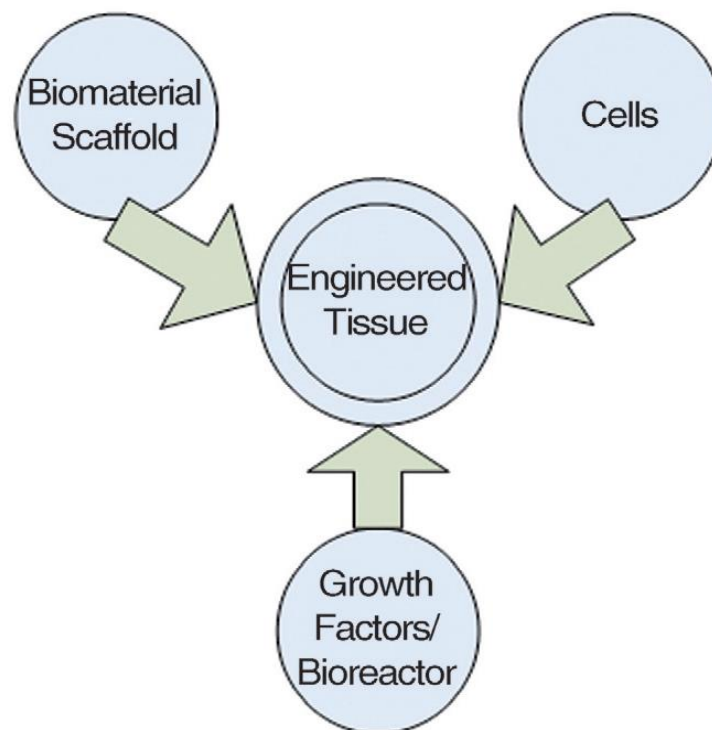
Figure 1.6. Tissue engineering triad of cells, signals and scaffold that acts as a template for tissue formation (Courtesy: O'Brien, 2011)

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The basic process of tissue engineering involves seeding cells onto a scaffold that can act as a support for growth and proliferation of cells. It provides the needed architecture on which the cells can organize and produce or replace a desired tissue or organ. This is accompanied by gradual and eventual degradation of the scaffold material as the new fully functional engineered tissue is formed. The process is sketched in Figure 1.7.
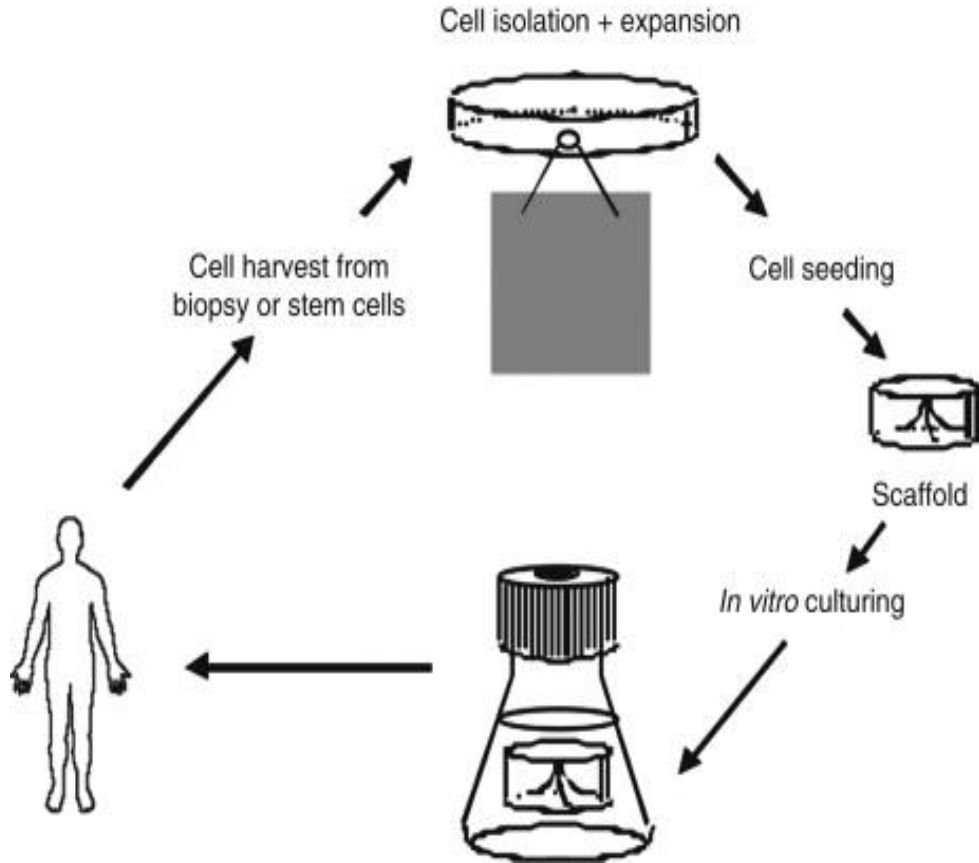
Figure 1.7. The basic principle of tissue engineering (Courtesy: Stock and Vacanti, 2001)

There is a plethora of information available on biodegradable scaffolds used for tissue engineering. These scaffolds can be composed of natural or synthetic polymers but the material that can be used as a scaffold needs to satisfy a number of key considerations. These include: i. Biocompatibility, wherein the cells can adhere, proliferate and migrate through the scaffold without inducing any adverse immune response; ii. Biodegradability, such that the scaffold degrades to non-toxic products and is able to exit the body within the time frame required for tissue

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regeneration; iii. Mechanically robust, such that the mechanical strength is consistent with the anatomical site to which it is implanted to; and, iv. Scaffold architecture should be such that the scaffold should have an interconnected pore structure with high porosity so that there is an adequate diffusion of nutrients into and waste material out from the cells within the scaffold (Gunatillake and Adhikari, 2003).

1.4.1. Natural Polymers in Tissue Engineering Applications

Polymers derived from natural sources such as plants, animals and microorganisms are useful as biomaterials in regenerative medicine owing to their similarity to the extracellular matrix and bioactive properties that aid in better interactions with the cells (Dhandayuthapani, 2011; Mano *et al.*, 2007; Nugent and Iozzo, 2000). Natural polymers that are used in tissue engineering can be divided into three classes: i. Polysaccharides; ii. Proteins; and, iii. Polyhydroxyalkanoates (Gomes, 2008; Malafaya *et al.*, 2007).

Polysaccharides are a class of natural bio-polymers consisting of monosaccharides linked together by O-glycosidic linkages which enable them to form linear as well as branched structures. Their physical properties are dependent on the differences in the monosaccharide composition, chain linkages and molecular weight (Malafaya *et al.*, 2007). Alginate, chitosan, cellulose, starch hyaluronic acid, and other polysaccharides are popular options that have been used as scaffold materials. Alginates can readily cross-link by addition of divalent ions such as Ca^{2+} and have a pH- dependent anionic nature. Due to its ease of processing into scaffolds, biocompatibility, non-antigenicity and chelating ability, alginate is a popular candidate for a wide range of biomedical applications such as drug delivery, wound dressings, cartilage repair, bone regeneration, etc. (Sun and Tan, 2013). Chitosan is one of the most promising natural material for tissue engineering due to its biocompatibility, antibacterial activity, ability to bind to mammalian and microbial cells

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(Dutta, 2004). Its scaffolds are most commonly used to engineer bone, cartilage, skin, liver or trachea (Gomes, 2008). Hyaluronan is a major extracellular matrix component (ECM) and has been popularly used in cartilage, bone and osteochondral applications (Malafaya *et al.*, 2007).

Proteins are another class of natural polymers and are the most abundant components of ECM. They can direct growth and organization of cells, and find application in tissue engineering. Proteins are complex polymeric structures made up of amino acids linked by amide bonds (Gomes, 2008). Collagen, a major protein of ECM has high mechanical strength, good biocompatibility, low antigenicity, ability to be cross-linked, and has been used for a wide range of tissue engineering products (Malafaya *et al.*, 2007). It is used in bone and cartilage reconstruction, cardiovascular tissue engineering, dermal tissue engineering, etc. (Parenteau-Bareil *et al.*, 2010). Gelatin is derived from collagen and has been widely used in drug delivery for tissue engineering applications targeting bone, cartilage, skin, etc. (Malafaya *et al.*, 2007). Silk fibroin is a naturally occurring fibrous protein produced by domestic silk worms (Gomes, 2008). It is an attractive biomaterial due to its slow degradability, low inflammatory response, excellent mechanical properties, and cell adhesion properties (Altman *et al.*, 2003).

Polyhydroxyalkanoates (PHA) are a group of naturally occurring polyesters that are biodegradable thermoplastics with a high degree of biocompatibility (Gomes, 2008). PHAs have been used in numerous medical applications such as sutures, cardiovascular patches, cartilage repair scaffolds, bone graft substitutes, stents, and wound dressings, etc. (Chen and Wang, 2013; Chen and Wu, 2005). PHA can be blended, surface modified and used in combination with other polymers to enhance their biocompatibility and mechanical properties (Malafaya *et al.*, 2007).

Natural polymers have the advantage of biocompatibility, biodegradability, similarity to ECM, cellular interactions, etc. that make them promising tissue engineering scaffolds (Gomes,

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2008; Malafaya *et al.*, 2007; Mano *et al.*, 2007). However, they suffer from some drawbacks such as batch to batch variability, limited processability, narrow range of mechanical properties, and high cost (Dhandayuthapani, 2011; Malafaya *et al.*, 2007). Synthetic polymers, on the other hand can be tailored for specific applications, are cheaper than natural scaffolds and can be made on a large scale with desired mechanical properties (Gomes, 2008; Gunatillake and Adhikari, 2003).

1.4.2. Biodegradable Synthetic Polymers for Tissue Engineering

The advantages of biodegradable synthetic polymers are the ability to precisely control their mechanical, chemical and biological properties, pore size and porosity, and predict their degradation rates (Zanetti, 2011). These scaffolds can be fabricated into desired shapes with required morphology conducive to tissue growth as well as synthesized by incorporating a functional group that induces growth of a desired tissue (Gunatillake and Adhikari, 2003). The greater control over their quality allows for production of these scaffolds on a large scale with no batch to batch variations as in natural polymers (Gomes, 2008).

Numerous biodegradable synthetic polymers such as poly(glycolic acid), poly(lactic acid) and their copolymers, poly(p-dioxanone), poly(ε-caprolactone), polyanhydrides, poly(amino acids), polyorthoesters have been used in clinical applications for tissue engineering and drug delivery (Gunatillake and Adhikari, 2003). Polylactic acid (PLA) is an aliphatic ester prepared by condensation of lactic acid or by ring opening of the lactide dimer. It consists of two isomeric forms: poly(L-lactide) (PLLA) and poly(D-lactide). For most applications, PLLA is chosen as it is easily metabolized in the body (Gunatillake and Adhikari, 2003). Poly(glycolic acid) (PGA) is one of the most commonly used scaffolds for tissue engineering *in vitro* as well as *in vivo*. While PGA has higher degradation rates and more rapid deterioration of mechanical properties over time, PLA degradation produces more acidic compounds detrimental to cell compatibility. But the co-

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polymer of PLLA and PGLA termed poly(lactic acid–co-glycolic acid) (PLGA) is found to have lower degradation rate and higher mechanical strength compared to PLLA and PLGA. The degradation rates of these polyesters are affected by polymer composition, copolymer ratio, crystallinity, molecular weight, porosity, and site of implantation (Hacker, 2011). These are one of the most frequently used polymers in tissue engineering applications (Nelson *et al.*, 1977; Zanetti, 2011) and are among the few that have FDA approval for human clinical use (Hacker, 2011). Poly(ε-caprolactone) (PCL) is an aliphatic semicrystalline polymer prepared by polymerization of cyclic monomer ε-caprolactone. Since it degrades at a slower rate than PLA it is utilized in developing long term, implantable drug delivery applications (Gunatillake and Adhikari, 2003; Hacker, 2011). It can be easily co-polymerized with other polymers which makes it an attractive candidate for polymeric scaffolds (Zanetti, 2011) and is widely used in bone tissue engineering application (Porter *et al.*, 2009; Schantz *et al.*, 2002). Polyanhydrides are a class of thermoplastic polymers synthesized by dehydration of the diacids by melt polymerization (van Dijkhuizen-Radersma, 2008). They are biocompatible and demonstrate excellent controlled release characteristics and find their major application in drug delivery (Gunatillake and Adhikari, 2003).

1.4.3. Biodegradable Polyesters of Aconitic Acid, Glycerol and Cinnamic Acid in Tissue Engineering

Polyesters of aconitic acid, glycerol and cinnamic acid can be synthesized to yield cross-linked, high molecular weight hyperbranched structures. Polymerization occurs via a simple polycondensation reaction where the by-product water is eliminated. The double bond present in the cinnamic acid moiety can be exposed to UV light to give a highly cross-linked structure. Aconitic acid can be recovered from molasses after sucrose has been removed by crystallization, while cinnamic acid can be derived from the phenolic compounds recovered from the lignin waste

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stream generated during pretreatment of sugarcane bagasse. Glycerol, a by-product of biodiesel production can also be recovered from the fermentation of pretreated sugarcane bagasse to yield bio-ethanol. To obtain different monomer end-capped polyesters, the feed ratio of reactants can be varied in such a way that the number of free hydroxyl and carboxylic acid groups varies in the end product. In this work, novel class of these polyesters were synthesized and characterized to evaluate their potential use as scaffolds in tissue engineering applications.

1.5. Human Adipose Derived Stem Cells in Tissue Engineering Applications

The ability of stem cells to self-renew and differentiate make them an interesting and promising cell source for tissue engineering and regenerative medicine applications. Adult stem cells can be isolated from many human tissues such as bone marrow, adipose tissue, amniotic fluid, umbilical cord blood, etc. (Bajada *et al.*, 2008). Adult stem cells are responsible for growth, tissue maintenance, and regeneration and repair of damaged tissue (Bianco, 2008). For application of stem cells in regenerative medicine, they should be available in abundant quantities and easily harvestable by a minimally invasive procedure. Stem cells should also be readily differentiable into multiple cell lineage pathways and easily transplantable to an autologous or allogeneic host in a safe and effective manner (Gimble *et al.*, 2007).

In this study, human subcutaneous adipose tissue depots were used as a potential source of adult stem cells. Adult stem cells can be easily obtained from the stromal vascular fraction of subcutaneous adipose tissue with a minimal invasive procedure, liposuction. The increased obesity in the US has led to an increase in liposuction surgeries and hence abundance of the subcutaneous adipose tissue material which is otherwise discarded (Gimble *et al.*, 2007; Yang *et al.*, 2010). It has been reported that relative to bone marrow derived stem cells, adipose tissue contains 100-1000 times more number of cells (Natesan *et al.*, 2011). Human adipose derived stem cells (hASC)

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are also self-renewing and possess multi-lineage development potential (Gimble *et al.*, 2007). All these factors make hASC a promising candidate for therapeutic and regenerative medicinal applications.

1.6.   Skin Tissue Engineering

Skin is the largest organ in the human body accounting for about 8% of total body mass. It is an effective and protective barrier against microbial invasion, mechanical, chemical, physical, and thermal damage (Ravichandran *et al.*, 2012). Skin injuries can sometimes result in severe skin loss or damage. It is composed of superficial epidermis and deeper layer called dermis. The epidermis is composed mainly of keratinocytes and its main function is to protect against infection and moisture loss. The dermal layer consists mainly of fibroblasts and its function is to provide nutrition to the epidermal layer and to regulate the growth and differentiation of epidermal keratinocytes (Lu *et al.*, 2012). Skin tissue engineering has been studied by many researchers (Chan *et al.*, 2012; Lu *et al.*, 2012; Natesan *et al.*, 2011; Trottier *et al.*, 2008). When contemplating regeneration of skin, the most important aspect is replacing the whole skin, dermal as well as epidermal layer. Split thickness grafts comprising of autologous cells are preferable for skin regeneration but the extent of skin loss may limit the availability of healthy skin. Also, introducing a secondary injury to the patient's skin to harvest skin cells may add to the trauma that a person is already facing (Price, 2008). Another way would be to use *ex vivo* cultured autologous cells. The advantage of the former autologous split thickness graft is that there would be no immunological rejection by the patient. While the advantage of the *ex vivo* approach is that it would allow coverage of larger surface wounds with a relatively small area of unaffected skin, the extended amount of time required to grow confluent dermal cell population on scaffolds *in vitro* makes this application challenging (Heng *et al.*, 2005).

Numerous studies have reported different pathways to induce keratinocyte/fibroblasts differentiation of stem cells (Bagutti *et al.*, 2001; Coraux *et al.*, 2003; Görmar *et al.*, 1990; Heng *et al.*, 2005; Hinsenkamp *et al.*, 1997; Supp *et al.*, 1999). One way to guide hASC to keratinocyte lineage *in vitro* would be by exposing the cells to a mixture of exogenous cytokines, growth factors, chemicals, and extracellular matrix (ECM) for a prolonged period of time (Heng *et al.*, 2005). In a study by Coraux *et al.* (2003), murine embryonic stem cells were differentiated to keratinocytes by the addition of bone morphogenetic protein-4 (BMP-4), ascorbate and ECM derived from human normal fibroblasts and murine NIH-3T3 fibroblasts. However, a high degree of non-specific differentiation into multiple uncharacterized lineages was observed. A co-culture of murine embryonic stem cells with human dermal fibroblasts (HDF) and HDF-conditioned media induced differentiation into the keratinocyte lineage (Bagutti *et al.*, 2001). Differentiation to other lineages was also observed. Different cytokines and growth factors, and various non-proteinaceous chemicals that can induce keratinocyte differentiation are listed in Table 1.1.

Table 1.1. Cytokines and growth factors that promote keratinocyte proliferation (adapted from Heng *et al*., 2005)

Cytokines/Growth Factors	Chemical Factors (Non-Proteinaceous)
Epidermal growth factor	Ascorbic acid
Epiregulin	Sodium butyrate
Insulin-like growth factor	Staurosporine
Fibroblast growth factor	Ceramide
Keratinocyte growth factor	Oxysterol
Transforming growth factor	Famesol
Bone morphogenetic protein	NPS R-467
Stem cell factor	Vitamin D and analogs
Angiopoietin-related growth factor	Retinoic acid
Granulocyte/macrophage colony-stimulating factor	Prostaglandin 12 and analogs
Growth differentiation factor-5	Nicotine
Thymocyte-activating factor	Arotinoid ethyl ester
Tumor necrosis factor-α	Ca^2 concentration
Interleukin-1	
Nerve growth factor	

Physical contact between cells and the autocrine and paracrine factors secreted by one cell type (in this case fibroblasts or keratinocytes) interact with other cell type (hASC) and in turn have stimulatory effect on differentiation to dermal lineage (Heng *et al.*, 2005). hASC express paracrine effects when transplanted into injured tissue affecting cells in the vicinity which is important in case of skin regeneration (Yang *et al.*, 2010). Some studies have reported that physical stimuli such as electrical and magnetic fields, heat treatment, mechanical forces, UV irradiation, reduced humidity levels, and hypoxic conditions also play a significant role in skin healing process by promoting growth and differentiation of keratinocytes (Görmar *et al.*, 1990; Hinsenkamp *et al.*, 1997; Manni *et al.*, 2002; Supp *et al.*, 1999).

The use of synthetic and natural polymeric materials as scaffolds for skin tissue engineering has garnered a lot of attention in the recent years (Dhandayuthapani *et al.*, 2010; Enrione *et al.*, 2010; Franco *et al.*, 2013; Tangsadthakun *et al.*, 2006; Yang *et al.*, 2009; Zhu *et al.*, 2008). An ideal scaffold should be able to support and guide cellular growth, provide mechanical and structural support, allow transfer of nutrients, and regulate cellular activities. Many synthetic biodegradable polymers such as poly (caprolactone) (PCL), poly (D,L-lactide) (PDLLA), poly(glycolide) (PGA) and poly[lactic-co-(glycolic acid)] (PLGA) have been studied as scaffolds for skin tissue engineering (Enrione *et al.*, 2010; Pramanick and Ray, 1988; Yang *et al.*, 2009). Naturally occurring materials such as collagen, chitosan, gelatin, hyaluronic acid, dextran, amongst others, have also been investigated as skin scaffolds for regeneration and repair (Enrione *et al.*, 2010a; Yang *et al.*, 2009). Collagen, a component of skin ECM when incorporated in primary keratinocyte culture is reported to be advantageous for keratinocyte growth under *in vitro* conditions (Auger *et al.*, 1998; Horch *et al.*, 2000). Several groups have used a bilayer scaffold approach by blending synthetic and naturally occurring materials to combine the best of both

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techniques (Dhandayuthapani *et al.*, 2010; Duarte *et al.*, 2010; Enrione *et al.*, 2010; Franco *et al.*, 2013; Ravichandran *et al.*, 2012; Tangsadthakun *et al.*, 2006; Yang *et al.*, 2009). Natural biomaterials mimic the molecular properties of the tissue, while synthetic biomaterials impart necessary mechanical strength to the scaffold. Composites of collagen-based substratum with materials such as polylactide-glycolide, alginate, fibronectin, elastin, poly L-lysine, and chitosan are used to enhance structural properties of scaffolds (Enrione *et al.*, 2010; Heng *et al.*, 2005; Ravichandran *et al.*, 2012; Tangsadthakun *et al.*, 2006; Trottier *et al.*, 2008; Yang *et al.*, 2009). Table 1.2 reports synthetic products that are used for dermal/epidermal wound repair. The future of epidermal and dermal replacements for skin tissue engineering relies on the stem cell technology for skin tissue regeneration and repair.

Table 1.2. Commercial synthetic products used for wound repair (Courtesy: Boateng *et al*., 2008)

Product	Type	Major Components
Integra™	Artificial skin	Collagen/chondroitin-6 sulphate matrix overlaid with a thin silicone sheet
Biobrane™	Biosynthetic skin substitute	Silicone, nylon mesh, collagen
Alloderm™	Acellular dermal graft	Normal human dermis with all the cellular material removed
Dermagraft™	Dermal skin substitute	Cultured human fibroblasts on a biodegradable polyglycolic acid or polyglactin mesh
Epicel™	Epidermal skin substitute	Cultured autologous human keratinocytes
Myskin™	Epidermal skin substitute	Cultured autologous human keratinocytes on medical grade silicone polymer substrate
Trancyte™	Human fibroblast derived skin substitute (synthetic epidermis)	Polyglycolic acid/polylactic acid, extracellular matrix proteins derived from allogenic human fibroblasts and collagen
Apligraf™	Epidermal and dermal skin substitutes	Bovine type I collagen mixed with a suspension of dermal fibroblasts
Hyalograft 3-D™	Epidermal skin substitute	Human fibroblasts on a laser-microperforated membrane of benzyl hyaluronate
Laserskin™	Epidermal skin subsitute	Human keratinocytes on a laser-microperforated membrane of benzyl hyaluronate
Bioseed™	Epidermal skin substitute	Fibrin sealant and cultured autologous human keratinocytes

1.7. Bone Tissue Engineering

Bone is the main supportive system of human body having a unique combination of minerals and highly vascularized tissue that provides structural support to the body (Gaalen, 2008; Salgado *et al.*, 2004). It is a composite material which consists of 65-70% hydroxylapatite (a mineral part), and an organic part that is composed of glycoproteins, proteoglycans, etc. which makes up the rest of the 25-30% of the bone matrix (Sommerfeldt and Rubin, 2001). Bone matrix is made up of 3 distinct cell types: osteoblasts, osteoclasts and osteocytes. Osteoblasts are matrix producing cells that synthesize and regulate ECM deposition and mineralization while osteoclasts are responsible for resorption of boney tissue. Osteocytes are characteristic bone cells that are specialized and differentiated osteoblasts. They are the most abundant cells in the bone responsible for calcification of the osteoid matrix (Gaalen, 2008; Salgado *et al.*, 2004; Sommerfeldt and Rubin, 2001). Structurally, adult bone tissue is arranged in two forms: cancellous (trabecular, spongy) and compact (dense, cortical). Cancellous bone has a porosity of 50–90% and is the most active part of the bone responsible for growth, homeostasis and hematopoiesis. Compact bone on the other hand is only 10% porous, and is the stronger and more static component of the bone (Gaalen, 2008; Salgado *et al.*, 2004). Roughly 1 million of skeletal defects occur every year and the current treatments that are favored and clinically available are the use of autologous or allogeneic bone grafts or as an alternative, the use of bone transport (Ilizarov technology) (Mastrogiacomo *et al.*, 2005; Salgado *et al.*, 2004; Schieker *et al.*, 2006). Autograft harvesting requires second operation for the harvesting of healthy tissue from the patient that can result in complications such as limited amount of autograft available, donor site morbidity, infections, etc. Allografts on the other hand have a high risk of infections, transmission of pathogens from donor to host and possibilities of

immune rejection (Gaalen, 2008; Mastrogiacomo *et al.*, 2005; Salgado *et al.*, 2004; Schieker *et al.*, 2006).

To overcome these problems, as an alternative, tissue engineering approach can be used where three-dimensional scaffolds made from polymeric materials can be used to induce growth of normal bone tissue. Bone tissue engineering has been studied in detail by many researchers. Natural biodegradable polymers such as collagen (Lee *et al.*, 2001), fibrin (Bensaïd *et al.*, 2003), chitosan (Zhao *et al.*, 2002), starch (Salgado *et al.*, 2004), and hyaluronic acid (Liu *et al.*, 1999) are widely used as scaffolds for bone tissue engineering. They exhibit good biocompatibility, capacity of interacting with host's tissue, and some osteoinductive properties (Liu *et al.*, 1999). As described earlier, poly (α-hydroxy acids) (Mooney *et al.*, 1996), poly (ε-caprolactone) (Hutmacher *et al.*, 2001) and poly (anhydrides) (Ibim *et al.*, 1998) are widely used in bone tissue engineering. Table 1.3 summarizes the polymers that are used in bone tissue engineering.

Hydroxyapatite HA is major constituent of natural bone and is considered osteogenic when added to synthetic polymer scaffolds (Hollinger *et al.*, 1996; Marra *et al.*, 1999). It is added to bone scaffolds not only for its osteoconductive and osteoinductive properties but also to enhance mechanical strength of the scaffolds. It has been extensively studied in bone tissue engineering applications. The osteoconductivity of polymers can be enhanced by attachment of proteins derived from ECM, particularly Arg-Gly-Asp (RGD). RGD peptides have shown to promote growth, attachment and migration of osteoprogenitor cells as well as osteoblasts (Gaalen, 2008; Shin *et al.*, 2003; Yang *et al.*, 2001).

Table 1.3. Natural and synthetic polymers used in bone tissue engineering (Courtesy: Salgado *et al.*, 2004)

Material	Origin	Characteristics
Collagen	Natural	Low immune response Good substrate for cell adhesion Chemotactic Scaffolds with low mechanical properties
Fibrin	Natural	Promotes cell migration and vascularization Promotes Osteoconduction Usually is used as a cell carrier for cell seeding on scaffolds
Chitosan	Natural	Hemostatic Promotes osteoconduction and wound healing
Starch	Natural	Thermoplastic behavior Good substrates for cell adhesion Non-cytotoxic and biocompatible Bone bonding behavior when reinforced with hydroxylapatite Scaffolds based on these materials have good mechanical properties
Hyaluronic acid (HA)	Natural	Minimal immunogenicity Chemotactic when combined with appropriate agents Scaffolds with low mechanical properties
Poly(hydroxybutyrate)	Natural	Natural occurring β-hydroxyacid Adequate substrate for bone growth Usefullness is limited due to brittle nature
Poly(α-hydroxy acids)	Synthetic	Extensively studied aliphatic polyesters Degradation by hydrolysis Already approved for other health related applications Acidic by products (e.g. lactic acid, glycolic acid), that enter the tricarboxylic acid cycle or in alternative (e.g. glycolic acid) are excreted in the urine It can present problems regarding biocompatibility and cytotoxicity in the surrounding area of the implantation site
Poly(ε-caprolactone)	Synthetic	Aliphatic polyester Degraded by hydrolysis or bulk erosion Slow degrading Degradation products incorporated in the tricarboxylic acid cycle Low chemical versatility Some problems related with withstanding mechanical loads
Poly(propylene fumarates)	Synthetic	Unsaturated polyester consisting on alternating propylene glycol and fumaric acids. Main degradation products are fumaric acid and propylene glycol Satisfactory biological results
Poly(BPA iminocarbonates)	Synthetic	Good biocompatibility when implanted in a bone canine chamber model
Poly(phosphazenes)	Synthetic	Contain alternating nitrogen and phosphorous with no carbon athoms in the backbone structure Degradation through hydrolysis
Poly(anhydrides)	Synthetic	Mainly developed as drug delivery carriers Biocompatible Support both endosteal and cortical bone regeneration

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Other factors such as transforming growth factors (TGF)-β, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), fibroblastic growth factor (FGF), and platelet-derived growth factor (PDGF) have also been associated with stimulation of bone formation and fracture healing (Gaalen, 2008). These can be either released to a specific site to stimulate bone formation via a delivery vehicle mechanism or covalently linked to a biomaterial surface that are used as implants for bone formation (Jennissen, 2002; Yasko *et al.*, 1992). Thus, several osteoconductive and osteoinductive components can be supplemented along with the bio scaffold to induce osteogenesis.

1.8.   Goal of this Study

The overriding goal of this project is to utilize sugarcane components and its by-products for the manufacture of bio-polymers that can serve as a value addition step to the sugarcane industry. The other aspect of this research is to evaluate the potential of these synthesized polymers in tissue engineering applications. The ultimate goal of this project is to use sugarcane industry as a biorefinery where all its by-products are utilized to generate several value added streams. In this work, molasses, a by-product of sugarcane processing will be utilized to isolate one of the monomers of polyester synthesis. We propose to synthesize polyesters of aconitic acid, cinnamic acid and glycerol. Aconitic acid, a major organic acid present in sugarcane can be extracted from sugarcane molasses and is investigated in this project. Polyesters of different compositions shall be synthesized and evaluated for their tissue engineering potential by a variety of tests and analyses. In this work, we propose to use these synthesized polyesters as scaffolds to promote differentiation of human adipose derived stem cells in wound healing and bone tissue engineering applications. This can be achieved by the addition of appropriate growth/differentiation factors to the cell culture medium and analyzing their effects on the cultured stem cells. Chapter 2 of this

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dissertation focusses on the extraction and purification of aconitic acid from molasses using solvent extraction. The aim is to isolate high purity, polymerization grade aconitic acid that can be directly used as a monomer for polymer synthesis. Chapter 3 involves synthesizing polyesters of aconitic acid, cinnamic acid and glycerol (materials that can be isolated from sugarcane industry by-products) and testing their potential as biodegradable scaffolds in skin tissue engineering applications, especially wound healing using hASC. Chapter 4 describes in detail the use of these polyesters as scaffolds for bone tissue engineering by the addition of osteogenic factors, dexamethasone, glycophosphate, and ascorbate-2-phosphate to the cell culture medium. The future direction of this research project is discussed in Chapter 5.

1.9. References

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CHAPTER 2. THE RECOVERY OF POLYMERIZATION GRADE ACONITIC ACID FROM SUGARCANE MOLASSES[1]

2.1.   Introduction

The processing of sugarcane to yield raw sugar generates several streams like residual molasses, which, in addition to sucrose and reducing sugars, contains an economically recoverable quantity of aconitic acid (Haines and Joyner, 1955). The sugars can be fermented to yield ethanol and vinasse. Molasses is a low-cost source of sugar and significant research has been aimed at the addition of value to agricultural by-products via fermentation to ethanol (Ghorbani *et al.*, 2011). Aconitic acid is an unsaturated, tribasic organic acid that exists as two isomers (trans- and cis-). Trans-aconitic acid is the favored form. For simplicity, 'aconitic acid' is used throughout the text instead of 'trans-aconitic acid'. In sugarcane, the amount of aconitic acid (free or as the Ca^{2+}/Mg^{2+} salt) varies widely. Accordingly, the molasses produced has been found to contain amounts of aconitic acid ranging from 0.9% to 5.5% (on dry solids basis) (Collier, 1950). The recovery of aconitic acid from molasses is important because the acid has several industrial uses. To name a few, the esters of aconitic acid are used in vinyl polymers, plasticizers and wetting agents (Azzam and Radwan, 1986; Reece, 2003). Thus, extracting this valuable component from molasses would be a value-addition step to the sugarcane industry.

In Louisiana, cane harvesting occurs between September and December so the sugarcane mills are only operational for 3 months of the year. Because molasses can be processed after the grinding-season, the extraction of aconitic acid from molasses can create a profit-based incentive to isolate feedstock chemicals that can be used to manufacture high-value products during the rest of the year (McMurray and Griffin, 2002; Zapata, 2007). Sugar prices have fluctuated between 5 and 30 US cents per lb over the past 20 years. In 1980–2000, they varied between 5 and 15 US

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cents per lb, while over the next decade, they ranged between 7 and 26 US cents per lb (FAO, 2012). Due to the volatility in the commodity-cost of sugars (apparently increasing with time), we sought to recover aconitic acid from molasses without degrading the value (either as-is or as ethanol) of the sugars contained therein.

An industrially feasible isolation of aconitic acid via precipitation of the Ca^{2+}/Mg^{2+} salt was made commercial in Louisiana Raceland Raw Sugar Corp., (Haines and Joyner, 1955), but a lack of demand for the chemical led the industry to abandon the practice. The extraction of aconitic acid from molasses via liquid-liquid extraction and ion-exchange has also been studied. In particular, the extraction by precipitation has been studied by many, but it suffers from low yield and requires an added acidification/isolation step to convert the salt to the free acid (Zapata, 2007). Although the isolation of aconitic acid via ion-exchange resin has been studied thoroughly, (Hanine *et al.*, 1992; Regna and Bruins, 1956; Saska M., 2006; Zapata, 2007) the lifespan of the resin and the regeneration cost needs to be studied in detail before extraction of aconitic acid via this method can be considered as a commercially viable option.

Solvent extraction of aconitic acid from molasses has been studied and developed by several researchers (Azzam and Radwan, 1986; Barnes *et al.*, 2000; Malmary *et al.*, 1995; Regna and Bruins, 1956; Zapata, 2007). Various solvents such as methyl-isobutyl ketone (MiBK), methyl ethyl ketone (MEK), tributyl phosphate (tBOP), ethyl acetate (EtOAc), and various alcohols have been tested (Azzam and Radwan, 1986; Barnes *et al.*, 2000; Malmary *et al.*, 1995; Regna and Bruins, 1956; Zapata, 2007). Malmary *et al*. (1995) investigated the extraction of aconitic acid from synthetic aqueous solutions composed of aconitic acid, citric acid and malic acid in varying proportions using tBOP. They studied the influence of solvent ratio and the effect of pH on the efficiency of trans -aconitic acid extraction. Back-extraction of aconitic acid from the tBOP phase

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with pure water was followed by stripping with sodium hydroxide. Unfortunately, it was not clearly stated whether the free form or salt of aconitic acid was isolated. In another study conducted by Barnes *et al.* (2000), the liquid- liquid extraction of an aqueous solution of trans- aconitic was evaluated using tBOP diluted with hexane. The phase diagrams of water: t-aconitic acid: tBOP were obtained. When tBOP is used as the extracting solvent, an additional step (back extraction of aconitic acid from organic phase) needs to be performed to obtain the free form of acid. Regna and Bruins (1956) studied the extraction of aconitic acid from molasses with MEK- water solution. The solvent was evaporated to obtain aconitic acid in its free form. They performed a cost analysis of the different methods (precipitation, solvent extraction, use of ion exchange resins) and concluded that solvent extraction process was 24% cheaper than the ion exchange resin process. Azzam and Radwan (1986) studied the extraction of trans-aconitic acid with EtOAc wherein molasses was acidified prior to extraction. In their study, a distillate was collected and ethyl acetate was separated by evaporation. The extraction efficiency of n-butanol (n-BuOH), EtOAc and tBOP was investigated by Zapata (2007) and it was concluded that while n-BuOH extracted the greatest quantity of aconitic acid, it also demonstrated the poorest selectivity. In contrast, while suffering low yield, EtOAc was the most selective of the solvents tested.

In this research, ethyl acetate was the solvent of choice for extraction of aconitic acid from molasses. The motivation behind selecting ethyl acetate as the solvent was the final application of aconitic acid in synthesis of bio-polymers. Being the most selective solvent for aconitic acid, ethyl acetate had the advantage of extracting higher purity aconitic acid. For good yields of polymerization reaction, high purity of reactants is a must. Hence, ethyl acetate was the obvious choice based on previous studies (Zapata, 2007). The present study aims at extracting aconitic acid of high purity from molasses that can be directly used for polymerization. This study, for the first

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time, also reports extraction of aconitic acid from vinasse (material left behind after fermentation of molasses to ethanol). In the first step, ethanol is fermented from the sugars present in the molasses and in the subsequent step, aconitic acid is extracted from the vinasse. The yields and purity of the extracted acid were analyzed and ethanol was an additional stream generated that can be used to synthesize the extracting solvent, ethyl acetate.

2.2.   Material and Methods

2.2.1.   Preparation of Molasses Prior to Extraction

A 50 gal drum of molasses was obtained and stored at 4°C for future studies. Molasses (150g) of 80°brix (g per 100 g refractive dry solids) was diluted to 40°brix with distilled water. The amount of aconitic acid present in the molasses was determined via high performance liquid chromatography (HPLC, Agilent1200) with a BioRad Aminex HPX-87H (300mm $\times$ 7.8 mm) column, using a 0.0025M H_2SO_4 mobile phase with diode- array ultraviolet- visible detector at a wavelength of 210 nm.

Diluted molasses (40°brix) was acidified to pH 2 with sulfuric acid and heated to 60°C for half an hour. The acidified molasses was centrifuged (Dupont Sorvall RC-5, Newtown, Connecticut) at 8000 rpm (7464g) for 20 min to remove the impurities. The samples were subjected to a two-stage extraction with ethyl acetate (EtOAc) (extraction with two consecutive portions (450 g) of ethyl acetate), at a solvent to molasses ratio of 3:1 (450g of ethyl acetate to 150 g 80°brix molasses). Extractions were carried out in 500 mL shake-flasks at 30 and 40°C for 1, 3 and 6 h in a shaker- incubator (Amerex Instruments Inc. Lafayette, CA). Once extractions were completed, the molasses-ethyl acetate mixture was centrifuged to separate the phases. The EtOAc was evaporated using a rotary evaporator (Buchi Rotovapor R-200, Brinkmann Instruments Inc., Westbury, NY) and the concentrated mixture was dissolved in acetic acid and allowed to

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crystallize. The recovered EtOAc was used for subsequent extractions and the crude aconitic acid was dissolved in water and decolorized using granular activated carbon at 45°C for 45 min. The water was evaporated and the product subsequently recrystallized. The aconitic acid crystals were weighed to determine the yield and the purity was analyzed by HPLC as previously described. All the experiments were performed in triplicate.

2.2.2. Preparation of Molasses for Fermentation Followed by Extraction

A 150g portion of molasses (80°brix) was diluted to 20°brix with water (450 g) and used as-is (pH= 5.4). It was treated in two different ways prior to fermentation. In the first case, the molasses (20°brix) was autoclaved at 121°C for 20 min and then fermented. In the other case, molasses (20°brix) was sterilized via filtration through a 0.2 µm membrane (Pall Corporation, Ann Arbor, Michigan). Both samples were fermented using S*accharomyces cerevisiae* D_5A at 30°C for 30 h. The cell loading was 4% with an inoculum of $1x10^{10}$ CFU ml^{-1} per flask. After 30 h fermentation, samples were centrifuged at 8000 rpm (7464g) for 20 min and filtered (0.2µm filters, Pall Corporation, Ann Arbor, Michigan) to remove the yeast. The content of both sugars (sucrose, glucose and fructose) and ethanol was measured by HPLC Agilent 1200 Series using a BioRad Aminex HPX-87 K (300mm × 7.8 mm) column with 0.01 mM K_2SO_4 at 0.6 mL min^{-1} and a differential refractive index detector. The resulting molasses was extracted using ethyl acetate as previously described.

2.3.   Results and Discussion

2.3.1. Extraction of Molasses with Ethyl Acetate (no fermentation)

Diluted molasses (40°Brix) was acidified using concentrated sulfuric acid to a pH of 2. The pKa values for the 3 hydrogen atoms of aconitic acid are 2.80, 4.46 and 6.30 (Zapata, 2007). Hence, the pH of molasses was reduced to a value below the lowest pKa of the acid. The molasses used

in the study had an initial aconitic content of 2.8% (HPLC). Table 2.1 shows the recoveries and purity of the aconitic acid extracted at the various combinations of time and temperature.

Table 2.1. Yield and purity of aconitic acid extracted from molasses

	Temperature					
	30° C			40° C		
Time	1h	3h	6h	1h	3h	6h
% Yields	34.44±1.84	43.40±0.67	49.91±0.63	43.30±0.53	62.95±1.79	68.81±0.53
% Purity	99.85±0.18	99.62±0.06	99.53±0.36	99.68±0.17	99.89±0.18	99.62±0.25

The yield (in % of maximum present in feedstock) of aconitic acid increased with both time and temperature. The highest yield of 68.8% was obtained with extraction at 40°C for 6 h. For all conditions a range of 34–69% of the aconitic acid was extracted with a product purity of 99% (HPLC). The high purity aconitic acid thus obtained can be easily utilized as a starting material for synthesis of bio-polymers. Experiments were also carried out longer than 6 h and at higher temperatures, but no increase in yields was observed after 40°C- 6h. Hence, 68.8% was the highest yield that can be achieved under the given set of experimental conditions from molasses. The increase in yields with increase in time and temperature indicate that the process is mass transfer controlled with 69% being the equilibrium point for the extraction. At higher temperatures, the viscosity of the solution decreases, increasing the rate of mass transfer. Emulsions (molasses–ethyl acetate) also separate faster at higher temperatures due to lower viscosity of the mixed phase. These factors contribute to increase in yields at higher temperatures.

In a similar study conducted by Azzam and Radwan (1986), the results were reported in the form of a 'recovery ratio'. This was defined as $\frac{x-y}{x} \times 100$, where x is the mean amount of the acid content and y is the mean amount of the acid recovered per 100 g (Azzam and Radwan, 1986). The recovery ratio for their process was ~90%, while our recovery ratio was 85%. The study also

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reports that with their experimental set up, vapors of ethyl acetate and aconitic acid were entrained rather than a liquid- liquid extraction set up (where only the vapors of ethyl acetate would separate leaving behind aconitic acid).The aconitic acid was subsequently separated from ethyl acetate by distillation. In our study, we analyzed the ethyl acetate vapors that were condensed from the rotary evaporator using HPLC for any aconitic acid entrapment and none was detected. Similar observations were made by Saska and Zapata (2006) where they found none or mere traces of aconitic acid in the condensate along with ethyl acetate and it was concluded that liquid carry-over must have led to the observations by Azzam and Radwan (1986). In a study by Regna and Bruins (1956), with methyl-ethyl ketone as a solvent, yields of 90% were assumed in the process design and the aconitic acid purity detected was 96.8%. Zapata (2007) utilized butanol as the solvent for extraction of aconitic acid from molasses and its subsequent esterification to produce tributyl aconitate. They obtained higher yields of aconitic acid with butanol as a solvent (~85%) as compared to ethyl acetate (~62%). Though the yields were higher, the purity of aconitic acid was lower in case of butanol (~30% dry solids in the extract) as compared to (~55% dry solids in the extract) for ethyl acetate. They also reported that among the solvents tested for extraction, ethyl acetate was the solvent that had faster separation from the aqueous phase after extraction and lower color transfer. Since ethyl acetate is highly selective compared to other solvents, it had lowest extraction of color bodies in the extract as compared to butanol and tri-butyl phosphate.

The major achievement in this process was the extraction of aconitic acid of very high purity as compared to other studies. A simple decolorization operation using activated carbon gave aconitic acid purity as high as 99.89%. The purity of aconitic acid remained unaffected irrespective of the extraction parameters (time/temperature). About 70% of aconitic acid originally present in the molasses was extracted in a simple liquid-liquid extraction set up described here. The high

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purity, free form of aconitic acid obtained in this study can be directly used for the synthesis of polymers without any further treatment. By extracting the free form of acid directly, the additional step of acidifying the salt of aconitic acid to its free form and generating an acid effluent is avoided in this method. The sugar equivalent in this case (unfermented, aconitic acid depleted molasses) does not change and the molasses can still be used as a feed material.

2.3.2. Extraction of Molasses with Ethyl Acetate (after fermentation)

2.3.2.1 Fermentation of Molasses

The initial concentration of sugars in the molasses (80°brix) was 35.5, 2.3 and 7.3% (g per 100 g of molasses) of sucrose, glucose and fructose, respectively, as determined by HPLC. The consumption of sugars with time is graphically represented in Figure 2.1. It should be noted that the trends depicted in Figure 2.1 are for diluted molasses (20°brix). Similar trends were observed for autoclaved and filter sterilized molasses. Therefore, to avoid repetition, only an average of the two is represented.

The concentrations of sucrose and glucose were rapidly depleted to undetectable levels in about 6 h of fermentation using *S. cerevisiae.* The concentration of fructose declined much more slowly. A similar trend in sugar consumption was observed by (Ghorbani *et al.*, 2011). Figure 2.1 also represents the yield of ethanol as the concentration of sucrose, glucose and fructose declined throughout the period of fermentation. The synthesis of ethanol began between 1-2 h post-inoculation. A maximum ethanol yield of 12.4% (g per 100 g of molasses) was obtained after 30 h of fermentation, which was 75% of the theoretical. Similar yields of were observed by (Jones *et al.*, 1994) with 13% ethanol generated after fermentation at 30°C for 96 h. In a study by (Limtong *et al.*, 2007), using the yeast strain *Kluyveromyces marxianus*, an ethanol concentration of 8.7% and yield of 77.5% of theoretical yield was obtained at 37°C. The ethanol yields obtained in this

study were similar to those previously observed (Jones *et al.*, 1994; Limtong *et al.*, 2007; Ngwenya *et al.*, 2012).
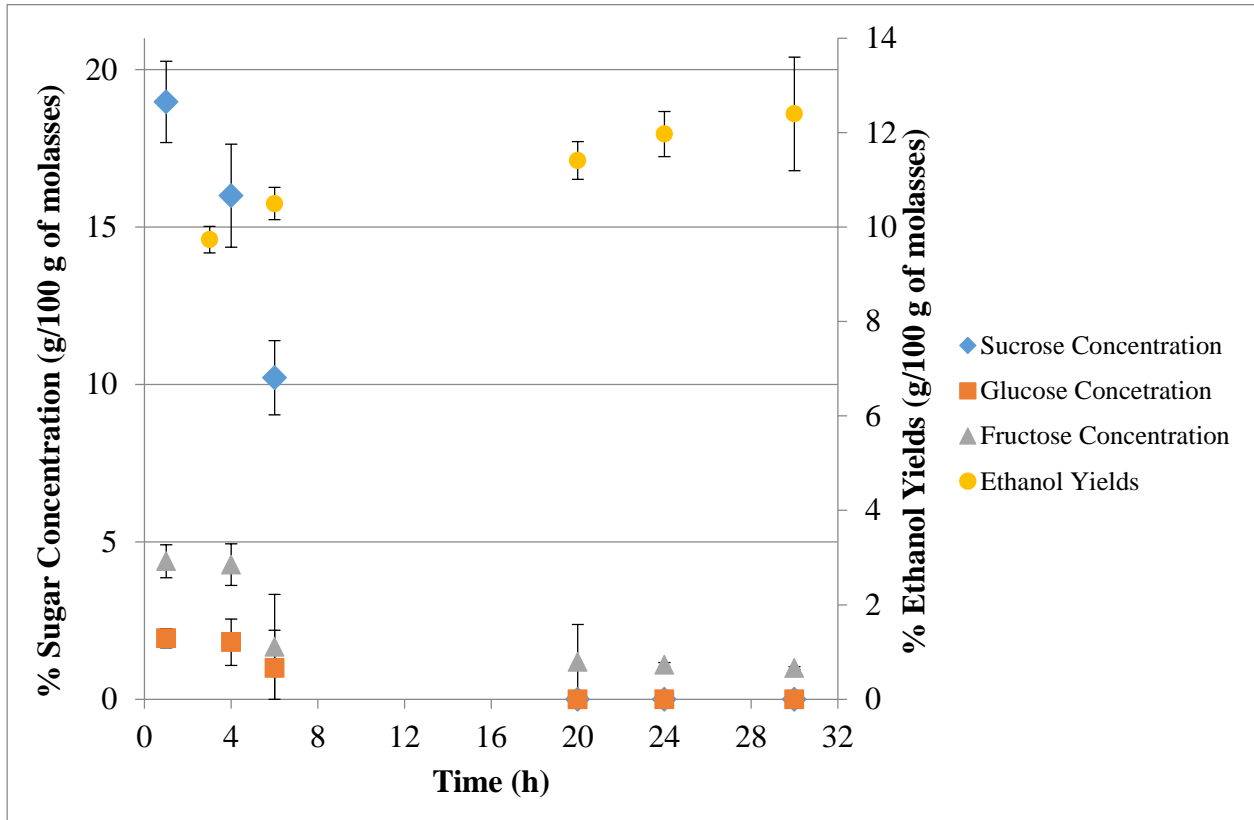
Figure 2.1. Change in sugar (sucrose, glucose fructose) concentration and ethanol yields over fermentation period

2.3.2.2. Aconitic Acid Yields of Fermented Molasses

After fermentation, the molasses (20°brix) were extracted with EtOAc. The yields and purities of aconitic acid obtained are listed in Table 2.2. A slight decrease in aconitic acid yield was observed when the extractions were carried out post-fermentation. To investigate whether fermenting the molasses prior to extraction depleted its aconitic acid levels, molasses prior to and after fermentation were analyzed for aconitic acid content. No loss of aconitic acid was observed in the fermentation broth after the completion of fermentation. The decrease in yields might be due to the formation of inhibitory products after fermentation that prevents complete or efficient recovery of aconitic acid from molasses. The yields ranged from 26–62%, lower than those

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observed with unfermented molasses (34–69%). The purity of the extracted acid was similar in both cases (fermented vs. unfermented molasses) indicating the high selectivity of ethyl acetate towards aconitic acid extraction. No difference in yields and purity was observed between the two methods (autoclaved vs. filter sterilized). Thus, the molasses preparation, e.g. heating the molasses to 120°C (autoclaved molasses) did not have an effect on the aconitic acid yield and purity.

Table 2.2. Yields and purity of aconitic acid extracted from molasses after fermentation

	Temperature					
	30° C			40° C		
Time	1h	3h	6h	1h	3h	6h
	Autoclaved Molasses					
% Yields	25.75±2.86	39.38±3.10	46.89±1.13	38.99±0.31	55.69±2.22	61.33±0.95
% Purity	99.49±0.22	99.60±0.11	99.61±0.25	99.57±0.27	99.45±0.18	99.58±0.23
	Filter Sterilized Molasses					
% Yields	26.26±3.17	38.27±2.55	46.66±0.63	39.38±0.63	57.86±0.89	62.26±0.66
% Purity	99.82±0.09	99.76±0.18	99.83±0.12	99.87±0.09	99.90±0.08	99.89±0.05

The EtOAc used for all the extractions was recovered and used for consecutive extractions. About 75–80% of solvent was recycled for following extractions, minimizing the total solvent consumption. The advantage of fermenting molasses prior to extraction is the generation of ethanol. Molasses is a low cost agricultural byproduct that is a rich source of sugars. A great deal of research into utilizing ethanol from renewable resources as an alternative source for energy has been conducted over the years. In this study, extracting aconitic acid as well as generating ethanol from molasses, a by-product of the sugarcane industry, not only adds value to this industry but also promises to be an economical route to obtain ethanol and high purity aconitic acid. Efforts are being devoted to synthesize a biopolymer of which aconitic acid is one of the components. The biopolymer can later be used as a tissue scaffold or for drug delivery in biomedical applications.

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Ethanol finds application as an alternative fuel or can be used for synthesizing ethyl acetate, the solvent used in the study. Thus, in this research, molasses which is a by-product of the sugarcane industry has been utilized to generate two valuable streams, aconitic acid and ethanol. These two products can serve as a medium to generate economic returns for sugarcane growers and processors.

2.4.   Conclusions

Extraction of aconitic acid, a major organic acid present in sugarcane molasses was investigated in this study. Molasses as is and after fermentation were extracted using ethyl acetate as a solvent. The extractions were carried out at temperature of 30 and 40 °C for 1, 3 and 6 h. The yields of aconitic acid from untreated molasses varied from 34–69%, depending on the extraction conditions, with purity of extracted acid being 99.9%. Fermented molasses gave aconitic acid yields of up to 62% and purities that reached 99.9%. The added advantage of the latter process included generation of ethanol as an additional product. The yield of ethanol was found to be 12.5% (g/100 g of molasses). The extracted aconitic acid is of a quality sufficient (99%) to synthesize polymers that hold promising potential in tissue engineering applications.

2.5.   References

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CHAPTER 3. SYNTHESIS OF NOVEL POLYESTERS FOR POTENTIAL APPLICATIONS IN SKIN TISSUE ENGINEERING

3.1. Introduction

Tissue engineering is a promising alternative to surgical procedures for the replacement or restoration of damaged or traumatized tissue (Enrione *et al.*, 2010; Shaker *et al.*, 2012; Zhu *et al.*, 2008). Traditional treatments to repair damaged and degenerated tissues involved autografting (transplanting tissue from one site to another of the same patient) or allografting (transplanting tissue from one individual to another). Autografting is expensive, painful and has anatomical constraints, while allografting suffers from the risk of rejection by the patient's immune system, and chances of infections and disease transmission from the donor to the patient (Heng *et al.*, 2005; Tangsadthakun *et al.*, 2006). Developing a tissue engineered scaffold laden with stem cells can prove to be a promising approach for tissue regeneration and repair.

In this study, the synthesis and characterization of a novel class of polyesters of glycerol, aconitic acid and cinnamic acid is reported. Glycerol, aconitic acid and cinnamic acid can be recovered from the by-products of sugarcane industry as detailed in Chapter 1. Synthetic polyesters have been a popular material of choice for tissue engineering applications and therapeutic delivery among the class of synthetic polymers. The advantages of polyesters are the ease of their degradation by the hydrolytic scission of the ester bond, the degradation products being non-toxic and easily metabolized by the body, and the ability to tailor the degradation rates by altering the chemical structure of the esters (Bettinger, 2011; Gunatillake and Adhikari, 2003)

The use of synthetic and natural polymeric materials as scaffolds for skin tissue engineering has garnered a lot of attention in the recent years (Dhandayuthapani *et al.*, 2010; Enrione *et al.*, 2010; Franco *et al.*, 2013; Tangsadthakun *et al.*, 2006; Yang *et al.*, 2009; Zhu *et al.*, 2008). An ideal scaffold should be able to support and guide cellular growth, provide mechanical and

structural support, allow transfer of nutrients, and regulate cellular activities. Many synthetic biodegradable polymers such as poly (caprolactone) (PCL), poly (D,L-lactide) (PDLLA), poly (glycolide) (PGA), and poly[lactic-co-(glycolic acid)] (PLGA) have been studied as scaffolds for skin tissue engineering (Enrione *et al.*, 2010; Pramanick and Ray, 1988; Yang *et al.*, 2009). Naturally occurring materials such as collagen, chitosan, gelatin, hyaluronic acid, dextran, amongst others have also been investigated as skin scaffolds for regeneration and repair (Enrione *et al.*, 2010; Yang *et al.*, 2009). Several groups have used a bilayer scaffold approach by blending synthetic and naturally occurring materials to combine the best of both techniques (Dhandayuthapani *et al.*, 2010; Duarte *et al.*, 2010; Enrione *et al.*, 2010; Franco *et al.*, 2013; Ravichandran *et al.*, 2012; Tangsadthakun *et al.*, 2006; Yang *et al.*, 2009). Natural biomaterials mimic the molecular properties of the tissue, while synthetic biomaterials impart necessary mechanical strength to the scaffold.

A great deal of research has been done on combining stem cells with bio scaffolds for engineering tissues and cellular delivery. Adipose 'fat' tissue is one of the richest sources of stem cells that have the ability to differentiate into various skin cells and possess immense potential to regenerate damaged skin (Bajada *et al.*, 2008; Chan *et al.*, 2012; Lu *et al.*, 2012). The idea of using adipose tissue as source for stem cells is attractive, since hASC can be easily isolated from the liposuction aspirates obtained from subcutaneous adipose tissue of the subjects undergoing liposuction surgeries. It is an abundant source of mesenchymal stem cells that can be differentiated into fibroblasts/keratinocytes using a bio-polymer scaffold (Trottier *et al.*, 2008).

In this study, the synthesis and characterization of a novel class of polyesters of glycerol, aconitic acid and cinnamic acid has been reported. Glycerol, aconitic acid and cinnamic acid can be recovered from the by-products of sugarcane industry. Utilizing the by-products of sugarcane

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industry not only creates incentives for growers/processors by generating value added products (bio-polymers) but also paves way for synthesis of novel biomaterials that hold promising potential in tissue engineering scaffolds. The synthesized scaffolds were also studied for their ability to support growth and differentiation of human adipose derived mesenchymal stem cells (hASC) by the addition of basic fibroblast growth factor (bFGF), and analyzing the amount of collagen synthesized.

3.2. Materials and Methods

3.2.1. Synthesis and Characterization of Polyesters for Tissue Engineering

3.2.1.1. Synthesis of Polyesters

Glycerol (≥99%), trans aconitic acid (98%) and trans cinnamic acid (97%) were purchased from Sigma-Aldrich and used as is. All the three components were stirred continuously in a beaker using an overheard stirrer. The reaction was carried out for 5 h at 120°C using a constant temperature oil bath. Few drops (0.5 ml) of concentrated sulfuric acid were added as a catalyst. The amount of reactants was varied to synthesize polymers having different compositions (varying amounts of free –OH and –COOH groups). Table 3.1 shows the different polymer compositions synthesized and used for further tests in this study. It was observed that the amount of cinnamic acid (discussed later in the chapter) present in the polymer had an effect on hASC growth and proliferation. Hence, polymers having varying amount of cinnamic acid were investigated. After the completion of reaction, polymer containing solution was knife casted into thin films on an aluminum foil and cured overnight in the oven at 105°C. Cured polymers were given water washings to remove the catalyst and any unreacted monomers present in them. The number of washings were determined based on the pH of the washed water (until it reached a neutral value). The samples were further cross-linked under UV light for 30 min using a UV transilluminator.

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Table 3.1. Different polymer compositions tested in the study

	Glycerol	Aconitic Acid	Cinnamic Acid
	(mol %)		
Excess Glycerol (–OH)(EG)	60	30	10
Excess Acid (–COOH) (EA)	40	50	10
Equal –OH and –COOH (EQ)	40	30	30
Low Cinnamic Acid (AGC)	63	31	6
Low Cinnamic Acid (AGC$_1$)	64	27	9
No Cinnamic Acid (AG)	63	37	0

3.2.1.2. Fourier Transform Infrared Spectroscopy- Attenuated Total Reflectance (FTIR-ATR) Analysis

The FTIR spectra of reactants and fine powdered samples were recorded at room temperature (25°C) using a Bruker Tensor 27 FTIR and OPUS data collection program. It was equipped with a room temperature DTGS detector, mid-IR source (4000 to 400 cm^{-1}, and a KBr beamsplitter.

3.2.1.3. Mechanical Testing

Compression testing of cylindrical polymeric discs 5 mm x 10 mm was performed at room temperature (25°C) using a hydraulic universal testing machine (Instron Model 5969, Canton, MA, USA) at an extension rate of 0.5 mm/min to a maximum compression strain of 90%.

3.2.1.4. Micro-CT Analysis

Samples were cut to a length of 10-15 mm and 1-2 mm thickness and imaged with 12 keV monochromatic X-rays with 2.5µm/pixel resolution at the tomography beam line at the Center for Advanced Microstructures and Devices (Louisiana State University, Baton Rouge, LA). Total number of projections were 720 corresponding to 0.25 increment in the angle of rotation (Δθ=0.25). Projection exposure time varied from 3-4 sec, though reconstruction algorithms ensured normalized data. To analyze the three dimensional data, two dimensional slices were read

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into Image Processing Toolbox in MATLAB (MathWorks, Natick, MA). For each slice, the gray scale image was converted to a binary image. Three-dimensional (3-D) files were reconstructed from 2-D images. Measurements of 2-D and 3-D images were performed with AVIZO® Standard packages (FEITM Visualization Sciences Group). Avizo 7.0.1(Visualization Services Group) generated the volume renderings from the 3D data of the different samples. A red-orange colormap was used to present 3D structure of all types of scaffolds. Image J generated 2-D orthogonal slices possessing grey colormap settings using the same data with a scale equivalent to the 3-D rendering. Morphological operations were performed to remove small imaging artifacts, and isolate interior and exterior pores. After quantifying solid and void pixels, porosity was calculated as follows:

$$\phi = \frac{Vpores}{Vpores + Vsolid} \times 100\%$$

where, V_{pores} = Total volume of all pores and V_{solid} = Total volume of solid (polymer)

3.2.1.5. hASC Isolation and Culture

All tissues were obtained with informed consent under a clinical protocol reviewed and approved by the Institutional Review Board at the Pennington Biomedical Research Center. Isolation of human adipose derived stem cells (hASC) was performed as described elsewhere (Gimble *et al.*, 2010). Liposuction aspirates from subcutaneous adipose tissue were obtained from three different donors. "Passage 0", p0 refers to the initial passage of the primary cell culture. p0 cells were cultured in T125 flasks with stromal media (Dulbecco's modified eagle medium, 10% fetal bovine serum and 1% triple antibiotic solution). The cells were passaged after trypsinization and plated (Passage 1) for expansion in T125 flasks in order to attain 80% confluence. Passage 2 (p2) of each individual was used for further tests.

3.2.1.6. Mass Loss Test

The mass loss of polyester samples in stromal media over 7 days and 14 days period was

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analyzed. Polymer samples were placed in 5 ml of stromal media in a 15 ml centrifuge tube. All samples were incubated at 37°C on an orbital shaker incubator at 200 rpm/min for the specified time period (1 week/2 weeks). After each time period, polymer samples were removed from the stromal media and freeze dried to constant weight. The stromal media extracts were refrigerated for cell viability tests described below. Difference in weight of polymer samples indicates the degradation rate in stromal media. All samples were normalized to the initial weight (before exposure to the stromal media).

3.2.1.7. Extract Cytotoxicity (Cell Viability)

The stromal media extracts from the mass lost test were filtered using 0.2 µm syringe filters (Thermo Scientific, Rochester, NY). Approximately, 100 µl of filtered media extract was pipetted into a 96-well plate previously sub cultured with hASC (25,000 cells/well). The 96-well plate containing hASC-stromal media extract was incubated at 37°C in 5% CO_2 for 24 h. The toxicity of the stromal media extracts towards hASC was determined using alamarBlue® assay. 10 µl of alamarBlue® reagent (10% of the volume of stromal media in each well) was added to each well containing hASC in stromal media extracts and re-incubated at 37°C in 5% CO_2 environment for 24 h. At the end of 24 h, fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a Perkin Elmer Wallac 1420 Victor2 microplate reader (Shelton, CT). hASC cultured with stromal media in tissue cultured 96-well plate served as positive control, while ethanol (70%) added wells served as negative control. Stromal media extracts without cells served as blank to subtract background fluorescence.

3.2.1.8. hASC Loading on Scaffolds

Prior to loading of cells, polymer samples were soaked in stromal media for 2 h. Later the stromal media was removed and 5 µl (1 x 10^4 cells/µl) of p2 cells from three different donors were

pooled together and loaded on top of each sample. After 30 min of incubation at 37°C and 5% CO_2, the other side of each sample was loaded with the same number of cells. Stromal media was added to each well and polymers loaded with hASC were incubated at 37°C and 5% CO_2 for 7 days and 14 days. Samples were collected to assess cell viability with alamarBlue® stain after each time period.

3.2.1.9. *In Vitro* hASC Viability on Scaffolds with alamarBlue® Stain

The viability of stem cells on the scaffolds was analyzed using alamarBlue® indicator after 7 and 14 days period. The cell cultured scaffolds were removed and washed in phosphate buffer saline (PBS) three times. Later 10% alamarBlue® reagent in Hank's balanced salt solution (HBSS) (pH 7) without phenol red was added to the scaffolds and incubated for 24 h at 5% CO_2. 100 µl of incubated alamarBlue®/HBSS was placed in a 96 well plate and the fluorescence was measured using plate reader as described earlier. Scaffolds without cells served as blank to subtract background fluorescence from all the readings.

3.2.1.10. *In vitro* hASC Viability on Scaffolds with Quant-iT ^{TM} Picogreen® dsDNA Reagent

Total dsDNA amount was used to determine the number of hASC on each polymer sample as previously described (Liu *et al.*, 2008). Samples seeded with cells were removed from stromal media and washed with PBS three times. These were then crushed using forceps and incubated with 0.5 ml proteinase K (0.5 mg/ml) at 56°C overnight. The mixture was later centrifuged for 5 min at 108 g. 50 µl of supernatant was mixed with 50 µl Quant-iT ^{TM} Picogreen® dsDNA reagent in 96-well plates. Fluorescence was measured using Perkin Elmer Wallac 1420 Victor2 microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Scaffolds without cells served as blanks to subtract background fluorescence from all the readings.

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3.2.2. *In Vitro* characterization of synthesized scaffolds for skin tissue engineering

3.2.2.1. Synthesis of Polyesters

Thin film of the polyesters were fabricated as described earlier for further studies. Based on the cytotoxicity and picogreen analysis, polymers AGC (63, 31 and 6 mol % of glycerol, aconitic acid and cinnamic acid respectively) and AG (63 and 37 mol % of glycerol and aconitic acid respectively) were used for differentiation studies. Poly-caprolactone (PCL) scaffolds were prepared through thermal-precipitation method as described elsewhere (Zanetti, 2011) and were used as control in this study. Briefly, a 10% PCL solution in 8 ml of 1,4-dioxane was prepared in a glass bottle. The mixture was molded into a polydimethylsiloxane template and immediately transferred to a freezer at -20°C overnight. Post freezing, the samples were immediately freeze-dried for 48 h. These samples were then used for differentiation studies.

3.2.2.2. hASC Culture and Loading on Scaffolds

p2 of hASC were cultured and loaded on scaffolds as described earlier. The same amount of second cell passages from all donors (n = 3) were pooled and directly loaded on a single face of each scaffold type at a concentration of 1.0×10^4 cells/µL for total volume of 5 µL. After 30 min of incubation in a saturated humidity atmosphere at 37°C and 5% CO_2, the same volume of hASCs containing solution were directly applied on the opposite side of each scaffold as previously described (Zanetti *et al.*, 2012). Control groups included PCL scaffolds while the experimental groups included AG and AGC scaffolds. Scaffolds loaded with hASCs were immediately transferred to 48-well plates and cultured in stromal medium or stromal medium with basic fibroblast growth factor (bFGF) at the concentration of 2 µg/ml for 14 days. Cell medium were changed every 2-3 days. Triplicates were performed for each assay.

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3.2.2.3. *In vitro* Quantification of DNA on Scaffolds

Total dsDNA amount was used to determine the number of hASC on each scaffold as previously described. Scaffolds cultured in stromal and bFGF supplemented media were analyzed for their DNA content.

3.2.2.4. *In vitro* Quantification of Collagen on Scaffolds

Sirius red staining method was used to analyze the amount of collagen synthesized using the 'Sirius Red Collagen Detection Kit' purchased from Chondrex Inc. (Redmond, WA) and following their protocol. A standard curve was plotted by measuring the absorbance values of various standard solutions at 510-550 nm. Collagen concentration (µg/ml) was calculated for test samples using regression analysis from the standard curve. Cells without scaffolds were used as control and the difference between the stromal and bFGF supplemented media was studied.

3.2.3. Statistical Analysis

All results were reported as mean ± standard deviation. Data was evaluated with one or two way analysis of variance (ANOVA), and then analyzed by Tukey's minimum significant difference (MSD) post hoc test for pairwise comparisons of main effects. For all comparisons a p-value < 0.05 was considered significant.

3.3. Results and Discussion

3.3.1. Synthesis of Polyesters

Polyesters of different compositions were synthesized and tested in the study for their biocompatibility and cytotoxicity towards stem cells, of which the ones listed in Table 3.1 were used for further studies. Hyperbranched polyesters are an interesting class of materials and can be synthesized easily by one step condensation reaction (Stumbé and Bruchmann, 2004). Similar hyperbranched polyesters from multifunctional monomers have been reported (Cao *et al.*, 2011;

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Pramanick and Ray, 1988; Stumbé and Bruchmann, 2004). In this study, aconitic acid (A_3), glycerol (B_3) and cinnamic acid (a double bonded aromatic species) were polymerized to get a hyperbranched polyester product. To obtain different monomer end-capped polyesters, the feed ratio of reactants was varied in such a way that the number of free hydroxyl and carboxylic acid groups differed in the end product. Polymers with excess –OH groups, –COOH groups, and equal number of both groups were synthesized, and its effect on the growth of stem cells was analyzed. It was observed and as evident by alamarBlue® and picogreen assays (discussed later), the amount of cinnamic acid seemed to have a profound effect on cell attachment and growth profiles. Since multifunctional groups are present, a cross-linked, hyperbranched polyester structure as depicted in Figure 3.1 is expected. As seen in Figure 3.1, the three hydroxyl groups present on the glycerol molecule react with the carboxyl groups of either the aconitic acid and/or cinnamic acid. Glycerol serves as a branching unit where all the three OH groups react. The polymer can be further cross-linked by taking advantage of the double bond present in the cinnamic acid moiety that undergoes [2+2] photocycloaddition under UV-light.

For the initial set of experiments, polymerization reactions were carried out for shorter times in a beaker equipped with a magnetic stirrer. Reactions were stopped when the viscosity of the solution was high enough such that the stirrer could no longer mix the polymer solution. Thin film of polyesters were casted and cured overnight in the oven at 105°C as described. The rate of degradation for these polymer samples was rapid. Due to formation of acidic degradation products by the hydrolytic scission of the ester bonds present in the polymer matrix, there was a rapid decrease in pH of the stromal medium when polymers were suspended in it. This in turn had an adverse effect on the growth and proliferation of hASC as evident by their alamarBlue® assays.
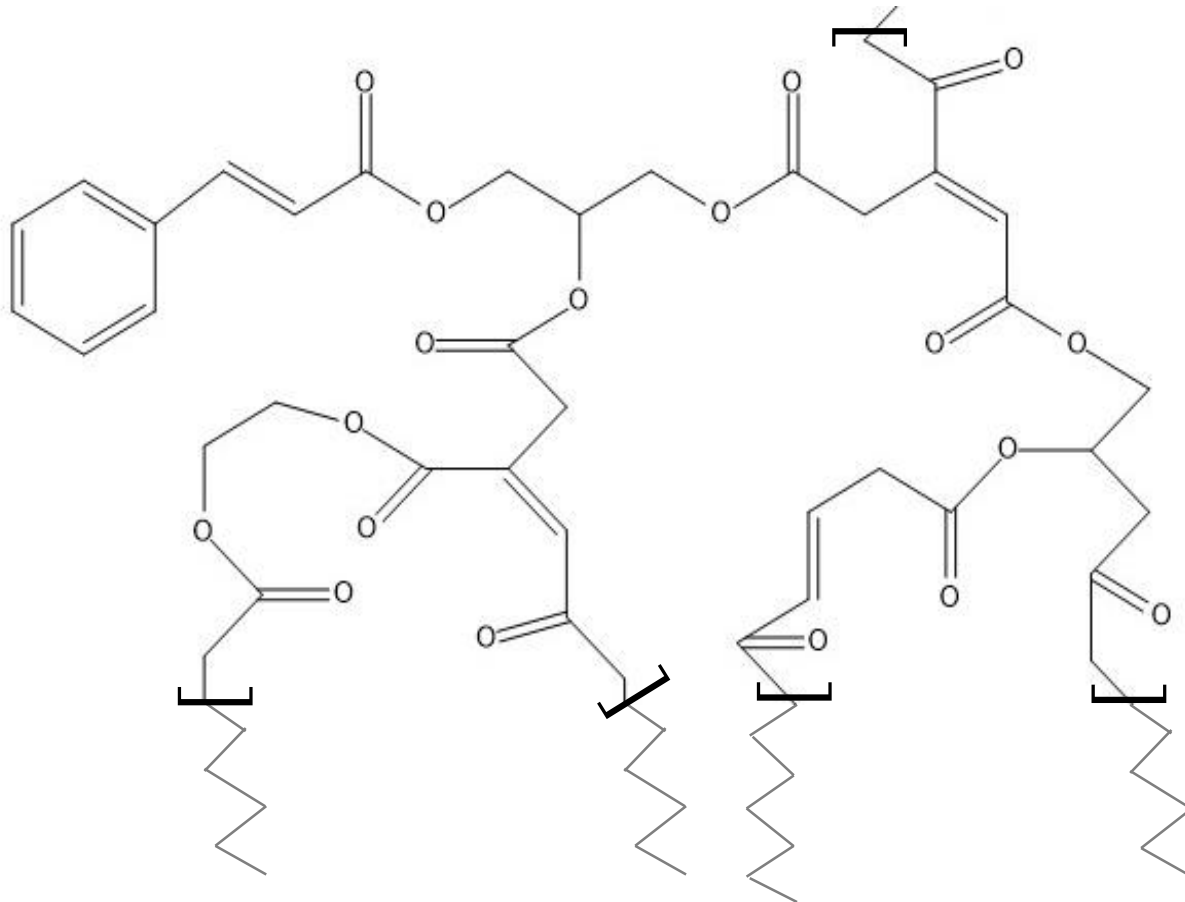
Figure 3.1. Hyperbranched polyester of aconitic acid, glycerol and cinnamic acid

To monitor their degradation rates, polymers were suspended in various solvents such as phosphate buffer saline (PBS), 1:1 ethanol-water and 1:1 tetrahydrofuran (THF)-water and their pH was monitored as shown in Table 3.2. A rapid drop in the pH of the solutions in which the polymer samples were suspended was observed. The pH of the solutions dropped to as low as 2 within a day of suspension of polymer samples in them. For ethanol and tetrahydrofuran solutions, pH drop was even faster with values reaching to 3–4 almost instantaneously. Cinnamic acid has a higher solubility in these solvents as opposed to aqueous solutions indicating that it was a major degradation component in this case that accounts for the low pH. The pH profiles indicated that the sudden hydrolytic scission of the ester linkages present in the polymer matrix led to the drop in the pH affecting hASC in a negative way.

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A gradual degradation of polyesters is favored such that there is no abrupt increase in the local acidity that can lead to cell death (Sung *et al*., 2004). To overcome this degradation problem, polymerization reactions were run for a longer time using an overhead stirrer that caused an efficient mixing of the reactants. Polymer samples were washed thoroughly with water and ethanol until a neutral pH value was attained. This step ensured removal of catalyst and any unreacted

Table 3.2. Rate of degradation of polymer samples in different solvents

Sample	Solvent	pH				
		t= 30 min	Day 1	Day 2	Day 3	Day 4
Excess Glycerol (– OH) (EG)	1M PBS	6.20	2.27	2.28	2.21	2.20
	0.5M PBS	6.41	2.03	2.08	2.06	2.05
	1:1Ethanol-water	3.55	2.30	2.27	2.33	2.30
	1:1 THF-water	3.22	2.41	2.34	2.38	2.35
Excess Acid (–COOH) (EA)	1M PBS	6.42	2.19	2.13	2.17	2.17
	0.5M PBS	6.30	2.28	2.22	2.23	2.20
	1:1Ethanol-water	3.51	2.35	2.39	2.39	2.38
	1:1 THF-water	3.30	2.42	2.38	2.25	2.26
Equal (–OH and –COOH) (EQ)	1M PBS	6.42	2.19	2.13	2.17	2.17
	0.5M PBS	6.30	6.30	2.28	2.22	2.23
	1:1Ethanol-water	3.51	3.51	2.35	2.39	2.39
	1:1 THF-water	3.30	3.30	2.42	2.38	2.25

monomer present in the polymer samples. Polymerizations were carried out at 120°C in order to strip off the water formed during the polycondensation reaction and drive the reaction forward.

The reaction procedure was verified by FTIR. The typical FTIR spectra for the synthesized polyester is shown in Figure 3.2. The characteristic peaks for the reactants: glycerol (3300 cm^{-1}), aconitic acid (1690 cm^{-1}) and cinnamic acid (1630-1670 cm^{-1}) are shown. For the polyesters, (AG and AGC), the intense stretch around 1730 cm^{-1} due to the carbonyl functional group (O–C=O)

stretching vibrations and the disappearance of the intense –OH peak (due to presence of glycerol) around 3300 cm^{-1} confirmed the formation of ester bonds. Similar FTIR spectra for ester bond formations have been reported (Cao *et al.*, 2011; Thomas and Nair, 2011).
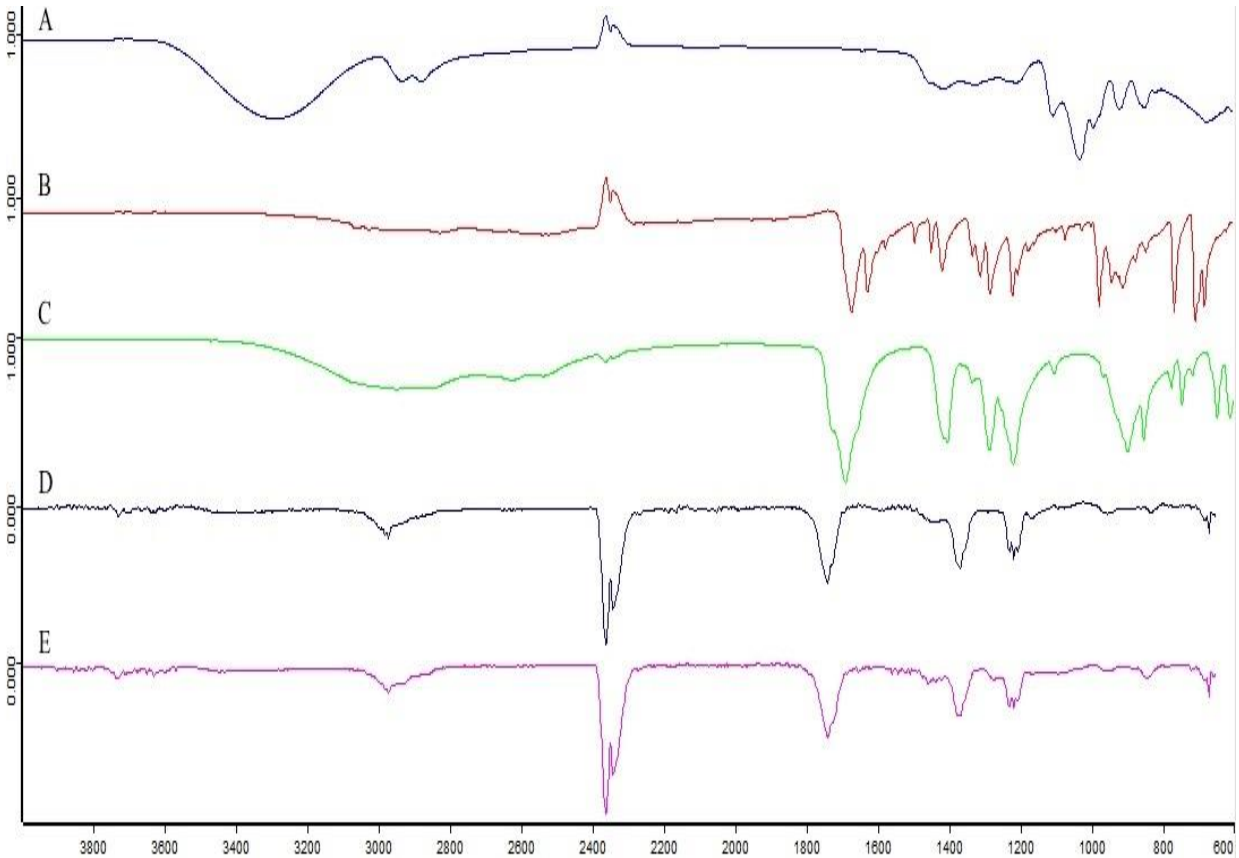
Figure 3.2. FTIR spectra of A] Glycerol; B] Cinnamic Acid; C] Aconitic Acid; D] AG; E] AGC (AG: Polyester of Aconitic Acid and Glycerol; AGC: Polyester of Aconitic Acid, Glycerol and Cinnamic Acid)

3.3.2. Mechanical Testing

A novel biomaterial that has the potential to be used as a scaffold in tissue engineering applications needs to be mechanically robust. Mimicking the complex mechanical properties of native tissues is difficult since each tissue has unique non-linear viscoelastic properties and strain-dependent moduli (Freed *et al.*, 2009; Garber *et al.*, 2013). Table 3.3 lists the compressive strength values for the synthesized polyesters. The compressive strength for all the polymer samples was

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found to be in the range of 5-6 MPa. However, the modulus of elasticity was found to vary for different polymer compositions. It was found to be lower (indicating more flexibility) for samples having excess of –OH groups and lower cinnamic acid content. For polymer samples containing relatively higher amount of cinnamic acid, the stiffness (higher Young's modulus) can be attributed to a highly cross-linked structure due to photocycloaddition of the cinnamic acid moeity. Thus the mechanical properties of these scaffolds can be finely tuned by varying the amount of monomers such that a final polymer with desired mechanical properties is obtained.

Table 3.3. Mechanical Strength for synthesized polyesters

Compressive Strength (MPa)	Modulus of Elasticity (MPa)	
Excess Glycerol (–OH)(EG)	5.30±0.63	21.92±4.92
Excess Acid (–COOH) (EA)	5.58±0.42	40.46±3.39
Equal –OH and –COOH (EQ)	5.90±0.94	50.03±5.7
Low Cinnamic Acid (AGC)	5.89±1.1	10.32±2.92
Low Cinnamic Acid (AGC$_1$)	5.33±0.58	24.68±4.89
No Cinnamic Acid (AG)	5.28±0.78	20.92±3.87

In a study by Zhu *et al.* (2008), electrospun fibrous mats of poly (DL-lactide-glycolide) for skin tissue engineering applications showed a maximum tensile stress of 1.6-4 MPa. While the mechanical properties data for dermal tissue engineering scaffolds is limited, tensile strength of dermal tissue engineering scaffolds can range from 0.8-18 MPa (Dhandayuthapani *et al.*, 2010; Enrione *et al.*, 2010; Franco *et al.*, 2013; Yang *et al.*, 2009; Zhu *et al.*, 2008). In another study by (Agache *et al.*, 1980) on mechanical properties of human skin in vivo, measurements of Young's modulus were performed by means of a torque applied to human skin. They concluded that skin revealed a loss in elasticity and stretchability, and an increase in Young's modulus as a person ages. Young's modulus varied from 0.42-0.85 MPa which was about 100 times smaller than that reported by Grahame and Holt (1969). Based on the application desired, mechanical properties of

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the scaffolds can be tuned for a tissue-specific applications. Many studies have reported the use of composite scaffolds by the incorporation of an inorganic phase in the polymer matrix to improve their mechanical properties (Lu *et al.*, 2003; Moradi, 2013).

3.3.3. Micro-CT Analysis

Micro-CT images for polymer samples were generated from 3D data using Avizo 7.0.1 (Visualization Services Group) and are shown in Figure 3.3. At the end of the polymer reactions, thin film polyesters fabricated using knife casting method were cured at 105°C in the oven. This caused the residual amount of water entrapped in the polymer matrix to escape, which in turn generated pores in the sample (Figure 3.3). From the micro-CT images, the mean porosity values of the samples were calculated and are presented in Figure 3.4. The porosity values were found to be in the range of 35-40% for all the polyesters samples. The highest porosity of 40.6% was observed for AG scaffolds, while the lowest value of 28.6% was reported for EA (polyesters containing excess –COOH groups). Micro-CT images show wide variations in the pore size. The pore size for the samples varied from 10-150 μm. The wide range of pore sizes and high porosity values provides an inter connected pore structure that can support cell migration, differentiation and transport of nutrients (Lawrence and Madihally, 2008). Porosity is an important factor to be considered for the design of a scaffold since it facilitates nutrient migration and removal of waste material from the cells. An optimum pore size for supporting cell growth is suggested to be in the range of 100-150 μm (Chin *et al.*, 2008; Lawrence and Madihally, 2008). Pore size below 20 μm would not allow proper access to cells, while inhibiting cell attachment for pores greater than 125 μm (Enrione *et al.*, 2010). A wide range of pore size observed in the synthesized polyesters can be advantageous for nutrient and waste transport by providing an interconnected pore structure. Porosity can also influence the mechanical properties of a scaffold. While a high porosity is desired

for good transport of nutrients, it can worsen the mechanical properties of the scaffold by reducing

the strength of the scaffolds due to higher degree of void spaces in them (Garber *et al.*, 2013).
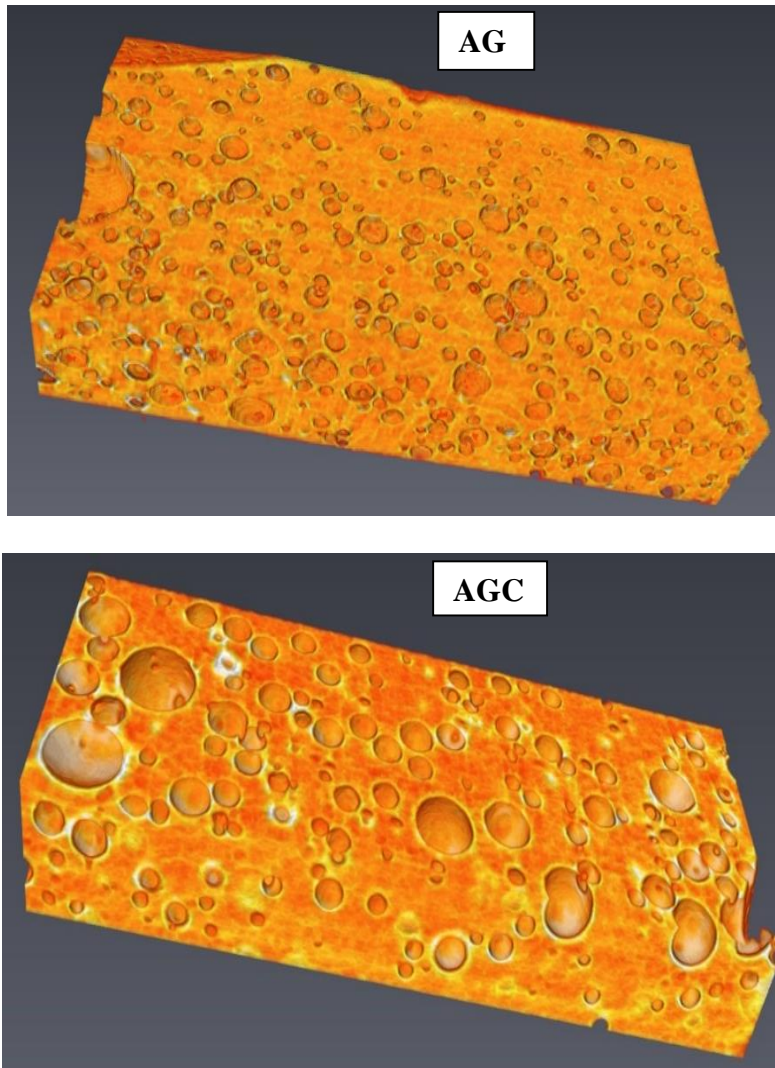
Figure 3.3. Avizo rendered 3D images of micro-CT data of scaffolds: AG (top) and AGC
(bottom)
(AG: Polyester of Aconitic Acid and Glycerol; AGC: Polyester of Aconitic Acid, Glycerol and
Cinnamic Acid)

In this study, the pores generated in the thin film polymer samples can be attributed to the

escape of water molecules from the bulk of the polymer matrix. Different techniques such as

solvent casting/particulate leaching, gas foaming, melt molding, fiber bonding, and phase

separation can be employed to create a well-defined porous scaffold material (Mikos, 2000;

Sachlos and Czernuszka, 2003). These can give a greater control over the porosity and pore structure of the scaffolds.
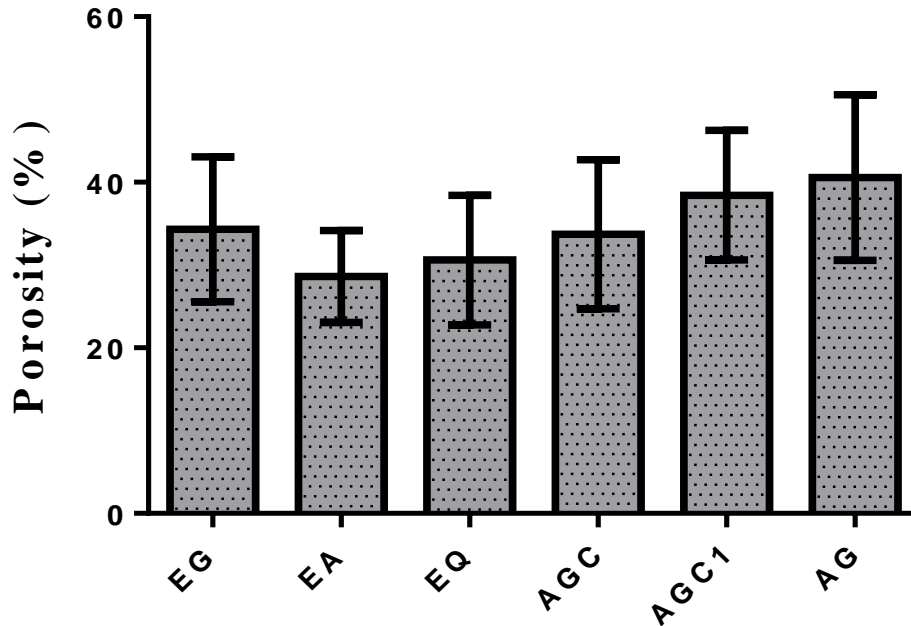
Figure 3.4. Porosity values for polyester samples
(EG: Polyester having excess glycerol (–OH groups); EA: Polyester having excess acid (–COOH groups); EQ: Polyester having equal moles of –OH and –COOH groups; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AGC1: Polyester of aconitic acid, glycerol and cinnamic acid and AG: Polyester of aconitic acid and glycerol (no cinnamic acid)

3.3.4. Mass Loss Test and Extract Cytotoxicity

The mass loss for the polymers exposed to physiological solution over a period of 7 days and 14 days in stromal media was determined and is represented in Figure 3.5. All the samples tested were found to retain their physical structure for 2 weeks without undergoing total degradation. For some samples, the increase in mass is likely to be a result of mineralization or non-specific protein deposition (Garber *et al.*, 2013). For tissue engineering applications, a gradual degradation of polyesters is favored rather than an abrupt hydrolysis of ester linkages. The sudden degradation of polyesters leading to higher concentration of degradation products may cause local tissue acidity and ultimately cell death. In this study, for some polyesters (shorter reaction times)

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(Table 3.2), total disintegration of polyester matrix occurred within 3 days leading to lower pH and hence decrease in viability of stem cells.
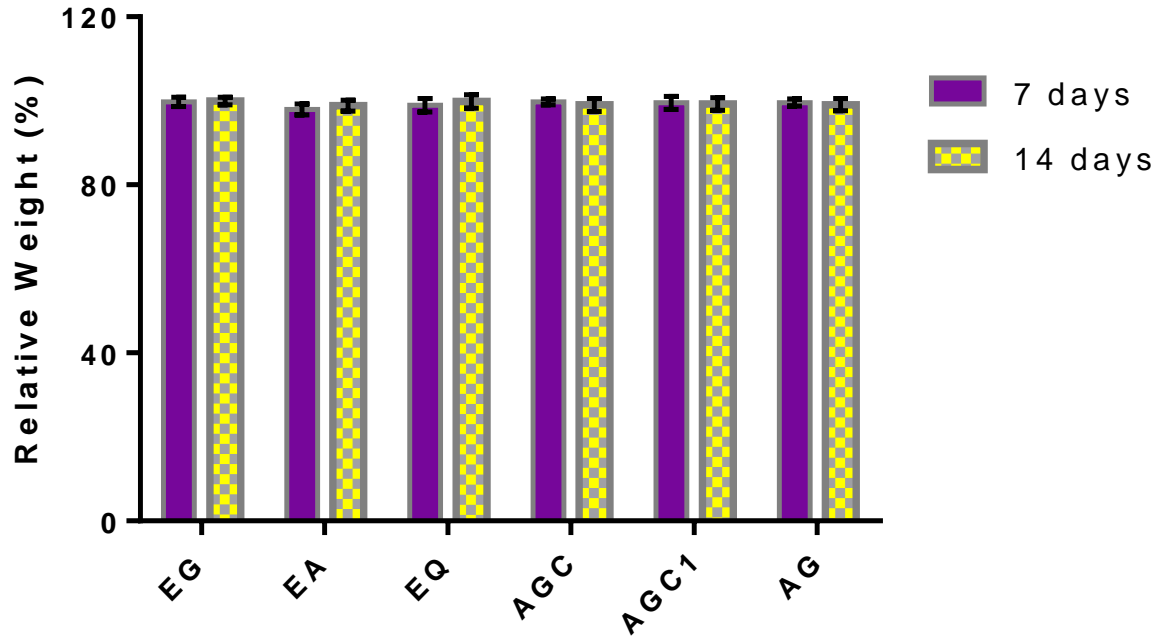
Figure 3.5. Mass loss of polyester samples after 7 and 14 days of incubation in stromal media. Samples are normalized to the starting mass of each sample
(EG: Polyester having excess glycerol (–OH groups); EA: Polyester having excess acid (–COOH groups); EQ: Polyester having equal moles of –OH and –COOH groups; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AGC1: Polyester of aconitic acid, glycerol and cinnamic acid and AG: Polyester of aconitic acid and glycerol (no cinnamic acid)

The extracts were later tested for cytotoxicity studies towards hASC. Figure 3.6 depicts the relative metabolic activity of polymers after 7 and 14 days as per the alamarBlue® analysis. The relative metabolic activity of polymers with higher –OH group content was found to be higher than the positive control for the 7 day period. At the end of 7 days, hASC on polymer with no cinnamic acid (AG) had significantly higher metabolic activity than the positive control. At the end of 14 days, cells exposed to all the polymer compositions had higher metabolic activity than the dead control or positive control. The metabolic activity for hASC on EG (excess –OH groups), AGC (lower cinnamic acid content) and AG (no cinnamic acid) was found to be significantly higher than the positive control at the end of 14 days. The ability of the polyesters to retain their structure

and be non-toxic to the hASC over a 14 day period implies their potential ability to support growth, proliferation and differentiation of stem cells for tissue engineering. In the next step, these polyesters were used as scaffolds and hASC were seeded on them, and their relative metabolic activity was analyzed (as discussed in the next section).
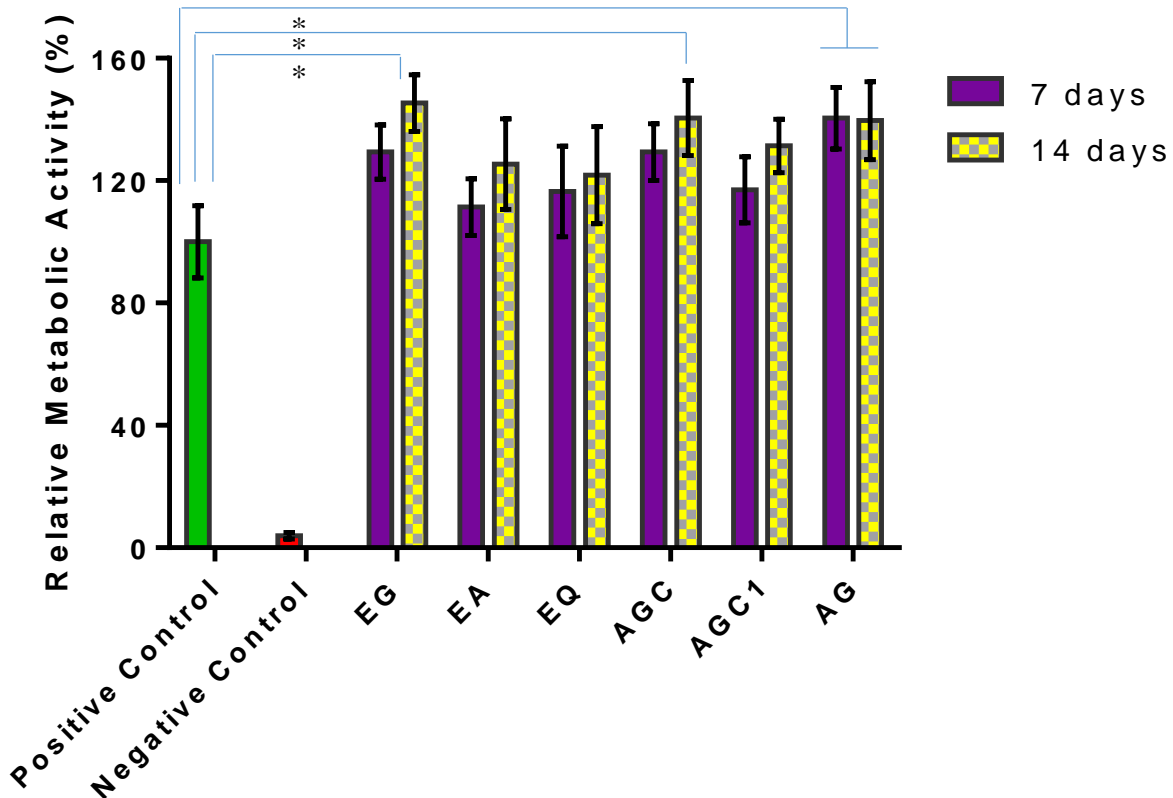
Figure 3.6. Relative metabolic activity of hASC exposed to 7 and 14 days stromal media extracts from respective polymers. The results are normalized to positive control.
*indicates statistical significance between two groups
(EG: Polyester having excess glycerol (–OH groups); EA: Polyester having excess acid (–COOH groups); EQ: Polyester having equal moles of –OH and –COOH groups; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AGC1: Polyester of aconitic acid, glycerol and cinnamic acid and AG: Polyester of aconitic acid and glycerol (no cinnamic acid)

3.3.5. Biocompatibility Tests of hASC on Biopolymer (hASC Loading on Scaffolds)

The ability of the polyesters to support adhesion and further promote growth of hASC was evaluated using alamarBlue® assay. The metabolic activity of hASC relative to positive control (cells with stromal media) was monitored for 7 and 14 days period and is illustrated in Figure 3.7.

Based on the prior results, stromal extracts exposed to polyester samples were non-toxic to hASC. alamarBlue® assay was performed to determine if the polyesters support the growth of hASC and to study the proliferation profiles of hASC by monitoring their relative metabolic activities on polyesters. At the end of 7 day period, cells cultured on polyesters with excess –OH groups and low cinnamic acid content had higher metabolic activity than the ones cultured on polyesters having higher –COOH groups. Similar trends were observed at the end of 14 days.

Figure 3.7. Relative metabolic activity of hASC cultured on different polymers for 7 and 14 days. The results are normalized to positive control.
*indicates statistical significance between two groups
(EG: Polyester having excess glycerol (–OH groups); EA: Polyester having excess acid (–COOH groups); EQ: Polyester having equal moles of –OH and –COOH groups; AGC: Polyester of aconitic acid, glycerol and cinnamic; AGC1: Polyester of aconitic acid, glycerol and cinnamic acid and AG: Polyester of aconitic acid and glycerol (no cinnamic acid)

Cells on polymers EA (excess of –COOH groups) and EQ (equal number of –OH and –COOH groups) displayed significantly lower metabolic activities than the positive control at the

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end of 7 and 14 days ($p < 0.05$). It appears that hASC cultured on polyesters having excess of –OH groups and lower cinnamic acid (hydrophobic) component demonstrated higher metabolic activity than the other two polyesters (excess acid and equal moles). These metabolic assays indicated that polymers EG, AGC, AGC$_1$ and AG can be utilized as scaffolds to support growth of hASC in tissue engineering applications since they do not undergo any disintegration in the structural integrity and have proven to be non-toxic to their growth.

3.3.6. DNA Quantification on Scaffolds (Picogreen Assay)

DNA content of hASC cultured on all polyester samples was measured as a relative measure of cell viability and proliferation. Figure 3.8 shows the DNA content of hASC cultured on all the polyesters at the end of 7 and 14 days. Similar trends for DNA content were observed, as evident by metabolic activity assays using alamarBlue®. Polyester samples having higher free hydroxyl groups and lower cinnamic acid indicated the most DNA content. After 14 days of culture, the highest DNA content of 90% relative to control was observed in the AG scaffold with no cinnamic acid followed by AGC and AGC1 polyesters with lowest cinnamic acid content. For the EG, EA and EQ samples, the total DNA content was found to be significantly lower than the positive control. As inferred from the relative metabolic activity data, polymers with no or lower cinnamic acid content seem to support cell growth in a more favorable way. This can be attributed to the presence of phenolic aromatic group in the cinnamic acid that adversely affects the growth of the cells. At the end of 14 days, AG and AGC scaffolds exhibited significantly higher amount of DNA ($p < 0.05$) than the rest of the polyester samples. Based on these results, polymers AG and AGC were used for further studies. PCL scaffolds were used as control and all the polymers were cultured in stromal medium and stromal medium containing bFGF.
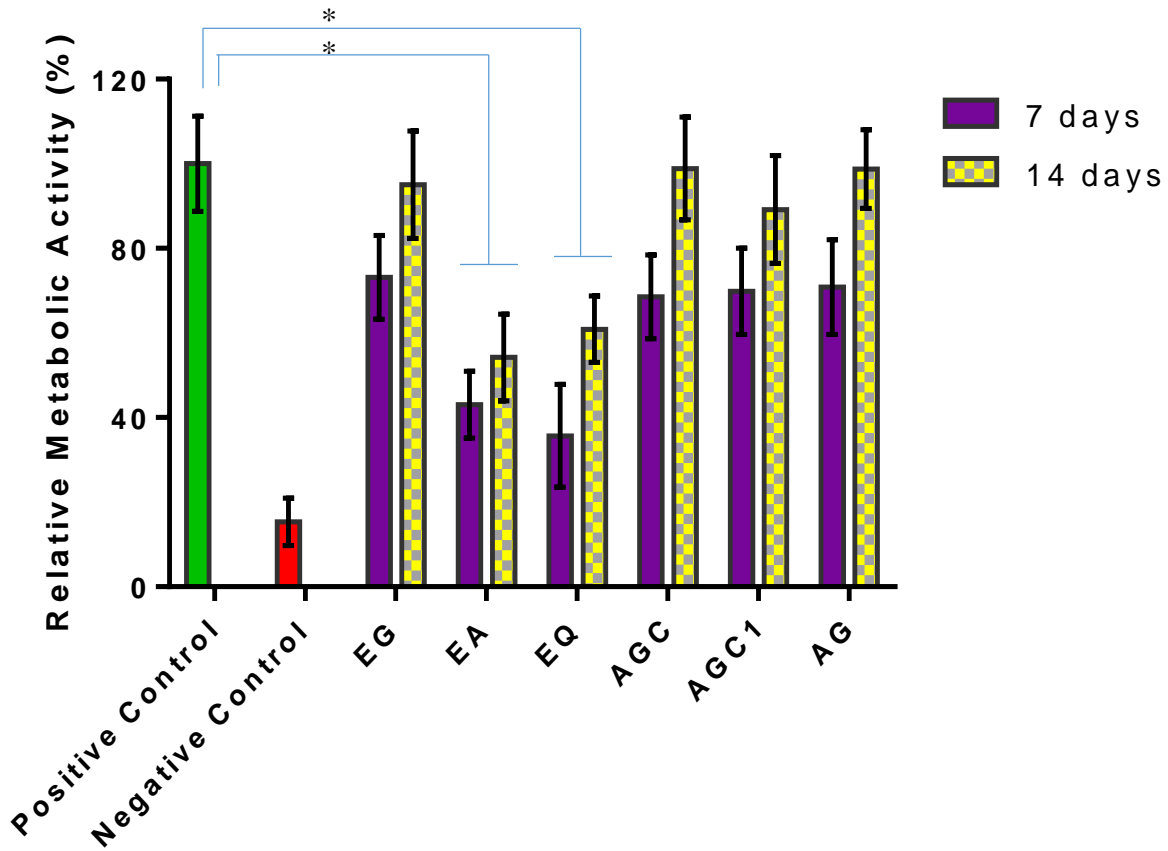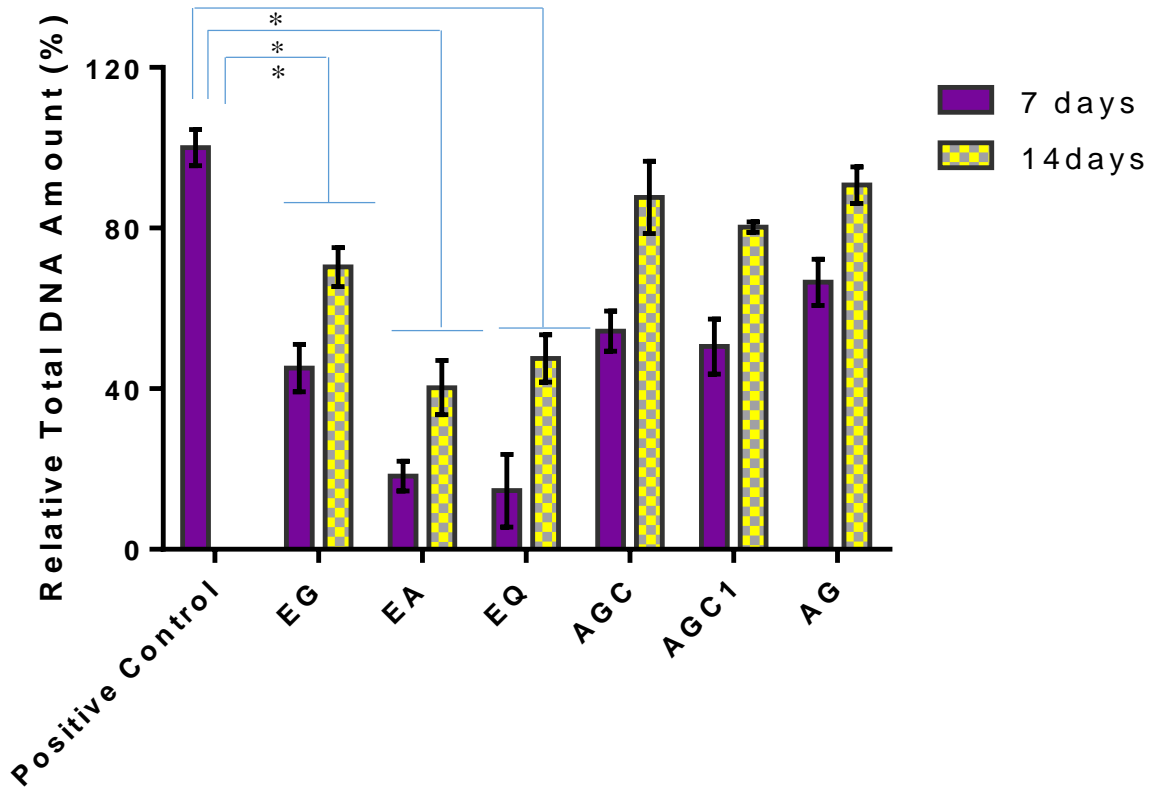
Figure 3.8. Relative total DNA amount for hASC cultured on different polymers for 7 and 14 days. All the results are normalized to positive control.
*indicates statistical significance between the two groups
(EG: Polyester having excess glycerol (–OH groups); EA: Polyester having excess acid (–COOH groups); EQ: Polyester having equal moles of –OH and –COOH groups; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AGC1: Polyester of aconitic acid, glycerol and cinnamic acid and AG: Polyester of aconitic acid and glycerol (no cinnamic acid)

3.3.7. *In vitro* Quantification of Collagen on Scaffolds

The intricate process of dermal wound healing involves ingrowth of cells, extracellular matrix (ECM) organization, inflammation regulation, and wound coverage among many other things (Ravichandran *et al.*, 2012). bFGF is present in tissues such as skin, skeleton and endothelium, and is considered to be a mediator of early wound angiogenesis which is important in the process of wound healing (Nissen *et al.*, 1996). It is one of the heparin-binding growth factors that helps in collagen production, neovascularization and epithelialization by regulating migration and replication of endothelial, epithelial, and fibroblast cells (Hom *et al.*, 2005; Nugent

and Iozzo, 2000). bFGF was used in the study since it contributes to normal wound healing and tissue development by stimulating growth and development of new blood vessels. It was added to the stromal medium and its effect on the amount of collagen synthesized by hASC cultured on polyester samples was analyzed. The amount of collagen synthesized was analyzed by Sirius Red assay at the end of 7 and 14 days. Sirius red is a dye which specifically binds to the helical structure on collagen (type I to V) and does not discriminate between collagen species and types, and therefore was used to detect total collagen present in the samples.

Figure 3.9 demonstrates the amount of collagen synthesized by hASC cultured in the stromal (control) medium and bFGF supplemented stromal medium. For all the scaffolds, the amount of collagen synthesized in bFGF supplemented medium was significantly higher than the scaffolds with hASC in control (stromal) medium ($p<0.05$). In a similar study by Ravichandran *et al.* (2012), there was an increase in the amount of collagen synthesized by the addition of bFGF media to poly-L-lactic acid/poly-(α,β)-DL-aspartic acid/collagen nanofibrous scaffolds.

In this study, maximum collagen amount of 124 µg/ml was synthesized by cells cultured on AG scaffolds in bFGF supplemented medium at the end of one week. PCL scaffolds with hASC cultured in bFGF supplemented medium had collagen amount of 113 µg/ml, while the lowest amount (103 µg/ml) was observed on hASC cultured on AGC scaffolds in bFGF supplemented medium at the end of 7 days. However, at the end of one week, there were no significant differences observed between amounts of collagen synthesized by hASC cultured in bFGF supplemented medium for different scaffold types. At the end of 14 days, the amount of collagen detected for hASC on scaffolds treated with bFGF was significantly higher than at the end of 7 days ($p<0.05$) for all scaffold types. The amount of collagen on PCL and AG scaffolds laden with stem cells cultured in bFGF treated medium was found to be significantly higher (293 and 306 µg/ml) than

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the hASC cultured on AGC scaffolds (223 µg/ml) in bFGF treated medium at the end of 14 days. This again supports the hypothesis that the presence of a phenolic (cinnamic) group in the AGC polymer structure probably impacts the growth of hASC in a negative manner.

*Solid bars: Stromal Medium
**Bars with Patterns: bFGF added Medium
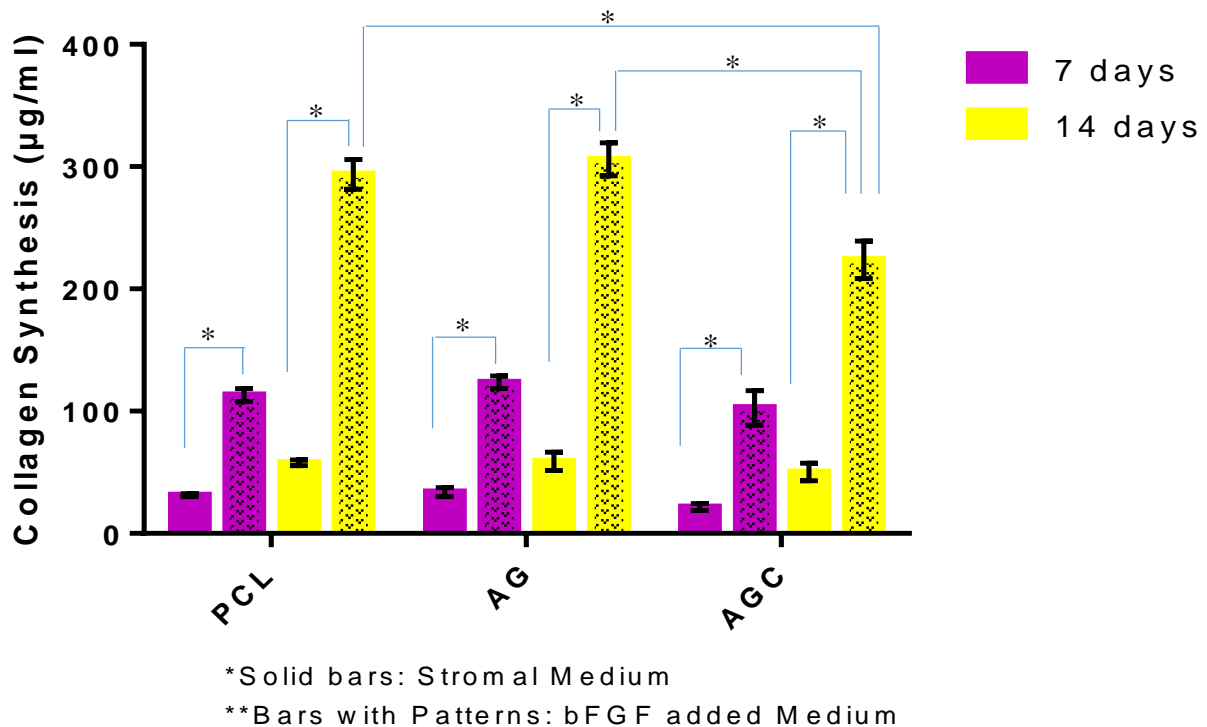
Figure 3.9. Amount of collagen synthesized on different scaffolds cultured in untreated (stromal medium) vs. bFGF added medium
*indicates statistical significance between the two groups
PCL: Poly (caprolcatone) scaffolds; AG: Polyester of aconitic acid and glycerol (no cinnamic acid and AGC: Polyester of aconitic acid, glycerol and cinnamic acid

In another study by Hom *et al.* (2005), decreased levels of bFGF in porcine skin caused a delay in wound healing process and when supplemented intravenously with bFGF, skin flap viability of irradiated tissue increased significantly. It is believed that bFGF is released from the cytosol of cells after cellular injury or damage (Nissen *et al.*, 1996; Nugent and Iozzo, 2000). After being released, it contributes to growth and development of new blood vessels, normal wound healing and tissue growth. By supplementing the hASC growth medium with this factor its effect on collagen synthesis was studied. In our study, the highest quantity of collagen (306 µg/ml) was

observed for hASC cultured in bFGF supplemented medium on AG scaffolds at the end of 14 days. The observed augmented collagen levels conclusively prove that these scaffolds can be used for hASC growth and proliferation that would stimulate wound healing. Thus by supplementing the scaffolds for skin tissue engineering with bFGF can help expedite the wound healing process by taking advantage of its angiogenic activity in early stages of wound repair.

3.3.8. *In vitro* Quantification of DNA on Scaffolds Supplemented with bFGF

DNA quantification on scaffolds supplemented with bFGF was performed and compared to their stromal medium treated counterparts and is shown in Figure 3.10. For all time points, the bFGF treated scaffolds had higher DNA content than the stromal medium ones. This can be attributed to the property of bFGF that stimulates cell proliferation and migration, an important phenomenon for wound healing (Karperien, 2008; Nugent and Iozzo, 2000). The highest DNA amount was observed in bFGF treated PCL scaffolds which was higher than AG and significantly higher ($p<0.05$) than the AGC scaffolds. The DNA amounts for PCL and AG scaffolds were significantly higher ($p<0.05$) than the AGC scaffolds under all the conditions. This supports the previous evidence where AG scaffolds supported the growth of hASC to a greater extent than the AGC ones. The elevated levels of DNA imply higher cell viability and proliferation, suggesting these polyesters can be used as scaffolds for growth and proliferation of cells. These polyester samples can be used as potential candidates for wound repair as evident by their synthesized collagen levels. Thus, addition of bFGF not only has the potential benefits of wound healing through angiogenesis but it also has an impact on cell proliferation which is important for wound healing.

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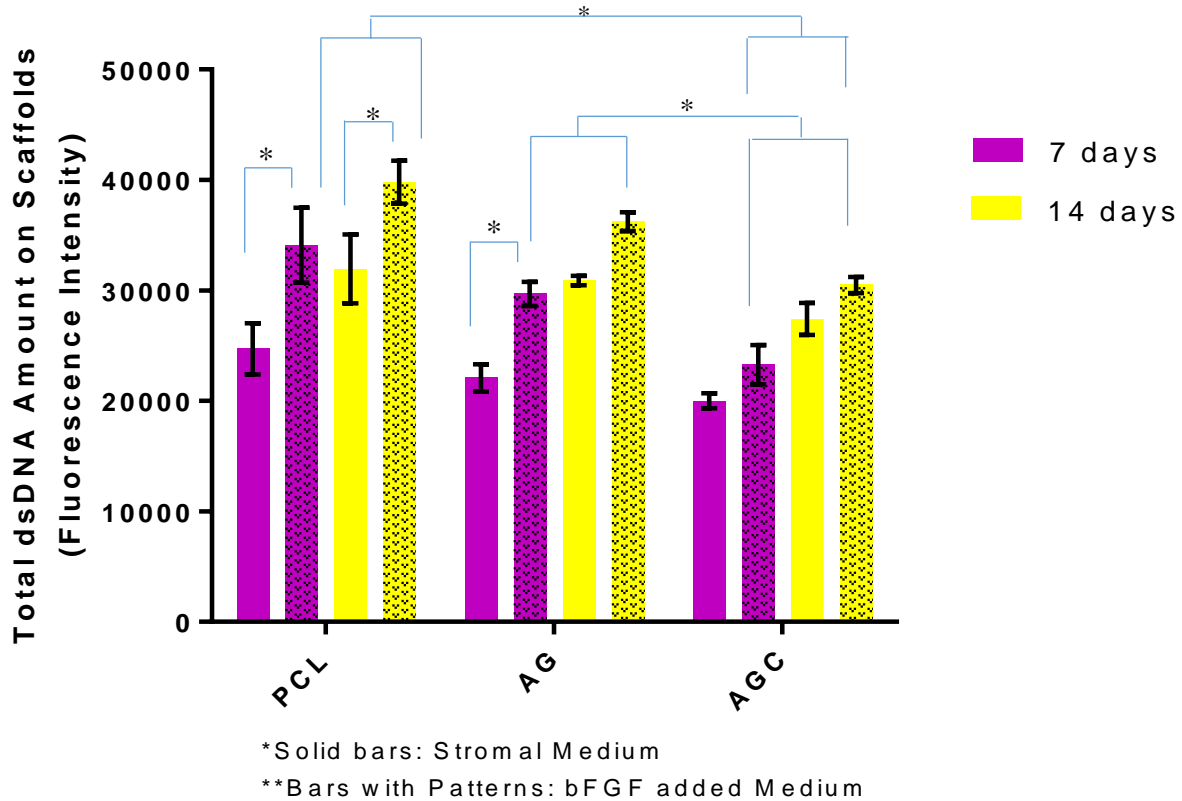
Figure 3.10. Total DNA amount for hASC cultured on different polymers for 7 and 14 days treated in stromal medium and bFGF added stromal medium
*indicates statistical significance between the two groups
PCL: Poly (caprolcatone) scaffolds; AG: Polyester of aconitic acid and glycerol (no cinnamic acid) and AGC: Polyester of aconitic acid, glycerol and cinnamic acid

3.4. Conclusions

Polyesters of aconitic acid, glycerol and cinnamic acid (by-products of sugarcane industry) were synthesized and characterized in this study. Several composition of polymers were tested for their biocompatibility and cell viability assays. It was observed that the amount of free –OH and –COOH groups present in the polymer samples influenced their cytotoxicity and biocompatibility properties. These polyesters were tested as scaffolds for growth and viability of hASC, and it was observed that the amount of cinnamic acid present in the polyester samples affected the growth and proliferation profiles of hASC. Overall, based on the metabolic activity assays by alamarBlue® analysis and total DNA content as evident by picogreen assay, it was concluded that

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these polyesters hold potential as scaffolds for tissue engineering applications. Polyesters AG (polyester of aconitic acid- 37 mol% and glycerol- 63 mol %) and AGC (polyester of aconitic acid- 31 mol%, glycerol- 63 mol% and cinnamic acid- 6 mol%) were compared to PCL (polycaprolactone) scaffolds (used as control), for their potential in skin tissue engineering, especially wound repair. Several bio-compatibility tests were performed on the polyesters to determine their toxicity towards human adipose derived stem cells. The scaffolds were tested for their potential application in wound healing by adding bFGF to the hASC culture medium and studying the amount of collagen synthesized on each scaffold type by the cultured hASC. At the end of 14 days, the highest collagen amount was observed on hASC cultured on AG scaffolds (306 µg/ml). At the end of 14 days, significantly lower amount of collagen synthesized and DNA quantification was observed for AGC scaffolds as compared to PCL and AG scaffolds. Based on the collagen and total DNA amount synthesized, these polyesters hold great potential as scaffolds for tissue engineering of skin, in particular wound healing.

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CHAPTER 4. *IN VITRO* CHARACTERIZATION OF POLYESTERS OF ACONITIC ACID, GLYCEROL AND CINNAMIC ACID FOR BONE TISSUE ENGINEERING

4.1.   Introduction

The process by which raw sugar is refined from sugarcane yields several by-products which may be utilized for increased commercial gain, increasing overall profit for the industry and decreasing waste by additional processing to yield chemicals and products. Two such by-products, sugarcane bagasse and molasses, in particular have been shown to yield commercially useful products and are the subject of much research concerning ethanol production (Cardona *et al.*, 2010; Cazetta *et al.*, 2007; Kim and Day, 2011). As a lignocellulosic material, pretreated sugarcane bagasse additionally yields phenolic acids including ferulic acid, p-coumaric acid, hydroxyl cinnamic acid, and others (Xu *et al.*, 2005). Through various biochemical processes molasses can be transformed into a wide range of products such as glycerol as well as numerous organic acids including citric acid, lactic acid and aconitic acid (McMurray and Griffin, 2002; Navarro *et al.*, 2000). These products may then be used to synthesize highly specific novel materials for the fabrication of bio-based polymers (FitzPatrick *et al.*, 2010). The processing of these by-products from sugarcane bagasse and molasses therefore holds implications for both the economic well-being of the sugarcane industry and polymer-based research.

Regeneration of bone tissue is currently and it has been for many years encouraged through the implantation of bone grafts (Zhang, 2011). Bone grafts, both allogeneic and autogenous, are sources of further complications. Allogeneic grafts, in addition to requiring costly donations and storage, create the potential for rejection and disease transmission. Autogenous grafts, although unlikely to cause any adverse immunological response, have limited availability and risk morbidity at the requisite additional donor surgery sites (Bohner, 2010). It has become a goal of tissue engineering in recent years to develop alternatives to bone grafts. Although much research has

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been conducted into tissue scaffolds (bio-derived, synthetic and hybrid), there have been little clinical applications (Nelson *et al.*, 2013).

Naturally occurring polymers such as collagen, chitosan, fibrin, and starch are attractive because of their high biocompatibility and biodegradability. However, low mechanical strength precludes widespread use of these materials in bone regeneration (Schieker *et al.*, 2006). Synthetic biocompatible polymers are desirable for increased morphological variability and mechanical strength. Polyesters, in particular poly (glycolic acid) and poly (lactic acid), are the most commonly studied in this group, desirable for biocompatibility and the ease of degradation by hydrolysis of the ester bond. However, mechanical strength is shown to decrease significantly early in the degradation process, accompanied by the release of acidic degradation products (Gunatillake and Adhikari, 2003).

A successful bone scaffold must balance the necessary bone-like mechanical strength with porosity and interconnectivity. Such biomimetic morphology facilitates cell attachment, growth, and nutrient and waste transport, as well as the development of the extracellular matrix (Hutmacher *et al.*, 2001; Karageorgiou and Kaplan, 2005; Leong *et al.*, 2003; Zeltinger *et al.*, 2001). Such porous scaffolds are commonly fabricated through solid freeform fabrication, thermal precipitation, gas foaming, and solvent casting with particulate leaching (Karageorgiou and Kaplan, 2005; Liao *et al.*, 2002; Taboas *et al.*, 2003).

Herein we describe the *in vitro* mechanical and osteoinductive properties of scaffolds derived from trans-aconitic acid, glycerol and trans-cinnamic acid, by-products of sugarcane bagasse and molasses processing. Solid polymer scaffolds were fabricated and studied for their potential application in bone tissue engineering. Morphology was confirmed using micro-CT, while compressive yield strength and modulus of the material were evaluated through mechanical

testing. The growth and differentiation of human adipose derived stem cells (hASC) was studied. Osteogenesis was analyzed by the amount of calcium deposition on scaffolds and gene expression by real time polymerase chain reaction (RT PCR).

4.2. Materials and Methods

4.2.1. Preparation of Scaffolds

Glycerol ($\geq$99%), trans-aconitic acid (98%) and trans-cinnamic acid (97%) were purchased from Sigma-Aldrich (St. Louis, Missouri) and used as is. All the three components were stirred continuously in a beaker using an overheard stirrer at 400 rpm (Caframo, Ontario, Canada). The reaction was carried out for 5 h at 120°C using a constant temperature oil bath (VWR Scientific, Niles, Illinois). Few drops (0.5 ml) of concentrated sulfuric acid were added as a catalyst. Based on prior studies, the composition for AG (aconitic acid-glycerol polyesters) and AGC (aconitic acid-glycerol-cinnamic acid polyesters) scaffolds were chosen as shown in Table 4.1. For osteogenic studies, composites of AG and AGC with hydroxyapatite (HA) (20% by weight) were also synthesized and compared. HA was added to the pre-polymer mix and the reaction was carried out for 5 h. After the completion of reaction, polymer solution was transferred to 96-well plate for molding and cured overnight in an oven at 105°C. The samples containing cinnamic acid were further cross-linked under UV light for 30 min using a UV transilluminator. Polymer samples were washed with water to remove catalyst and unreacted monomers present in them. The number of washings were determined based on the pH of the washed water (till it reached a neutral value).

Poly-caprolactone (PCL) scaffolds were prepared through thermal-precipitation method as described elsewhere (Zanetti, 2011) and used as a control in this study. Briefly, a 10% PCL solution in 8 ml of 1,4-dioxane was prepared. The mixture was poured into a polydimethylsiloxane mold and immediately transferred to a freezer at -20°C overnight.

Post freezing, the samples were immediately freeze-dried for 48 h. These samples were then used for differentiation studies.

Table 4.1. Composition of synthesized polyesters used in the study

	Glycerol (mol %)	Aconitic Acid (mol %)	Cinnamic Acid (mol %)
AG	63	37	0
AGC	63	31	6

4.2.2. Fourier Transform Infrared Spectroscopy- Attenuated Total Refletance (FTIR-ATR) Analysis

The FTIR-ATR spectra of fine powdered samples were recorded at room temperature using a Bruker Tensor 27 FTIR and OPUS data collection program. It was equipped with a room temperature DTGS detector, mid-IR source (4000 to 400 cm^{-1}), and a KBr beamsplitter.

4.2.3. Mechanical Testing

AG:HA (100:0,80:20) and AGC:HA (100:0, 80:20) scaffolds of 6 mm (diameter)×12 mm (height) cylinder shape were tested to determine maximal compressive strength and modulus. A universal testing machine (Instron Model 5696, Canton, MA, USA) was used at an extension rate of 0.5 mm/min.

4.2.4. Micro-CT Analysis

Two-dimensional (2-D) microcomputed tomography (μ-CT) imaging of all scaffolds was performed (40 kV and 540 ms) to obtain 0.04 mm slices every 0.9° throughout a 360° rotation (SkyScan 1074, Skyscan n.v., Belgium). Three-dimensional (3-D) files were reconstructed from 2-D images. Measurements of 2-D and 3-D images were performed with AVIZO® Standard packages (FEITM Visualization Sciences Group).

Avizo 7.0.1(Visualization Services Group) generated the volume renderings from the 3D data of the different samples. A red-orange colormap was used to present 3D structure of all types

of scaffolds. Image J generated 2-D orthogonal slices possessing grey colormap settings using the same data with a scale equivalent to the 3-D rendering. An approximate pore size was also measured using Image J.

4.2.5. Porosity Calculation Based on Micro-CT

To analyze the three-dimensional data, two dimensional slices were read into a custom MATLAB code. For each slice, the grayscale image was thresholded using Otsu's method (Otsu, 1975) and then converted into a binary image. Morphological operations were performed to remove small imaging artifacts, and isolate interior and exterior pores. After quantifying solid and void pixels, porosity was calculated as follows:

$$\phi = \frac{Vpores}{Vpores + Vsolid} \times 100\%$$

where, V_{pores} = Total volume of all pores and V_{solid} = Total volume of solid (polymer)

4.2.6. Adult Stem Cells Isolation and Culture

Liposuction aspirates from subcutaneous hip adipose tissue were obtained from three healthy adult subjects undergoing elective procedures. All tissues were obtained with informed consent under a clinical protocol reviewed and approved by the Institutional Review Board at the LSU Pennington Biomedical Research Center and used under an exempted protocol at LSU A&M College. Isolation of hASCs was performed as published (Zanetti *et al.*, 2012). Passage 2 of hASCs from each donor was used for *in vitro* hASC osteogenesis evaluation on scaffolds of different compositions. In both cases, hASCs were cultured in either stromal (control - DMEM, 10% FBS, and 1% triple antibiotic solution) or osteogenic (DMEM, 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 1% triple antibiotic solution) media for up to 21 days with media changed three times a week.

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4.2.7. hASC Loading on Scaffolds and Culture

All types of scaffolds were either molded or sculpted into 5 mm (diameter) $\times$ 10 mm (height) cylinder shape and gas sterilized afterwards. All the scaffolds were then soaked in stromal medium for 1 h before loading the hASCs. The same amount of second cell passages from all donors (n = 3) were pooled and directly loaded on a single face of each scaffold type at a concentration of 1.0×10^4 cells/µL for total volume of 5 µL. After 30 min of incubation in a saturated humidity atmosphere at 37 °C and 5% CO_2, the same volume of hASCs containing solution were directly applied on the opposite side of each scaffold as previously described. After 30 min of incubation, stromal media was added to each well and the samples were incubated at 37°C and 5% CO_2 environment. Control groups included PCL:HA (100:0 and 80:20) scaffolds while the experimental groups included AG:HA (100:0, 80:20) and AGC:HA (100:0, 80:20) scaffolds. Scaffolds loaded with hASCs were immediately transferred to 48-well plates and cultured in stromal or osteogenic media for 21 days. Cell medium were changed every 2-3 days. Triplicates were performed for each assay.

4.2.8. *In vitro* hASC Viability on Scaffolds with alamarBlue® Stain

The viability of cells within the scaffolds at 7, 14 and 21 days in control and osteogenic media was determined using an alamarBlue® metabolic activity assay. The scaffolds were removed from culture, washed three times with PBS, and incubated with 10% alamarBlue® in Hank's balanced salt solution (HBSS) without phenol red (pH 7) for 90 min. 100 µL aliquots of alamarBlue®/HBSS were placed in a 96-well plate and the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a fluorescence plate reader. All the measurements were done in triplicates.

4.2.9. *In vitro* Quantification of DNA on Scaffolds

Total dsDNA amount was used to determine the number of hASC on each scaffold as previously described (Liu *et al.*, 2008). All the scaffolds were first minced by a scalpel and then washed with PBS for three times to remove the residual dsDNA from dead cells. The dsDNA was released by adding 0.5 mL of 0.5mg/mL proteinase K (Sigma-Aldrich, St. Louis, Missouri) at 56ºC for enzymatic lysis of live cells overnight. Aliquots (50 µL) were mixed with equal volumes of 0.1 g/mL Picogreen dye solution (Invitrogen) in 96-well plates. Samples were then excited at 480 nm with a plate reader (Wallac 1420 multilabel hts counter). Scaffolds without cells were used as blank controls.

4.2.10. Alizarin Red Staining

hASC calcium deposition (scaffolds alone and cell-scaffolds) was measured after 7, 14, and 21 days of culture in stromal or osteogenic medium based on alizarin red staining. Wells were washed with 0.9% NaCl and fixed with 70% ethanol for 30 min. All scaffolds were stained with 2% alizarin red for 10 min and washed with DI water six times. Scaffolds were destained with 10% cetylpyridinium chloride monohydrate for 4 h at 25°C with constant mild agitation. The calcium deposition was then measured as optical density at 540 nm with a plate reader. Results were normalized to values from scaffolds cultured without cells for the same time periods.

4.2.11. Quantitative Real-time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from cell-scaffold constructs as previously described (Zanetti *et al.*, 2012). qPCR was performed using $2\times$ iTaq^{TM} SYBR$^{®}$ green supermix with ROX (Biorad, Hercules, California) and primers for alkaline phosphatase (*ALP*) and osteoscalcin (*OCN*) (Zanetti *et al.*, 2012) to quantify osteogenic target gene expression of hASC loaded to scaffolds and cultured in both stromal and osteogenic media for 21 days. Analyses were performed every 7 days.

All the reactions were conducted in a MJ Mini™ Thermal Cycler (BioRad, Hercules, CA). The sequences of PCR primers (forward and backward, 50-30) were as follows: ALP, 5'-AATATGCCCTGGAGCTTCAGAA-3' and 5'-CCATCCCATCTCCCAGGAA-3';OCN, 5'-GCCCAGCGGTGCAGAGT-3' and 5'-TAGCGCCTGGGTCTCTTCAC-3'. Samples were normalized (ΔCt) against the house keeping gene 18S rRNA. The - ΔΔCt value of *ALP* and *OCN* in scaffolds cultured in osteogenic and control media was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

4.2.12. Statistical Analysis

All results were reported as mean ± standard deviation. Data was evaluated with one or two way analysis of variance (ANOVA), and then analyzed by Tukey's minimum significant difference (MSD) post hoc test for pairwise comparisons of main effects. For all comparisons a p-value < 0.05 was considered significant.

4.3.   Results and Discussion

4.3.1. Synthesis of Polyesters

Different compositions of polyesters of aconitic acid, glycerol and cinnamic acid were synthesized and characterized in a prior study for their bio-compatibility and cytotoxicity towards human adipose derived stem cells (Chapter 3). Of those, certain compositions with and without the addition of hydroxylapatite (HA) were tested for osteogenesis in this study (Table 1). Due to its biocompatibility, poor water solubility and slow *in vivo* degradation, PCL is the most versatile scaffolding material used in bone tissue engineering (Leong *et al.*, 2008; Zanetti, 2011) and was used as a control in this study. Since HA is a major constituent of native bone, composites of synthetic polymers and HA particles have been widely used for bone scaffolds studies due to their osteoconductive and osteoinductive properties (Moradi *et al.*, 2013; Salgado *et al.*, 2004; Schieker

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et al., 2006). Synthetic biodegradable polyesters such as poly (glycolic acid), poly (lactic acid), and their copolymers have been used as biomaterials in a number of clinical applications (Sabir *et al.*, 2009). In this study, polyesters of aconitic acid, glycerol and cinnamic acid with and without the incorporation of HA were used. Their ease of fabrication via one step polycondensation, and ease of degradation through hydrolytic scission of the ester bonds make them one of the most utilized biodegradable synthetic polymers in tissue engineering (Gunatillake and Adhikari, 2003; Rezwan *et al.*, 2006). In addition, the degradation products (monomeric components) of polyesters are easily eliminated from the body by physiological metabolic pathways (Lam *et al.*, 1994; Sabir *et al.*, 2009).

4.3.2. Fourier Transform Infrared (FTIR) Analysis

The reaction procedure was verified by FTIR analysis as shown in Figure 4.1. For all the polyesters, the intense C=O stretch around 1730 cm^{-1} confirmed the formation of ester bonds. Similar FTIR spectra for ester bond formations have been reported (Cao *et al.*, 2011; Thomas and Nair, 2011). As described in the earlier chapter (Figure 3.1), the characteristic peaks for the reactants were observed at: 3300 cm^{-1} (due to –OH groups in glycerol), and 1630-1670 cm^{-1} (due to –COOH groups in aconitic and cinnamic acid). The appearance of the intense ester peak around 1730 cm^{-1} and the disappearance of the –OH and –COOH peaks in the polyester samples confirm the polymerization reaction.

For HA, the characteristic stretching bands for PO_4^{-3} are observed at 1000-1200 cm^{-1} (Arsad, 2011). In case of polyesters mixed with HA, the characteristic peak due to C=O stretch was observed around 1740 cm^{-1} with no significant shift due to the incorporation of HA into the polyesters. However, for the composite polyester, AGC: HA, a PO_4^{-3} stretching at 1030 cm^{-1} (typical of HA) along with the C=O stretch due to ester bond formation was observed. This

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indicates that there was some free residual HA in this sample. Similar HA peaks have been reported by other studies in case of nano-HA composites (Jose *et al.*, 2009; Moradi *et al.*, 2013). This can be due to the insufficient dispersion of HA throughout the polyester sample. As the polymerization reaction neared completion, there was a rapid increase in the viscosity of the polymer solution that might have led to the non-uniform dispersion of HA particles in the AGC sample. This is also evident in the mechanical strength data of the scaffolds where incorporation of HA led to reduction in the mechanical properties of the scaffolds.
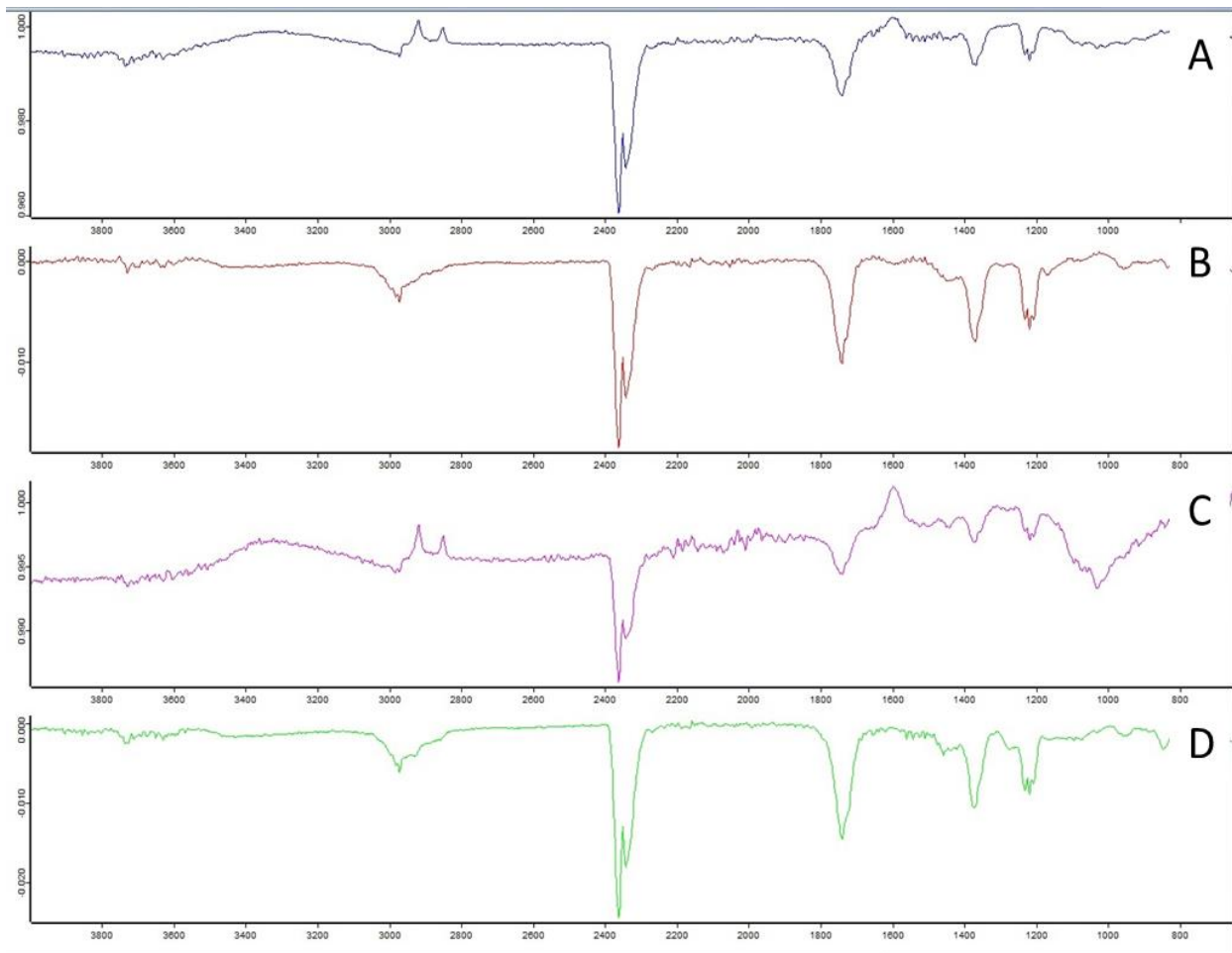
Figure 4.1. FTIR analysis of scaffolds. A] AG:HA (80:20); B] AG;
C] AGC:HA (80:20); D] AGC
AG:HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight) HA; AG:
Polyester of aconitic acid and glycerol; AGC:HA (80:20) Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight) HA; AGC: Polyester of aconitic acid, glycerol and cinnamic acid

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4.3.3. Mechanical Testing

Figure 4.2 shows the mechanical strength data for scaffolds with and without the incorporation of HA. For AGC and AG scaffolds with 20% HA, the compressive strength was found to be significantly lower than their non HA added counterparts. HA is a major constituent of natural bone and is considered osteogenic when added to synthetic polymer scaffolds (Hollinger *et al.*, 1996; Marra *et al.*, 1999). It is added to bone scaffolds not only for its osteoconductive and osteoinductive properties but also to enhance mechanical strength of the scaffolds.
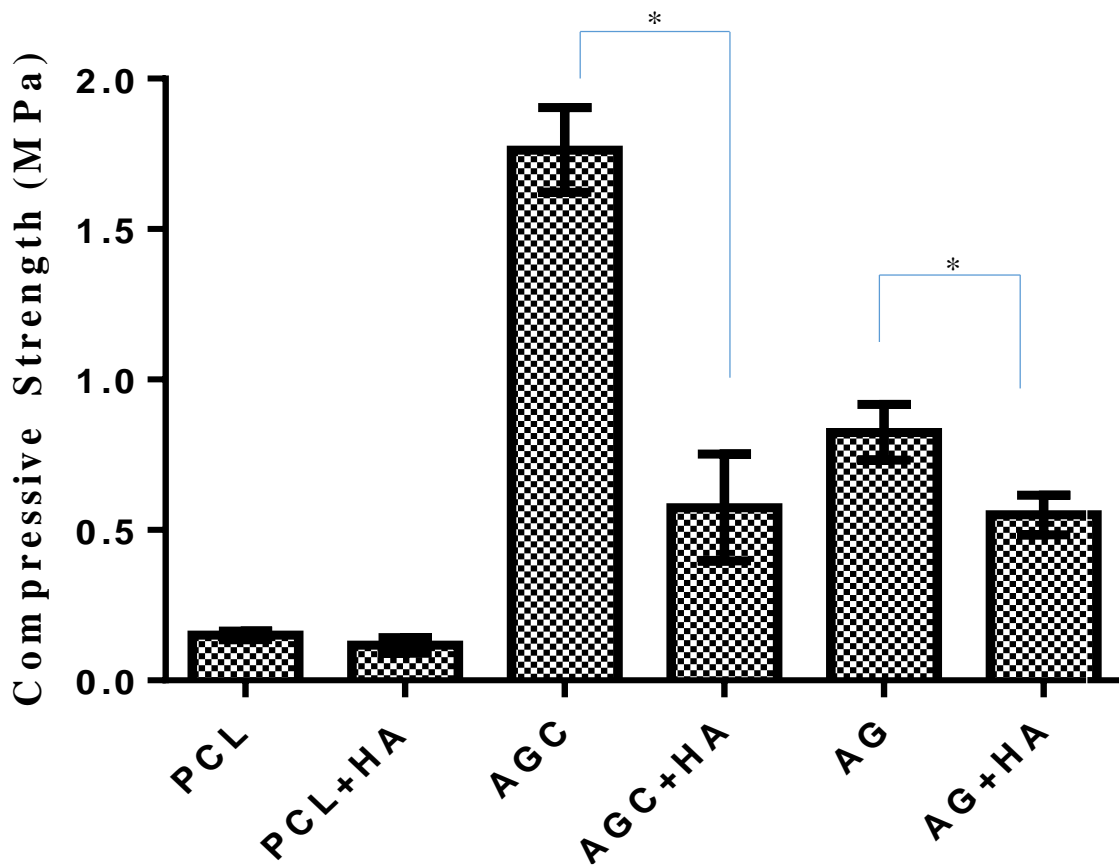
Figure 4.2. Mechanical strength of PCL, AGC, AG, PCL:HA (80:20), AGC:HA (80:20) and
AG:HA (80:20) scaffolds
*indicates statistical significance between the two groups
PCL: Poly (caprolactone); PCL+HA: Poly (caprolactone) with 20% (by weight) HA;
AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AG: Polyester of aconitic acid and
glycerol; AGC+HA: Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight)
HA; AG+HA: Polyester of aconitic acid and glycerol with 20% (by weight) HA.

Although many studies have reported reinforcement of polymeric scaffolds due to addition of HA (Attawia *et al.*, 1995; Jayabalan *et al.*, 2010), some studies have observed a trend of decrease in mechanical strength with an increase in HA concentration above a threshold (Jose *et al.*, 2009; Serbetci *et al.*, 2004; Yang, 2013; Zebarjad, 2011). In our study, the mechanical strength of the AGC and AG scaffolds decreased significantly due to incorporation of inorganic phase (hydroxylapatite) in them. In a study of hydroxyapatite-impregnated bone cement there was an improvement in the compressive strength of bone cement up to 8% HA inclusion. Further addition of HA (14%) caused a decrease in compressive strength (Serbetci *et al.*, 2004). In another study by Yang (2013), there was a steady decrease in compressive strength with an increase in HA concentration and a significant reduction was observed at, and above a 20% HA inclusion. The loss of mechanical strength at increased HA concentration can be attributed to non-homogenous distribution of particles in the polymer matrix leading to structural inhomogeneity and hence a decrease in compressive strength. At lower concentrations, HA particles act as load carriers increasing the mechanical strength of the matrix (Serbetci *et al.*, 2004). At higher concentration of HA coupled with increased viscosity of the polymerization solution, inefficient distribution of HA particles in the sample can create void spaces (increased pore size) as evident by the micro-CT images and porosity values. This increase in porosity due to incorporation of HA explains the loss of mechanical properties for the polyester-HA composites. The mechanical strength for AGC scaffolds was found to be significantly higher than the AG scaffolds. This can attributed to the highly cross-linked structure of AGC as compared to AG. The cinnamic acid moiety in the AGC scaffolds was further cross-linked by UV radiation imparting higher mechanical strength to the scaffolds due to higher degree of cross-linking.

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4.3.4. Micro CT Analysis and Porosity Calculations

3D images of all the scaffolds with and without HA incorporation were reconstructed using micro-CT analysis as shown in Figure 4.3. Micro CT image analysis is a sensitive, non -destructive analytical tool for measurements of porosity, pore interconnectivity and ceramic phase distribution in polymer composites (Ho and Hutmacher, 2006). Volume renderings (Figure 4.3) were generated from AG, AGC and PCL composite foam 3-D data using Avizo 7.0.1 (Visualization Services Group). Based on the micro-CT results, the 3D structures of AG and AGC scaffolds were different than the PCL scaffolds due to the difference in their fabrication methods. Furthermore, AG: HA (80:20) and AGC:HA (80:20) also displayed overall larger pores and porosity, indicating that the addition of HA to the AG and AGC scaffolds affected the structure of scaffolds, also indicated by the mechanical strength data of the scaffolds. As evident in Figure 4.3, especially for the AGC sample, AGC:HA (80:20) scaffold had bigger pores and highest increase in porosity compared to the respective non-HA counterpart. This could be due to insufficient mixing of HA particles in the overall matrix of the AGC:HA (80:20) sample. Based on the FTIR spectra for AGC:HA (80:20), the residual amount of free HA in the sample contributed to larger pores (higher porosity) and hence significant lower mechanical strength as compared to other scaffolds. As discussed further, the osteogenic potential of AGC:HA (80:20) was found to be lowest as compared to PCL:HA (80:20) and AG:HA (80:20) samples.

This is in accordance with the mechanical strength data where inclusion of HA in the polyester led to decrease in mechanical strength that can be explained by the increase in porosities of the HA-polyester samples. For the AGC sample, highest change in porosity was observed. The porosity of AGC:HA (80:20) sample increased to 48.3% as compared to 33.7% for AGC sample without any HA. The porosity of AG scaffolds was found to be 40% that increased to 48.5% for

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the AG:HA (80:20) sample. The highest porosity of 87.5% was observed for PCL:HA (80:20) scaffolds followed by PCL (78%).
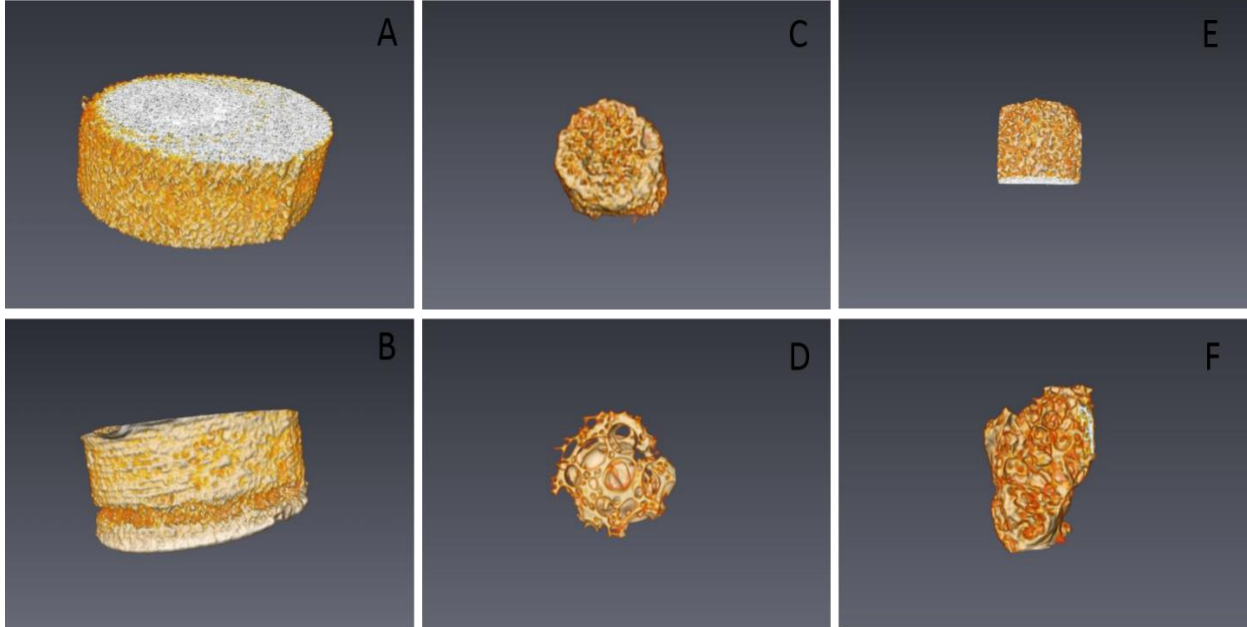
Figure 4.3. Avizo rendered 3D images of micro-CT data of scaffolds. A] PCL; B] PCL:HA (80:20); C] AGC; D] AGC: HA (80:20); E] AG; F] AG: HA (80:20) scaffolds
PCL: Poly (caprolactone) scaffold; PCL:HA (80:20): Poly (caprolactone) with 20% (by weight) HA; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AGC:HA (80:20): Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight) HA; AG: Polyester of aconitic acid and glycerol; AG:HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight) HA.

The optimum concentration of 20% HA inclusion in the scaffolds was chosen based on prior studies, in such a way that a fine balance between mechanical strength and porosity of scaffolds was achieved. An apparent decrease in porosity was observed when the HA concentration in scaffolds was increased from 20% to 25%. Increased HA concentration not only resulted in a decrease in porosity but also a substantial reduction in pore size and interconnected void volume (Garber *et al.*, 2013). Further tests need to be performed to determine the impact of HA concentration on mechanical strength, porosity and osteoinductive capability of bone scaffolds synthesized in this study.

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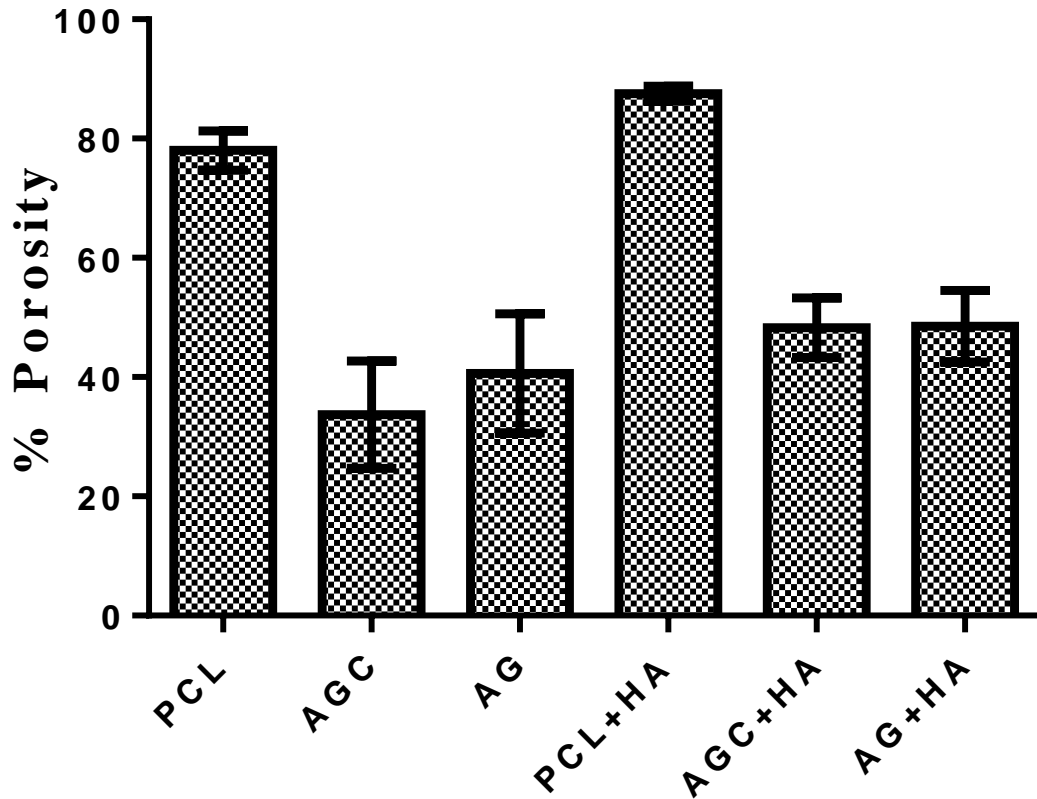
Figure 4.4. Porosity values (%) for PCL, AGC, AG, PCL:HA (80:20), AGC:HA (80:20) and
AG:HA (80:20)
PCL: Poly (caprolactone) scaffold; AGC: Polyester of aconitic acid, glycerol and cinnamic acid;
AG: Polyester of aconitic acid and glycerol; PCL+HA (80:20): Poly (caprolactone) with 20% (by
weight) HA; AGC+HA (80:20): Polyester of aconitic acid, glycerol and cinnamic acid with 20%
(by weight) HA; AG+HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight)
HA.

4.3.5. hASC Viability on Scaffolds Cultured in Control and Osteogenic Media

The viability of hASC loaded on different scaffold types cultured in both control and

osteogenic media is represented in Figure 4.5. For the same scaffold types, at a given time point,

there were no significant differences observed between the two media types except for PCL-7

days. At the end of 7 days, the metabolic activity for hASC cultured on PCL scaffolds in stromal

medium were significantly higher than hASC cultured on PCL scaffolds in osteogenic medium.

For the rest of the samples at a given time point, no significant differences were observed between

media types. In general, metabolic activity of hASC loaded to scaffolds cultured in stromal

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medium were higher than scaffolds cultured in osteogenic medium (except PCL at 14 days, AGC at 21 days and AG:HA at 21 days).
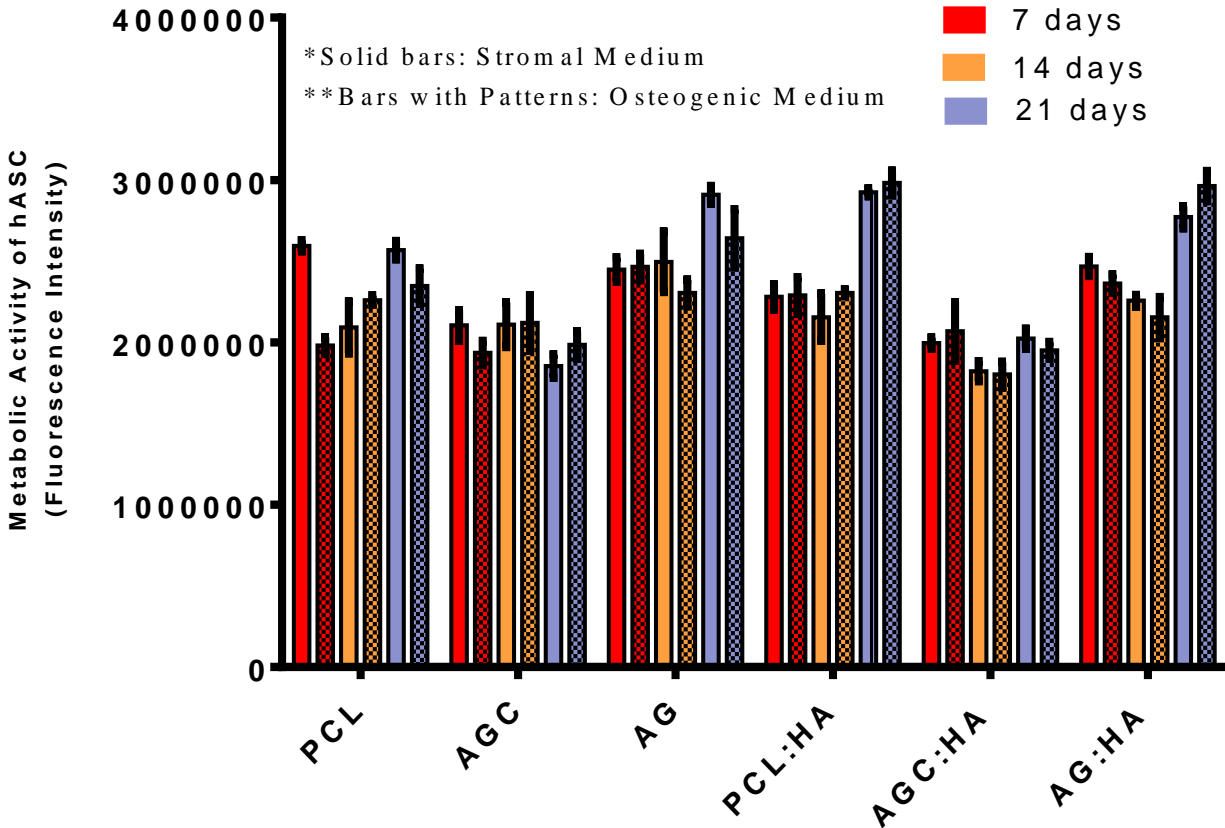
Figure 4.5. Relative metabolic activity for hASC cultured on PCL, AGC, AG, PCL:HA (80:20), AGC:HA (80:20) and AG:HA (80:20) scaffolds for 7, 14 and 21 days treated in stromal and osteogenic medium
PCL: Poly (caprolactone) scaffold; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AG: Polyester of aconitic acid and glycerol; PCL:HA (80:20): Poly (caprolactone) with 20% (by weight) HA; AGC:HA (80:20): Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight) HA; AG:HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight) HA.

For the samples containing HA there were no significant differences observed in the metabolic activity as compared to their non-HA counterparts. This indicates that the addition of HA did not affect the metabolic activity of hASC in a negative manner. For PCL, AG, PCL:HA (80:20), and AG:HA (80:20) scaffolds, at the end of 21 days the metabolic activity of cultured hASC was found to be significantly higher ($p < 0.05$) than the 14 days' time point. This trend however was not

observed for the AGC and AGC:HA (80:20) scaffolds. The cultured medium, number of days and presence of hydroxylapatite did not affect the relative metabolic activity of hASC cultured on these scaffolds. Based on relative metabolic activity data, no significant increase in metabolic activity (no significant cell proliferation) was observed for AGC and AGC:HA (80:20) scaffolds. This trend is also supportive of the lack of mechanical strength (presence of free HA particles) in the AGC:HA sample that contributed to lower cell proliferation on these scaffolds. The highest metabolic activities were observed for hASC cultured on PCL:HA and AG:HA scaffolds at the end of 21 days. However, there were no statistical differences in relative metabolic activities observed between these two scaffold types.

4.3.6. *In vitro* Quantification of DNA on Scaffolds

Total DNA content was quantified by Quant-iT™ PicoGreen® in order to compare cell proliferation between each scaffold type. The DNA content for all the scaffolds at 7, 14 and 21 days period in stromal and osteogenic media is reported in Figure 4.6. At a given time point (end of 7/14/21 days) for the same scaffold type, no significant differences were observed in total DNA content for hASC cultured in stromal or osteogenic medium. This was true for all scaffolds except for PCL, where at the end of 21 days highest DNA content was detected for PCL in osteogenic medium. Under given set of conditions, there were no significant differences observed between scaffolds with no HA and scaffolds with 20% HA except for PCL and PCL:HA (80:20) in osteogenic medium at the end of 21 days. For AG:HA (80:20) scaffolds cultured in stromal medium the DNA content was observed to be significantly higher than the non-HA counterpart ($p<0.05$). Based on relative metabolic activity and DNA amount data, HA does not seem to affect proliferation of hASC on scaffolds while promoting osteogenesis.
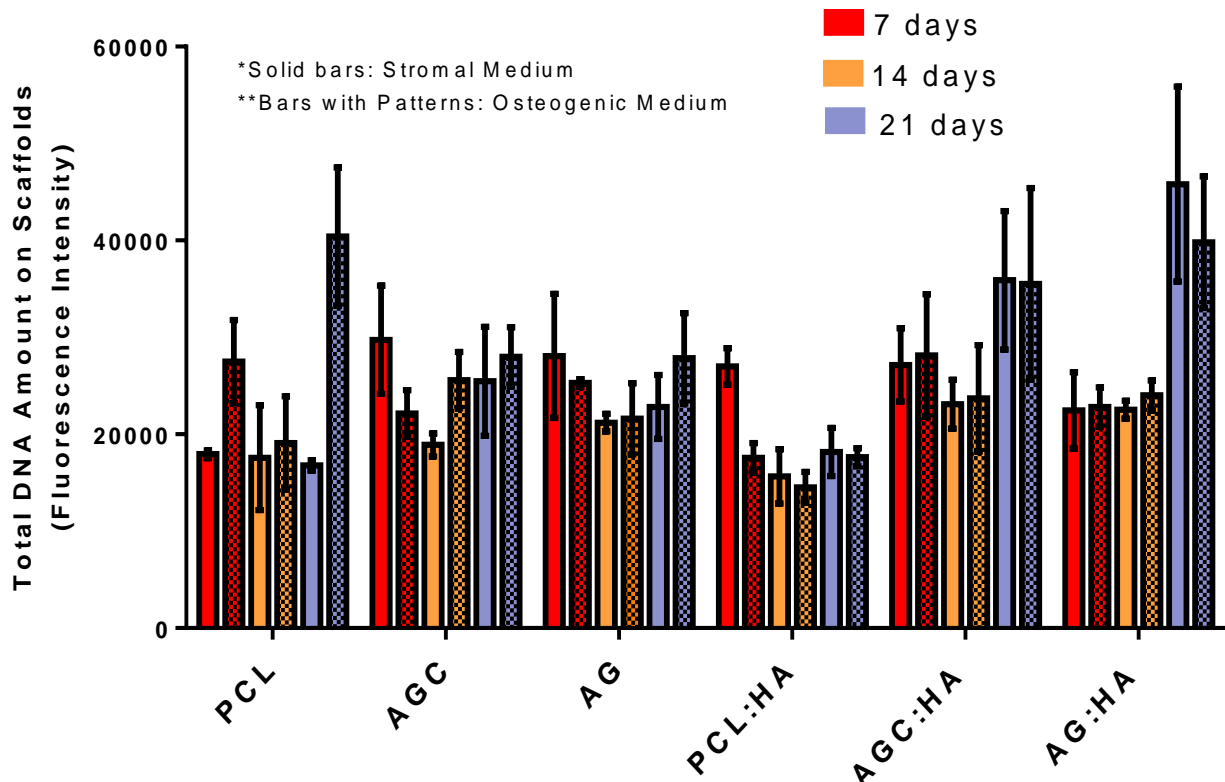
Figure 4.6. Total DNA amount of hASC cultured on PCL, AGC, AG, PCL:HA (80:20), AGC:HA (80:20) and AG:HA (80:20) scaffolds by picogreen analysis.
PCL: Poly (caprolactone) scaffold; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AG: Polyester of aconitic acid and glycerol; PCL:HA (80:20): Poly (caprolactone) with 20% (by weight) HA; AGC:HA (80:20): Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight) HA; AG:HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight) HA.

Overall, DNA content, similar to metabolic activities of hASC, decreased in all scaffold types between 7 day and 14 day time points. This trend is due to the increased differentiation observed around the 14 day time point, which leads to decreased cell proliferation. This data is in agreement with previous studies which indicate the metabolic activity of hASC is expected to decrease as cells commit to an osteogenic linage (Qureshi *et al.*, 2013; Zanetti *et al.*, 2012). There is an overall increase in DNA content between 14 day and 21 day time points. The highest DNA amount was observed on AG:HA (80:20) scaffold in stromal medium. This was followed by PCL in stromal medium at 21 days and AG:HA (80:20) in osteogenic medium after 21 days, though the difference was not statistically significant.

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4.3.7. Alizarin Red Staining

The calcium deposition of each scaffold type was assessed using alizarin red staining methodology. Calcium deposition, an indication of successful differentiation of hASC into osteoblasts can be detected quantitatively by alizarin red. Calcium deposits are stained bright orange-red by alizarin red. Each scaffold type was extracted from culture medium and stained at 7 day, 14 day and 21 day time points. The results are shown in Figure 4.7. For the scaffolds containing HA (PCL:HA, AGC:HA and AG:HA), a significantly higher mineralization was observed than the scaffolds without HA ($p<0.05$). Similar observations were reported by other studies (Kurashina *et al.*, 2002; Rahimi *et al.*, 1997). While there was no statistical differences in calcium deposition observed between osteogenic and stromal media in the non-HA containing scaffolds, the mineralization was observed to be slightly higher in the osteogenic medium. In general, the amount of calcium deposited increased with an increase in time. However, for AGC:HA (80:20) scaffolds cultured in osteogenic medium, a gradual drop in calcium deposition over 3 weeks period was observed. At the end of 21 days significantly lower calcium deposition for AGC:HA (80:20) scaffolds cultured in osteogenic medium was observed. This can be due to poor mechanical properties of these scaffolds that fail to promote growth and proliferation of cultured hASC as evident by their reduced metabolic activity rates (alamarBlue®). In osteogenic medium, there was no statistical difference between PCL:HA (80:20) and AGC:HA (80:20), and both showed the highest level of calcium depositions of the scaffold types tested at the end of 7 day period. But at the end of the study, the highest mineralization was observed in PCL:HA (80:20) and AG:HA (80:20) (at the end of 21 days). *In vitro* osteogenesis is characterized by increased cell proliferation and calcium deposition (Bauer and Muschler, 2000; Zanetti, 2011). These observations indicate that AG:HA (80:20) scaffolds have a greater potential to induce

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differentiation of cultured hASC to osteogenic lineage as compared to AGC:HA (80:20) scaffolds. At the end of 21 days, significantly higher mineralization was observed in AG:HA (80:20) scaffolds as compared to AGC:HA (80:20) scaffolds. Similar trends were also observed for relative metabolic activity and total DNA amount for hASC cultured on AG:HA (80:20) and AGC:HA (80:20) scaffolds.
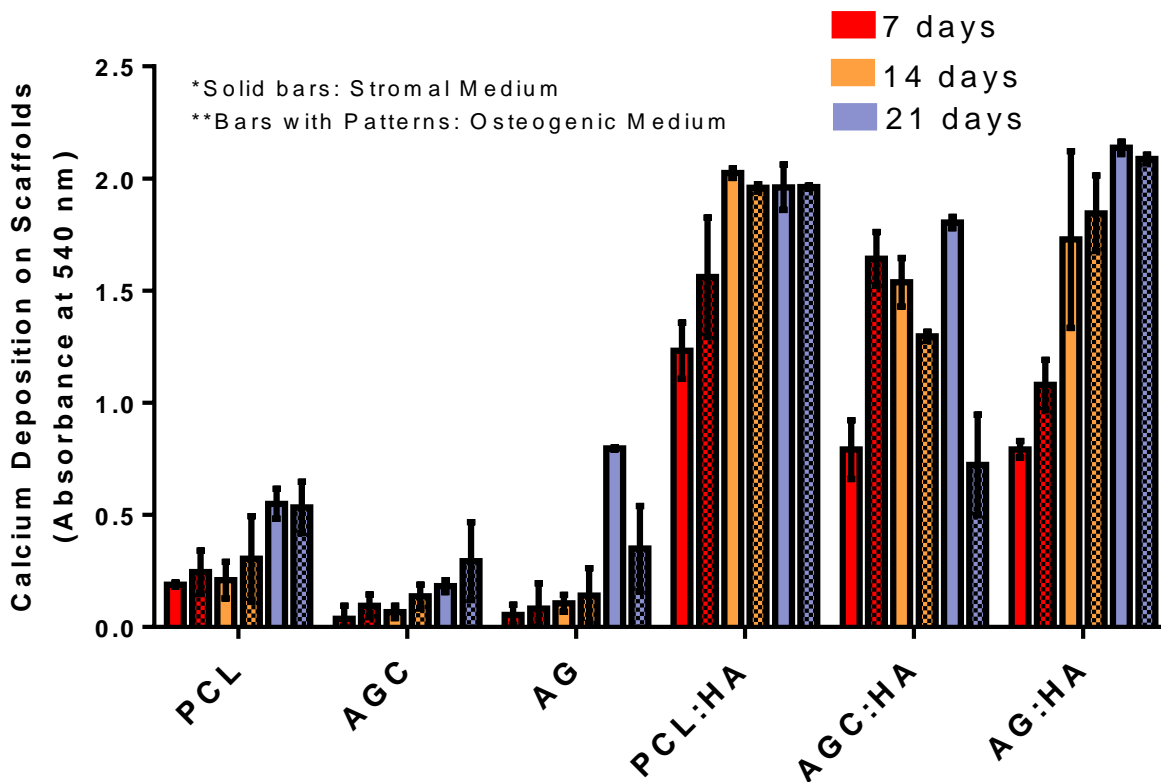
Figure 4.7. Alizarin red stain as a function of scaffold composition, media treatment and time. Quantitative analysis of staining on scaffolds loaded with hASC cultured in stromal and osteogenic media for 7, 14 and 21 days.
PCL: Poly (caprolactone) scaffold; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AG: Polyester of aconitic acid and glycerol; PCL:HA (80:20): Poly (caprolactone) with 20% (by weight) HA; AGC:HA (80:20): Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight) HA; AG:HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight) HA.

4.3.8. Quantitative Real-time Polymerase Chain Reaction (qPCR)

The differences in the expression of *ALP* and *OCN* in hASC loaded on different scaffolds cultured in stromal and osteogenic medium are represented in Figure 4.8. Bone morphogenic

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proteins (BMPs), known to regulate osteogenesis, stimulate the transcription factor core binding factor alpha1 (Cbfa1) that results in the activation of osteoblast-specific genes such as *ALP* and *OCN* (Liu *et al.*, 2008). The expression of these genes are commonly used as early (*ALP*) and middle (*OCN*) stage markers of osteogenesis, respectively. qPCR can therefore be used to assess early and middle stage markers of osteogenesis.
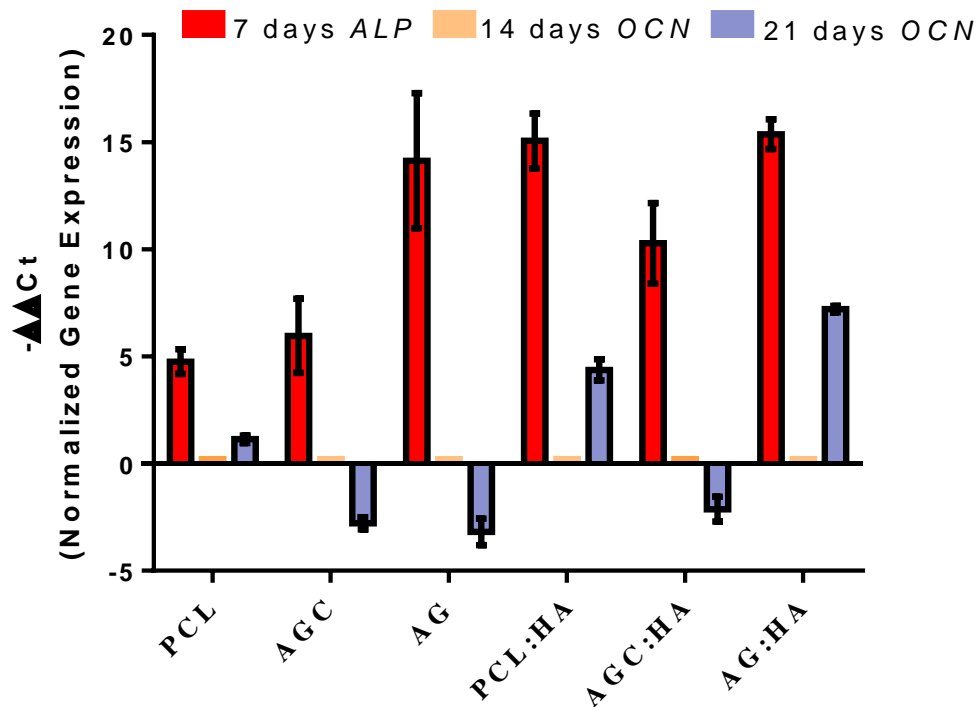
Figure 4.8. qPCR analysis of ALP (7 day) and OCN (14 and 21 day) expression from hASC on PCL, AGC, AG, PCL:HA (80:20), AGC:HA (80:20) and AG:HA (80:20) scaffolds. PCL: Poly (caprolactone) scaffold; AGC: Polyester of aconitic acid, glycerol and cinnamic acid; AG: Polyester of aconitic acid and glycerol; PCL:HA (80:20): Poly (caprolactone) with 20% (by weight) HA; AGC:HA (80:20): Polyester of aconitic acid, glycerol and cinnamic acid with 20% (by weight) HA; AG:HA (80:20): Polyester of aconitic acid and glycerol with 20% (by weight) HA.

ALP expression was measured at the 7 day time point, and *OCN* expression was quantified at 14 and 21 day time points. It is common for *ALP* expression to drop to a minimal level past the 7 day time point because it is an early stage marker for osteogenesis. Based on previous studies,

ALP expression in hASC decreased dramatically after 7 days in culture and was therefore not measured at the 14 and 21 day time points in this study (Zanetti *et al.*, 2012).

Based on Figure 4.8, for the 7-day time point, the greatest *ALP* expression was observed in AG, PCL:HA, and AG:HA, though none of these values were statistically different from each other. For the scaffolds containing no HA, significantly higher level of *ALP* expression was observed in AG scaffolds as compared to PCL and AGC scaffolds. For the polyester-HA composites, similar expression was observed in PCL:HA (80:20) and AG:HA (80:20) scaffolds that were higher than the AGC:HA (80:20) scaffolds (not significant). In general, higher *ALP* expression was observed in HA composites as compared to non HA scaffolds, indicating their greater osteogenic potential. These observations support the calcium deposition results, where due to presence of HA, greater mineralization (implying greater osteogenesis) was observed in scaffolds containing HA. At the 14 day time point there were low levels of OCN expression observed in all scaffold types. This trend parallels and further supports the decrease in DNA content observed at the 14 day time point described above. The expression of the OCN marker could only be observed at 21 days of culture, with little expression at 14 days regardless of the scaffold type. At the 21 day time point, OCN expression was upregulated only in PCL, AG:HA (80:20) and PCL:HA (80:20) scaffolds. AG:HA was observed to have the greatest OCN expression followed by PCL:HA (80:20). These results are supportive of the alizarin red staining, in which AG:HA was observed to contain the greatest calcium deposition at the 21 day time point. Similarly for the AGC:HA (80:20) scaffolds, though an upregulation in *ALP* marker was observed (indicating osteogenesis), none was detected for *OCN* marker. This can be attributed to the loss of mechanical strength of scaffolds over a 3 week period. This in turn affects the growth, proliferation and differentiation of hASC cultured on these scaffolds. As evident by calcium deposition and

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qPCR results, the AGC:HA (80:20) scaffolds support growth and differentiation of the hASC at the start of the study (end of 7 days) but the trend gradually decreases as time progresses (owing to loss of structural integrity of the scaffolds).

4.4.   Conclusions

In this study, polyesters of AG, AGC and their composites AG: HA (80:20) and AGC:HA (80:20) were synthesized and characterized for their potential use in bone tissue engineering. PCL and PCL:HA (80:20) scaffolds were used as control in this study. FTIR analysis was performed to confirm the polyesterification reaction. Mechanical testing on the synthesized scaffolds indicated decrease in mechanical strength due to incorporation of HA particles. The greatest drop in mechanical strength was observed for AGC:HA (80:20) scaffolds. The loss in mechanical properties of composite scaffolds was due to increase in porosity and pore size of the HA- added samples as evident by their micro-CT images. alamarBlue® assay and DNA quantification analysis of the scaffolds indicated higher cell proliferation and DNA content for hASC cultured on AG and AG:HA (80:20) scaffolds as compared to AGC and AGC:HA (80:20) scaffolds. Similar trends were observed for total amount of calcium deposited by hASC. Highest mineralization as analyzed by alizarin red staining was observed for AG:HA (80:20) compared to AGC:HA (80:20) scaffolds at the end of three weeks. *ALP,* an early stage osteogenic marker was expressed by all the scaffolds at the end of 7 days indicating the osteogenic potential of hASC cultured on them. However, *OCN,* which is a late stage marker for osteogenesis was expressed only by PCL, PCL:HA (80:20) and AG:HA (80:20) at the end of 21 days. The *in vitro* osteogenic study described herein indicates that AG:HA (100:0 and 80:20) can be used as potential scaffolds in bone tissue engineering applications. The calcium deposition by the hASC cultured on these scaffolds was indicated by Alizarin red staining protocol. The expression of *ALP* at 7 days and *OCN* at 21 days

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is an indication of the ability of the scaffolds to support growth and induce differentiation of hASC to the osteogenic lineage. Thus, the scaffolds synthesized by the monomers obtained from sugarcane industry by-products hold promising potential as tissue engineering scaffolds for bone.

4.5. References

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CHAPTER 5. SUMMARY AND FUTURE WORK

The aim of this work was to synthesize non-toxic, biodegradable polyesters utilizing the by-products of sugarcane industry, namely molasses and bagasse. The idea was to evaluate and characterize these polyesters as scaffolds for tissue engineering applications. In this study, these novel polyesters were tested for their potential in skin and bone tissue engineering. This rationale is not only profitable for the sugarcane industry as a means of value addition by the use of its by-products, but it also unfolds a path for generating novel biomaterials for tissue engineering applications.

The 2nd chapter of the dissertation work dealt with extraction of aconitic acid, a component of bio-polymer, from molasses, and analyzing its purity for its suitability in polymerization reactions. Extraction of aconitic acid using ethyl acetate as a solvent was studied. Yields of extraction varied from 26–69% at different time temperature combinations and purity values of the extracted acid were as high as 99.89% . Another approach taken in this study was fermentation of molasses prior to extraction of aconitic acid. The idea was to generate an additional stream of ethanol along with aconitic acid and to study its effect on aconitic acid yields. It was observed that aconitic acid yields decreased only marginally to about 62% though there was no compromise on the purity values of aconitic acid. Maximum ethanol yield of 12.4% (g per 100 g of molasses) at the end of 30 h of fermentation was observed. This route might be beneficial in the sense that two value added products can be generated by utilizing molasses from the sugarcane processing which is otherwise mainly used as a cattle feed. The high purity aconitic acid extracted from a simple liquid-liquid extraction set up can directly be used as a monomer for polyesters synthesis.

The next step in this research involved synthesizing polyesters of aconitic acid, glycerol and cinnamic acid, and evaluating their potential as tissue engineering scaffolds. Cinnamic acid

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can be derived from the different phenolic compounds recovered from the lignin waste stream generated during the production of bio-ethanol from sugarcane bagasse, while glycerol can be recovered from the fermentation broth from pretreated sugarcane bagasse used for bio-ethanol production. Efforts are undergoing to extract cinnamic acid from the lignin stream generated during pretreatment of sugarcane bagasse. Chapter 3 of this dissertation gave a detailed account of synthesis of polymers from aconitic acid, glycerol and cinnamic acid, and also their characterization using various techniques. Different compositions of polyesters were synthesized and studied for their mechanical properties, porosity, mass loss in stromal medium, and ability to support growth and proliferation of human adipose derived mesenchymal stem cells (hASC). The composition of polymers was varied so that polyesters with different end capped groups such as excess/equal number of hydroxyl/carboxyl groups were synthesized. Several biocompatibility tests including mass loss over time, alamarBlue® to analyze growth and viability of hASC on polyester scaffolds, and picogreen for total DNA content synthesized were performed to evaluate the potential of these polyesters as scaffolds in tissue engineering applications. It was observed that the synthesized polyesters were not only non-toxic to the cultured hASC but they also supported their growth and proliferation on them, as validated by their metabolic assays and DNA content profiles relative to positive control. Polyesters having lower or no cinnamic acid content in them were found to be relatively biocompatible and favorable to the growth of hASC. The relative metabolic activities of the hASC on these scaffolds were found to be higher than the positive control. The compressive strength of the polyesters were found to vary between 5–6 MPa and polyesters with lower cinnamic acid content displayed more flexibility (lower modulus of elasticity). The porosity values for the thin film polyesters as analyzed by micro-CT varied between 30–40 %. The lower metabolic activities for polyesters with higher cinnamic acid content

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suggests that higher concentrations of the phenolic acid have deleterious effect on the cell growth. This can be due to the fact that higher concentrations of the acid are toxic to the hASC or the surface is too hydrophobic for the cells to attach to the polyester surface containing higher amounts of cinnamic acid. Cinnamic acid addition to the polyesters is favorable due to the fact that it imparts cross-linked structure to the matrix imparting higher mechanical properties to the scaffolds. The double bond moiety present in the cinnamic acid can be cross-linked under the influence of UV light yielding cross-linked polyesters. Based on bio-compatibility and cell viability tests performed, it was concluded that the synthesized polyesters hold the potential to be used as scaffolds for tissue engineering. It was observed that the amount of cinnamic acid present in the polyesters influenced the growth and proliferation of hASC to a larger extent. Hence, for further studies polyesters with lowest cinnamic acid and polyesters without any cinnamic acid were investigated.

The next section of Chapter 3 discussed the use of these polyesters as potential scaffolds for tissue engineering of skin with potential use in wound repair. Thin film polyester scaffolds laden with hASC were grown in stromal medium supplemented with bFGF. The amount of collagen synthesized on scaffolds was analyzed by Sirius Red collagen detection kit. bFGF is considered to be a mediator of early wound angiogenesis and stimulates tissue development. Based on the amount of collagen synthesized and DNA quantification data it was concluded that the synthesized polyesters can be used as scaffolds for wound repair by the addition of bFGF and promoting collagen synthesis. After 7 days of culture, the amount of collagen synthesized by hASC on AG and AGC scaffolds was found to be similar (124 µg/ml and 113 µg/ml respectively). This indicates that both these scaffolds supported growth and differentiation of hASC to a similar extent by the end of first week. However, the amount of collagen synthesized by hASC cultured on AGC

scaffolds was significantly lower than AG scaffolds by the end of two weeks. For hASC on AG scaffolds the collagen amount synthesized was 306 µg/ml as opposed to only 223 µg/ml for AGC scaffolds. The significant decrease in collagen amount for AGC scaffolds indicates that the presence of cinnamic acid affects the growth and proliferation of hASC and its effect is pronounced as time in culture increases. From the degradation profiles of these polymer samples, it was observed that these polyesters retain their integrity and structure over 2 weeks in physiological solutions. An ideal scaffold should have the rate of degradation similar to the time frame in which the new tissue forms. For skin tissue engineering this time frame is typically 2 weeks. Since the synthesized scaffolds were found to be structurally intact for more than 2 weeks, they can be potentially used for applications where a longer time for tissue regeneration is required. Hence, it was proposed to use them as scaffolds for tissue engineering of bone where the rate of tissue development (new bone formation) spans over a period of several weeks.

Chapter 4 of this dissertation described in detail the use of these novel polyester scaffolds for bone tissue engineering. In this study, AGC, AG and PCL scaffolds along with incorporated HA in them were studied for their ability to support growth, and promote osteogenesis of the hASC. The addition of HA to polyesters increases the osteogenic potential of scaffolds and hence its effect on cell differentiation was also studied in this research. Scaffolds were analyzed for metabolic activity using alamarBlue®, total DNA synthesized as assayed by picogreen, and osteogenesis was confirmed by alizarin red detection and gene expression using qPCR. Calcium deposition on each scaffold type by hASC was analyzed using alizarin red detection method. It was observed that mineralization (calcium deposition) increased gradually over 21 days indicating osteogenic activity of the hASC on the scaffolds. Similar trends (as observed in collagen synthesized) were observed for AGC:HA (80:20) scaffolds where calcium deposition at the end of

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first week was similar to AG:HA (80:20) scaffolds, but with time a gradual decrease in the amount of calcium deposited was observed. AG:HA (80:20) had highest cell viability and proliferation (alamarBlue® and picogreen analysis) and highest calcium deposition. Quantitative real time PCR was performed to quantitate osteogenic target gene expression of hASC loaded to scaffolds and cultured in both stromal and osteogenic media for 21 days. Expression of *ALP* (early marker) at 7 days and *OCN* (late marker) at 14 days and 21 days was measured using qPCR. All scaffolds expressed *ALP* at the end of 7 day time point after which its expression dropped since it is an early stage marker for osteogenesis. At the end of 21 days highest *OCN* expression was observed in AG:HA (80:20) scaffolds followed by PCL:HA indicating higher osteogenesis in these samples. This study concluded that these polyesters in conjuction with HA can be used as scaffolds for bone tissue engineering.

The motivation behind this research was development of novel polymers utilizing by-products of sugarcane industry such that it can generate value and add to the profitability of the sugar industry. Aconitic acid was extracted from molasses as a part of this work and polyesters of aconitic acid, glycerol and cinnamic acid were synthesized. The polyesters through a number of bio-compatibility tests were proven to support growth and proliferation of hASC. The next phase of the research involved evaluating these polysters as scaffolds for skin tissue engineering. This was tested by growing the hASC on the polyester films and adding bFGF to the cell culture medium. The increase in the amount of collagen at the end of 14 days concluded that these polyesters can be used in applications requiring wound healing. Taking advantage of the lower degradation rates (over 2 weeks) of these polyesters, these were also evaulated for their potential as bone scaffolds. Calcium deposition and *ALP* and *OCN* gene expression at the end of 21 days implied that these polyesters can also be employed as scaffolds for bone tissue engineering. The

synthesis and application of polyesters as described in this work paves way for novel materials that can be employed in tissue engineering derived from biological feedstocks. It also provides an economical gain for the sugarcane industry by utilizing its by-products and creating novel value added commodities from them.

FUTURE WORK

Polyesters of aconitic acid, glycerol and cinnamic acid were synthesized in this study. Molasses is a rich source of aconitic acid, and cinnamic acid can be isolated from the lignin waste stream generated during the production of bio-ethanol from sugarcane. While this research discussed in detail the extraction of aconitic acid from molasses, efforts should be directed towards isolating cinnamic acid from the different phenolic compounds obtained after pretreatment of sugarcane bagasse to generate bio-ethanol. Research efforts should be directed not only to isolate cinnamic acid but other phenolic acids as well from the cocktail of compounds present in the hydrolysate stream generated during ethanol production. Different polymer structures can be synthesized using these various starting materials and tested for a wide range of applications.

The polyesters in this study were synthesized from the commercially purchased monomers. The next step should be isolating both the acids from their respective by-products (sugarcane molasses and bagasse) and synthesizing these polyesters from the monomers extracted in-house. This, in its true sense, will be a biorefinery where sugarcane is the main feedstock which generates not only raw sugar but also valuable commodities, such as these polyesters. Tuning the starting material compositions, mechanical properties as well as the degradation rates of the polymers can be varied and custom made according to tissue specific applications. One approach could involve making blends of these polyesters with naturally occuring material such as collagen, etc. Degradation rates, metabolic activity and bio-compatibility of these polyester blends can be

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studied and evaluated for potential in tissue engineering applications. In this research project, no specific fabrication method was employed to generate a a well-defined, interconnected pore structure. As a part of future work, different fabrication techniques (electrospinning, salt leaching, etc.) can be used to study their effect on scaffold architecture. Thus, different techniques can be used to enhance mechanical properties of the scaffolds, generate a scaffold with well defined pore structure, and also tune the degradation profiles of the scaffolds by changing their compositions. This would allow synthesis of a range of polyesters having varied structural, mechanical and biological properties.

All the research done in this dissertation was performed *in vitro*. For these polyesters to find clinical applications, *in vivo* animal studies need to be perfomed. The effect of these scaffolds in animal model wound healing and bone formation can be studied to know the performance of these polymers under actual physiological conditions.

APPENDIX: APPROVAL FROM JOURNAL OF CHEMICAL TECHNOLOGY & BIOTECHNOLOGY

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VITA

Akanksha V. Kanitkar was born in India in June 1986. She received her Bachelor of Technology in Chemical Technology with a concentration in oils, oleochemicals and surfactants in June 2007 from the Institute of Chemical Technology, Mumbai, India. In January 2008, she started her M.S. program at Louisiana State University and graduated in May 2010 with a Masters' in Biological and Agricultural Engineering with a concentration in biodiesel synthesis. In May 2010, she began her doctoral program in Engineering Science at LSU, minoring in polymer chemistry. She expects to obtain her doctoral degree in May 2014.