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Fabrication of nanofiber scaffolds by electrospinning and it's potential for tissue engineering

Shobana Shanmugasundaram
New Jersey Institute of Technology

ABSTRACT

FABRICATION OF NANOFIBER SCAFFOLDS BY ELECTROSPINNING AND ITS POTENTIAL FOR TISSUE ENGINEERING

by
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Electrospinning is a fabrication process that uses an electric field to control the deposition of polymer fibers on to a target substrate. This electrospinning strategy can be used to fabricate fibrous polymer mats composed of fiber diameters ranging from several microns down to tens of nanometers. This study assesses the potential of electrospinning, as an alternative scaffold fabrication technique for tissue engineering applications. In this study, electrospinning is adapted to produce tissue-engineering scaffolds of two different size ranges composed of non-woven poly-L-lactide (PLLA) nanofibers and as a first study, the potential use of these scaffolds as tissue engineering scaffolds was assessed with the cell proliferation of Mesenchymal stem cells. Electrospun fibers were characterized for fiber diameter, porosity, pore size and its distribution. The electrospun scaffolds achieved a high surface area and porosity. Mesenchymal stem cells (MSC) were seeded on to electrospun PLLA scaffolds having two different fiber diameters. The cell-polymer constructs were cultured under static culture conditions. Cell proliferation study was performed. The results showed that MSC tend to proliferate well on nanofibers than on microfibers.

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by
Shobana Shanmugasundaram

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FABRICATION OF NANOFIBER SCAFFOLDS BY ELECTROSPINNING AND ITS POTENTIAL FOR TISSUE ENGINEERING

Shobana Shanmugasundaram

Dr. Michael Jaffe, Thesis Advisor — Date
Research Professor of Biomedical Engineering, NJIT.

Dr. Treena Arinzeh, Thesis Advisor — Date
Assistant Professor of Biomedical Engineering, NJIT.

Dr. George Collins, Committee Member — Date
Visiting Scientist, Medical Device Concept Laboratory, NJIT.

BIOGRAPHICAL SKETCH

Author: Shobana Shanmugasundaram

Degree: Master of Science

Date: January 2004

Undergraduate and Graduate Education:

- Master of Science in Biomedical Engineering
  New Jersey Institute of Technology, Newark, NJ, 2004

- Bachelor of Engineering in Electronics and Communication
  VMKV Engineering College, Salem, India, 1997

Major: Biomedical Engineering

To my family,

With love…

For always being there to listen when things go wrong,

For hugs, for fun and laughter that we share, and

For all the countless ways you find to show how much you care…!

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CHAPTER 1

INTRODUCTION

Innovation is the hallmark of the manufactured fiber industry. Fibers more numerous and diverse than anything found in nature are now routinely created in the Industry's laboratories. Natural fiber has been available for centuries, synthetic fiber for less than a hundred years. Synthetic fibers are now pervasive in everyday life. There are more than a dozen generic types produced today. Increased knowledge of their properties and capabilities has resulted in a vast array of functions - some old, some new - throughout the broad range of products: from running shoes, home furnishings, medicine, aeronautics, energy, to innumerable industrial products. These fibers are produced from organic polymers and inorganic materials. Fibers can be combined, modified and tailored in ways far beyond the performance limits of fiber drawn from the silkworm cocoon, grown in the fields, or spun from the fleece of animals.

Engineered non-woven products of manufactured fibers are found in applications from surgical gowns and apparel interfacing to roofing materials, roadbed stabilizers, and floppy disk envelopes and liners. Non-woven fabrics, stiff as paper or as soft and comfortable as limp cloth, are made without knitting or weaving. As they always have, manufactured fibers continue to mean, "Life made better."

Though there are many methods of fiber production, Electrospinning is a much reported, but to date minimally commercialized process to generate smaller fibers. Electrospinning was first patented in 1897 and used in the textile industry in the 1930s [12].

The electrospinning process itself begins with a solution of polymers, long chains of molecules, pumped into an electrified metal nozzle. High voltage creates a cone of fluid outside the nozzle, and a tiny jet of material erupts, electrically attracted to a target surface a few inches below the nozzle. In a few milliseconds, the electric field aligns the polymer molecules in the jet stream into fibrous strands, pulling and stretching the jet 1,000 times thinner than the micron-sized nozzle opening, to about diameters in the nanometer range [12].

Oddly, the nanoscale fibers don't follow a straight path down from the jet stream to the target surface. In fact, they spiral downward to form an unwoven mesh like a fibrous web [26]. As the polymers travel in air, the liquid solvent they were suspended in evaporates. The resulting material has an unusual, ephemeral feel, a tactile blend of paper, plastic and rubber. It is dry, and is fairly strong and difficult to tear.

Unlike conventional fiber spinning techniques, which are capable of producing fibers with diameters down to the micron size range, electrostatic spinning, or electrospinning is capable of producing fibers in the nanometer diameter size range, or "nanofibers". In electrospinning, electrostatic forces are used in addition to mechanical forces to drive the fiber forming process. The resulting nanofibers are of substantial scientific and commercial interest, as they are thought to exhibit morphologies and properties different from conventional fibers.

The electrospun nanofibers provide a very large surface area/mass due to their small diameter, hence non-woven fabrics, with small fibers can be used for filtration of sub-particles in the separation industry. These can also be used for the adsorption of biological and chemical warfare gases, protective clothing, in medical industry as

artificial blood vessels, sutures, surgical facemask, fiber-reinforced materials, mono-directional composites and in agricultural field for control of pesticides. The electrospinning technique can be used in chemical and manufacturing industries for paint spraying, electro deposition, plasma deposition, and mining minerals. In general, polymer nanofibers are used in a variety of applications, including filtration, protective clothing, biomedical applications such as wound dressing and drug delivery systems, design of solar sails, light sails and mirrors for use in space, as well as for structural elements in artificial organs and in reinforced composites. Ceramic or carbon nanofibers made from polymer precursors make it possible to expand the list of possible uses for nanofibers.

Another promising field of application of nanofibers is Tissue engineering. Synthetic tissues help stimulate living tissues to repair themselves in various parts of the human body, such as cartilage, blood vessels, bones and so forth, due to diseases or wear and tear. Victims whose skin are burned or scalded by fire or boiling water may also find an answer in synthetic tissues. The newest generation of synthetic implant materials, also called biomaterials, may even treat diseases such as Parkinson's, arthritis and osteoporosis.

The uses of synthetic tissues are numerous. And there are several methods available to create them. One method is to make use of scaffold fabrication technology. Under this technology, synthetic tissues are cultured and placed in the scaffold that is shaped accordingly to, say a tendon or ligament, and then grafted onto the damaged part of the body. Once the new tissues grow over the damaged part of the organ or achieved sufficient structural integrity, the scaffolds would eventually degrade until only the

tissues remain. It is also possible to use biocompatible materials that do not degrade, in which case, the scaffolds remain harmlessly in the body.

In this work, tissue-engineering scaffolds of two different size range were fabricated by electrospinning a solution of Poly L - lactide. The electrospun scaffold was characterized for structure by measuring fiber diameter, porosity, pore size and its distribution. Mesenchymal Stem cells were seeded on to the scaffold and experiments were performed to study the cell proliferation on the scaffold.

CHAPTER 2

BACKGROUND

Fiber has a myriad of uses, totally unlike anything the world had previously known. Since its invention it has been produced by various methods. This chapter gives a brief summary of those methods as well explains the background and principle of electrospinning. It also elaborates on the process, various experimental setups and fiber-web structure.

2.1 Clinical Significance of Electrospun Fibers

From a biological viewpoint, almost all of the human tissues and organs are deposited in nanofibrous forms or structures. Examples include: bone, dentin, collagen, cartilage, and skin. All of them are characterized by well-organized hierarchical fibrous structures realigning in nanometer scale. As such, current research in electrospun polymer nanofibers has focused one of their major applications on bioengineering.

Polymer nanofibers fabricated via electrospinning have been proposed for a number of soft tissue prostheses applications such as blood vessel, vascular, breast, etc. In addition, electrospun biocompatible polymer nanofibers can also be deposited as a thin porous film onto a hard tissue prosthetic device designed to be implanted into the human body. This coating film with gradient fibrous structure works as an inter-phase between the prosthetic device and the host tissues, and is expected to efficiently reduce the stiffness mismatch at the tissue/device inter-phase and hence prevent the device failure after the implantation.

For the treatment of tissues or organs in malfunction in a human body, one of the challenges to the field of tissue engineering/biomaterials is the design of ideal scaffolds/synthetic matrices that can mimic the structure and biological functions of the natural extra cellular matrix (ECM). Human cells can attach and organize well around fibers with diameters smaller than those of the cells [20]. In this regard, nanoscale fibrous scaffolds can provide an optimal template for cells to seed, migrate, and grow. A successful regeneration of biological tissues and organs calls for the development of fibrous structures with fiber architectures beneficial for cell deposition and cell proliferation. Of particular interest in tissue engineering is the creation of reproducible and biocompatible three-dimensional scaffolds for cell ingrowth resulting in bio-matrix composites for various tissue repair and replacement procedures. Recently, people have started to pay attention to making such scaffolds with synthetic biopolymers and/or biodegradable polymer nanofibers [15]. It is believed that converting biopolymers into fibers and networks that mimic native structures will ultimately enhance the utility of these materials, as large diameter fibers do not mimic the morphological characteristics of the native fibrils.

Polymer nanofibers can also be used for the treatment of wounds or burns of a human skin, as well as designed for hemostatic devices with some unique characteristics.

Figure 2.1 Nanofibers for wound dressing.

With the aid of an electric field, fine fibers of biodegradable polymers can be directly sprayed/spun onto the injured location of skin to form a fibrous mat dressing [Figure 2.1], which can let wounds heal by encouraging the formation of normal skin growth and eliminate the formation of scar tissue which would occur in a traditional treatment [22].

2.2 The Electrospinning Process

Formhals first reported the manufacturing process of electrospinning [12] in 1934. From 1934 to 1944, Formhals published a series of patents, describing an experimental setup for the production of polymer filaments using an electrostatic force. A polymer solution, such as cellulose acetate, was introduced into the electric field. The polymer filaments were formed, from the solution, between two electrodes bearing electrical charges of opposite polarity. One of the electrodes was placed into the solution and the other onto a collector. Once ejected out of a metal spinneret with a small hole, the charged solution

jets evaporated to become fibers, which were collected on the collector. The potential difference depended on the properties of the spinning solution, such as polymer molecular weight and viscosity. When the distance between the spinneret and the collecting device was short, spun fibers tended to stick to the collecting device as well as to each other, due to incomplete solvent evaporation.

Conventional fiber spinning techniques, e.g., melt spinning, dry spinning or wet spinning, rely on mechanical forces to produce fibers by extruding polymer melt or solution through a spinneret and subsequently drawing the resulting filaments as they solidify or coagulate. Electrospinning offers a fundamentally different approach to fiber production by introducing electrostatic forces to modify the fiber formation process.

Although the idea goes back at least 65 years, the process of electrospinning has become a popular area of research. The increasing number of publications and patents in this field ascertain this. Gibson et al [13] describes electrospinning as a process in which high voltage is used to produce an interconnected membrane like web of small fibers. This method provides the capacity to lace together many types of polymers, fibers, and particles to produce ultra thin layers.

Electrospinning is a unique process that produces polymer fibers with diameters down to the nanometer range, which typically form non-woven fabrics. On the scale of the individual fibers, the beneficial structural features such as large surface-to volume and high aspect ratios result from the smallness of their diameters. On a larger scale, these properties of the individual fibers are further enhanced by the porous structure of the non-woven fabric. As a processing technique, electrospinning is characterized by its versatility. Nanofibers can be electrospun from a wide range of polymers at a reasonably

fast speed. In addition, since it requires only small quantities of polymer, it can be regarded as a 'micro processing' technique, and is therefore ideal for laboratory studies.

In the electrospinning process a high voltage is used to create an electrically charged jet of polymer solution or melt, which dries or solidifies to leave a polymer fiber [10,18,19]. One electrode is placed into the spinning solution/melt and the other attached to a collector. Electric field is subjected to the end of a capillary tube that contains the polymer fluid held by its surface tension. This induces a charge on the surface of the liquid. Mutual charge repulsion causes a force directly opposite to the surface tension [7,8]. As the intensity of the electric field is increased, the hemispherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape known as the Taylor cone [30-34]. With increasing field, a critical value is attained when the repulsive electrostatic force overcomes the surface tension and a charged jet of fluid is ejected from the tip of the Taylor cone. The discharged polymer solution jet undergoes a whipping process [25,26] wherein the solvent evaporates, leaving behind a charged polymer fiber, which lays itself randomly on a grounded collecting metal screen. In the case of the melt the discharged jet solidifies when it travels in the air and is collected on the grounded metal screen.

The morphology of the resultant polymer nanofibers are affected by the effects of various materials and system parameters that include the molecular weight, molecular-weight distribution and architecture (branched, linear etc.) of the polymer, solution properties (viscosity, conductivity and surface tension). The processing parameters, that are the electric potential, flow rate and concentration, distance between the capillary and

collection screen, and ambient parameters namely temperature, humidity and air velocity in the chamber, motion of target screen also have effects on the process.

2.2.1 Theory

Taylor's theory [30] proposed that fine jets of slightly conducting viscous fluids and thicker jets or drops of less viscous fluids can be drawn from conducting tubes by electric forces. As the potential of the tube rises, viscous fluids become nearly conical and fine jets come from the vertices [see Figure 2.2]. The stability of viscous jets depends on the geometry of the electrodes. When a liquid drop is subjected to an electric field, it will become slightly elongated in the direction of the field. Since the dielectric constant of the drop is larger than that of the surrounding air, the elongation of the drop effectively channels more of the field inside the dielectric material, lowering the overall energy stored in the electric field.

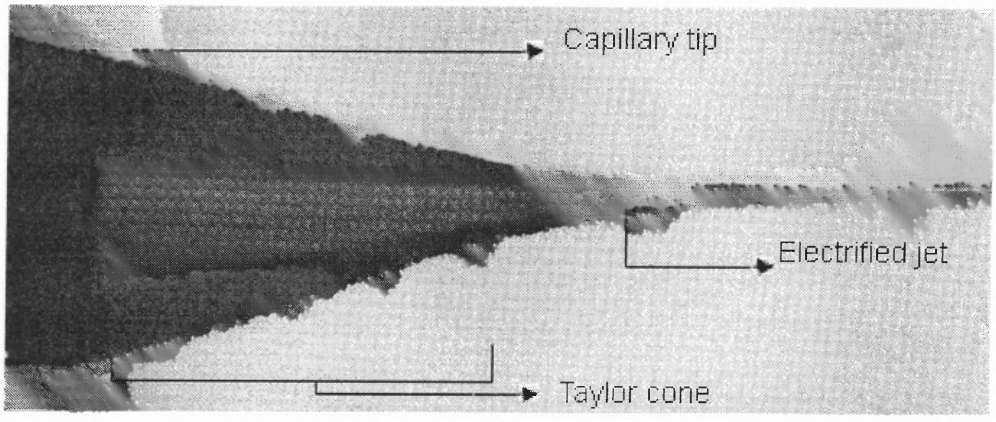

Figure 2.2 Formation of taylor cone.

This elongation is opposed by the surface tension of the drop, which will keep the drop shape close to spherical. As the field is increased, the drop will continue to deform.

As the tips of the drop reduce their radius of curvature, more electric field is concentrated at these points. This mechanism feeds back into further deformation. At some critical field and deformation, the rate of energy gained by raising the electric field inside the dielectric drop is no longer offset by the energy lost due to surface tension, and the drop unstably progresses toward a very sharp tip. Zeleny first studied this phenomenon around 1915 [37-40]. G.I. Taylor published a paper where he calculated the angle of the conical drop. Since then, the drops are called Zeleny-Taylor cones, or simply Taylor cones.

Theoretically, the tip of the drop will continue to grow infinitely sharp, but a new process takes over. For slightly conducting liquids a jet of fibers emerges from the tip. The jet transports accumulated charge away from the conical drop.

2.2.2. Principle

The principle of the electrospinning process emerges from the findings of Rayleigh, Zeleny and Taylor on electrically driven jets and their instabilities. Taylor observed that the electrosplayed jet is not always stable, but can shoot out of the cone intermittently. This instability was studied by Lord Rayleigh [23]. He showed that the droplet is unstable when electrical force is greater than or equal to force from surface tension i.e., when the electrostatic force overcomes the surface tension force, which is acting in the opposite direction of the electrostatic force, the unstable charged droplet breaks up into a series of charged droplets at this point and liquid is ejected in fine jets.

Rayleigh studied theoretically the stability of an isolated charged liquid droplet and predicted that it becomes unstable and fission takes place when the charge becomes sufficiently large compared to the stabilizing effect of the surface tension. Zeleny tried to

adapt this theory to the case of an electrified droplet losing its stability when jetting begins at its vertex. He studied the discharge from charged drops and from uncharged drops in electric fields and this showed that the surface electric intensities, when the discharge began, satisfied the theoretical relations for surface instability. He observed that the surface instability induced a cone on the liquid surface and the mode of spraying was from several sites from one cone. The liquid drop was stable at a value below the threshold for the discharge of the liquid. The process of ejection of small droplets occurs because of the surface instability that occurred when the internal pressure of droplet was same as external pressure.

2.2.2 Fiber Characteristics

Ultra-thin polymer fibers with diameters in the range of few microns down to 10 nm are accessible via the electrospinning process. The fiber size leads to vast differences in basic web properties such as fiber surface area, basis weights, thickness, permeability, and strength. While the calculations of nanofiber web physical properties are often straightforward from fiber counts and fiber diameters in scanning electron microscopes, the results, when expressed in the conventional units of the nonwovens industry, illustrate that electrospun nanofiber webs are categorically distinct from other non-wovens. Electrospun nanofibers have diameters that are 1 to 2 orders of magnitude smaller than melt spun fibers. This leads to a corresponding increase in fiber surface area and decrease in basis weight. This explains the need to study the fiber characteristics of electrospun fiber web. The property of having large surface area of the web enhances the interest.

For a variety of applications it is desirable to produce such fibers with well-defined surface topologies. Rough surfaces, pores or modulations of the chemical composition will not only contribute to larger surface to volume ratios, such features will affect wettability, adsorption and adhesion of the fibers. Fibers with specific surface topologies are also of interest as templates for the manufacturing of tubes [3,4].

In addition to round nanofibers, electrospinning a polymer solution can produce thin fibers with a variety of cross-sectional shapes which include branched fibers, flat ribbons, ribbons with other shapes, and fibers split longitudinally from larger fibers [Figure 2.3]. The fluid mechanical effects, electrical charge carried with the jet, and evaporation of the solvent all contributed to the formation of the fibers with different shapes [28].

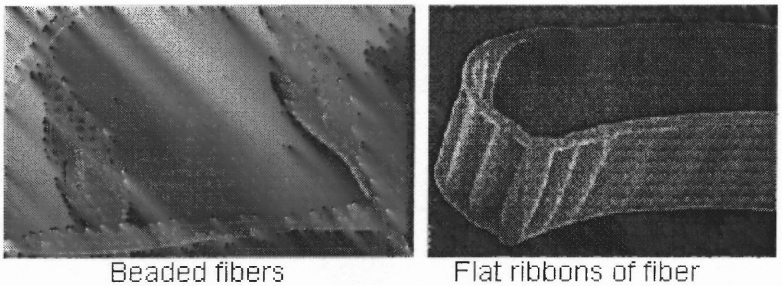

Figure 2.3 Thin Fibers with a variety of cross-sectional shapes.

Jaeger et al. [16,17] observed beaded electrospun fibers obtained from aqueous solution of poly (ethylene oxide) and they reported that the bead diameter and spacing depended on the fiber diameter i.e., thinner the fiber, shorter the distance between the beads and smaller the diameter of the beads. They explained that the formation of beads is the draw resonance phenomenon, which occurs if fibers are drawn to high draw ratio.

Entov et al. [9] developed a mathematical model for the break-up of jets of polymer solutions and they reported that the beaded electrospun fibers are related to the instability of the jet. Fong et al [11] carried out experiments on aqueous PEO and reported that the formation of the beaded structures can be considered as the capillary breakup of the jets by surface tension altered by the presence of electrical forces. They observed four reasons for the bead formation, viscosity, net charge density, surface tension, charges on the jets and gave probable solutions namely, as the viscosity is increased, beads become bigger, the average distance between beads longer, and the shape of the beads changes from spherical to spindle-like. The beads become smaller and more spindle-like when the net charge density is increased. Decreasing the surface tension results in bead disappearance and neutralization of the charge affects the formation of beads. Y K Luu et al., [36] showed in their work that it is possible to incorporate plasmid DNA into a polymer solution and to electrospin a nanostructured DNA/polymer matrix. As such, release profiles from electrospun membranes demonstrated sustained release for the duration of the study period, with plasmid DNA remaining structurally intact at all sample times.

Structured polymer fibers with diameters in the range from several micrometers down to tens of nanometers are of considerable interest for various kinds of applications. Examples are thin fibers for filter applications, fiber mats serving as reinforcing component in composite systems, biomedical applications or fiber templates for the preparation of functional nanotubes. Electrospinning has been shown to be an effective method for the production of such thin fibers. For selected applications it is desirable to control not only the fiber diameter, but also the internal morphology. Porous fibers are of

interest for applications such as filtration, tissue engineering scaffolds or the preparation of functional nanotubes by fiber templates. A given surface topology will affect, for instance, the wetting behavior as well as specific adsorption processes.

Bognitzki [4] found that electrospinning would directly yield porous fibers provided that the spinning parameters and the solvent are chosen appropriately. The basic concept was to control the formation of the fiber morphology in such a way that phase separation processes take place during electrospinning. This leads to spinodal or binodal types of phase morphologies within the fibers. The structure formation is apparently controlled by a rapid phase separation induced by the evaporation of the solvent and a subsequent rapid solidification.

High porosity and small pore size are potential advantages of electrospun fabrics compared to other materials. Scaffold fabrication using the electrospinning technology is becoming popular as electrospun nanofiber mats have a morphologic similarity to the extra cellular matrix of natural tissue. Pores in a tissue-engineered scaffold make up the space in which cells reside. Pore properties, such as porosity, dimension, and volume, are parameters directly related to the success of a scaffold. High porosity provides more structural space for cell accommodation and makes the exchange of nutrient and metabolic waste between a scaffold and environment more efficient.

Frank Ko et al. fabricated a novel scaffold for tissue engineering, a PLGA electrospun nanofibrous scaffold with more than 90% porosity, which indicated a highly porous structure. They observed that architecture of the structure was similar to that of natural extra cellular matrix, indicating that the electrospun nanofibrous scaffold is suitable as a tissue substitute. A wide range of pore size distribution, high porosity, and high surface

area-to-volume ratio characterizes these nanofiber-based scaffolds, which are favorable parameters for cell attachment, growth, and proliferation. The structures provide effective mechanical properties suitable for soft tissue, such as skin or cartilage.

2.3 Tissue Engineering

Tissue engineering is a new field based upon a relatively simple concept: Start with some building material (e.g., extra cellular matrix or biodegradable polymer), shape it as needed, seed it with living cells and bathe it with growth factors. When the cells multiply, they fill up the scaffold and grow into three-dimensional tissue, and once implanted in the body, the cells recreate their intended tissue functions. Blood vessels attach themselves to the new tissue, the scaffold dissolves, and the newly grown tissue eventually blends in with its surroundings.

2.3.1 Overview

Tissue engineering involves the design and fabrication of three-dimensional substitutes to mimic and restore the structural and functional properties of the original tissue. The term 'tissue engineering' is loosely defined and can be used to describe not only the formation of functional tissue by the use of cells cultured on a scaffold or delivered to a wound site, but also the induction of tissue regeneration by genes and proteins delivered in vivo.

Tissue engineering has emerged through a combination of many developments in biology, material science, engineering, manufacturing and medicine. The strategies developed in tissue engineering involve a range of approaches, the key element of which is the use of biologically based mechanisms to achieve the repair and healing of damaged

and diseased tissues. This application distinguishes it from the use of medical devices, and the delivery of an assembled 'tissue equivalent' distinguishes it from a pharmaceutical product. Tissue engineering addresses the problem caused by many injuries and disease processes that result in physically damaged tissues and organs in our bodies, which, if left unattended, repair imperfectly or not at all. It is the intervention with an engineered tissue that presents the prospect of achieving successful repair where it would not otherwise occur.

Tissue engineering is part of new wave of developments in biomedicine in which our scientific understanding of how living cells function will enable us to gain control and direct their activity to promote the repair of damaged and diseased tissues. The potential for medical intervention with a tissue engineering solution is seen nowhere better than with the chronic, persistent leg ulcer, which, in a patient with diabetes, provides a constant source of discomfort and incapacity. The patient does not lack the inherent capacity to heal a skin wound, but healing is failing to occur naturally at the site of the ulcer. What are lacking are the biological signals, chemical messengers and physical cues that initiate the events of cell migration, blood vessel formation and tissue assembly for normal wound healing. If we can provide these biological signals in a 'tissue engineered' package, we can kick-start a repair process that can be completed by the patient's own tissues.

The precise form will vary with the medical application for which it is designed. There are, however, two typical elements: one or more type(s) of living cell with particular tissue functions; and a material support that forms a structure for both culturing the cells in the laboratory and the surgical delivery of the tissue equivalent to the patient.

This support might be in the form of a lamella or tubular structure, or in a more complex three-dimensional structure, depending on the clinical application. The 'package' therefore contains several important, and quite different, material components and its assembly involves a manufacturing process that extends from the culture of living cells to the fabrication techniques for 3-dimensional structures, involving engineering and bioreactor design. It also has to be carried out in a regulatory framework that will ensure the monitoring and documenting of all stages of the process.

Although there is growing excitement in the field of tissue engineering, it is still in its infancy. Success will largely depend on the ability of scientists to figure out complex cellular interactions, then intervening with the right scaffold material and exact growth factors and cells.

Tissue engineering frequently involves stem cells, a kind of premature cell first isolated from the body in 1992 [35]; implanting stem cells in the appropriate location can generate everything from bone to tendon to cartilage. Stem cells have two important characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain physiologic or experimental conditions, they can be induced to become cells with special functions such as the beating cells of the heart muscle or the insulin-producing cells of the pancreas. All stem cells — regardless of their source are capable of dividing and renewing themselves for long periods.

Stem cells are important for living organisms for many reasons. In the 3 to 5 day old embryo, called a blastocyst, a small group of about 30 cells called the inner cell mass gives rise to the hundreds of highly specialized cells needed to make up an adult

organism. These inner cell mass cells are pluripotent. This means that they are able to give rise to many, but not all cell types necessary for fetal development (for example, they are able to give rise to fetal tissues, but not placental tissue). The pluripotent cells then further specialize into another type of stem cell, a multipotent stem cell.

Multipotent stem cells are less plastic and more differentiated stem cells. They give rise to a limited range of cells within a tissue type. The offspring of the pluripotent cells become the progenitors of such cell lines as blood cells, skin cells and nerve cells. At this stage, they are multipotent. They can become one of several types of cells within a given organ. For example, multipotent blood stem cells can develop into red blood cells, white blood cells or platelets [29].

The concept of stem cell technologies has been coined in recent years to describe a most promising alternative to conventional donor-dependent transplantation. It focuses on the study of stem cells, a vague label used to describe cells from various origins and functions that, to different extents, share two common properties: The ability of self-renewing in an undifferentiated state and the capacity to adopt many terminal fates. If harnessed, these unique properties of stem cells could be used to generate organs and tissues for transplantation from an unlimited supply of self-renewable cells.

Another potential application of stem cells is making cells and tissues for medical therapies. Today, donated organs and tissues are often used to replace those that are diseased or destroyed. About 128 million people suffer from chronic, degenerative, and acute diseases, and stem cell therapies hold great promise in the treatment of many of these diseases. Many different types of nerve cells are lost in injuries such as spinal cord injury, brain trauma, and stroke. Furthermore, glia, the cells that protect nerve fibers are

lost in multiple sclerosis. Creating new nerve tissue from pluripotent stem cells offers a potential therapy for these diseases. Pluripotent stem cells could be used to regenerate the missing immune cells of virtually all primary immunodeficiency diseases.

An adult stem cell is a multipotent stem cell in adult humans that is used to replace cells that have died or lost function. It is an undifferentiated cell present in differentiated tissue. It renews itself and can specialize to yield all cell types present in the tissue from which it originated. So far, adult stem cells have been identified for many different tissue types such as hematopoetic (blood), neural, endothelial, muscle, mesenchymal, gastrointestinal, and epidermal cells.

The advantages of embryonic stem cells are that they offer one cell source for multiple indications. They provide the potential for a wider variety of applications than do adult stem cells. While embryonic stem cells offer the potential for wider therapeutic applications, adult stem cells avoid the ethical issues roused by embryonic stem cell research. Therefore, many stem cell therapies are currently being tested using adult stem cells. Additionally, adult stem cells offer the potential for autologous stem cell donation, which may help to avoid issues of immune rejection in certain situations [29].

Adult stem cells have been identified in many organs and tissues. One important point to understand about adult stem cells is that there are a very small number of stem cells in each tissue. Stem cells are thought to reside in a specific area of each tissue where they may remain quiescent (non-dividing) for many years until they are activated by disease or tissue injury. A few stem cells that were recognized in humans are the hematopoietic stem cell, which produces all of the blood cell types; the gastrointestinal stem cell associated with regeneration of the gastrointestinal lining, the stem cell

responsible for the epidermal layer of skin, and germ cell precursors (in the adult human, the spermatogonial stem cell.) These stem cells were considered to have very limited repertoires, related to replenishment of cells within their tissue of origin. These limitations were considered to be a normal part of the developmental paradigm in which cells become more and more restricted in their lineage capabilities, leading to defined and specific differentiated cells in body tissues.

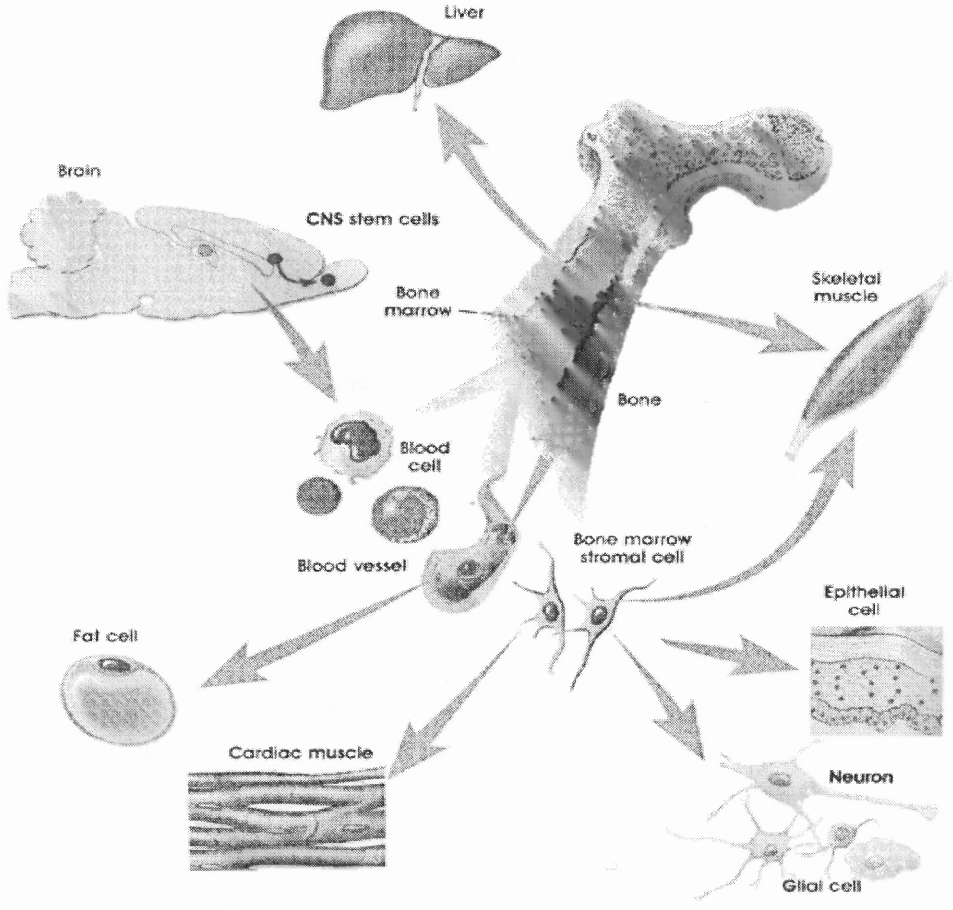

Figure 2.4 Plasticity of adult stem cells.

Bone marrow stromal cells (mesenchymal stem cells) give rise to a variety of cell types: bone cells (osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and

other kinds of connective tissue cells such as those in tendons. Neural stem cells in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells — astrocytes and oligodendrocytes. Epithelial stem cells in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells. Skin stem cells occur in the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer. The follicular stem cells can give rise to both the hair follicle and to the epidermis.

A number of experiments have suggested that certain adult stem cell types are pluripotent [29]. This ability to differentiate into multiple cell types is called plasticity or transdifferentiation. Hematopoietic stem cells may differentiate into: three major types of brain cells (neurons, oligodendrocytes, and astrocytes); skeletal muscle cells; cardiac muscle cells; and liver cells. Bone marrow stromal cells may differentiate into: cardiac muscle cells and skeletal muscle cells. Brain stem cells may differentiate into: blood cells and skeletal muscle cells.

2.3.2 Scaffolds in Tissue Engineering

Tissue engineering techniques generally require the use of temporary scaffolds to serve as a three-dimensional (3D) template for initial cell attachment and subsequent tissue formation. The ability of the scaffold to be metabolised by the body allows it to be gradually replaced by new cells to form functional tissues. Ideally, a scaffold should have the following characteristics: high porosity with an interconnected pore network for cell growth and transport of nutrients/metabolic waste, biocompatible and bioresorbable with

controllable degradation and resorption rates to match tissue replacement, suitable surface chemistry for cell attachment, proliferation and differentiation, mechanical properties such as stiffness to match those of the tissues at the site of implantation and load bearing characteristics.

Research on the biomaterials associated with tissue engineering was initiated ahead of the developments in cell biology. There is currently much research and innovation in the development of new biomaterials [1]. These extend from well-tried polylactates and polyglycolates to novel ceramics, caprolactones and hydrogels. Techniques are being developed for their formation into scaffolds, felts and weaves. Matrix printing devices are being explored for the fabrication of three-dimensional structures with micro architecture that might mimic complex living organs, such as liver or kidney tissues. Different applications require different material properties to cope both with the required tensile and compressive forces and with elastic deformation and compliance. There is also much innovation occurring in polymer chemistry, but each new polymer or biomaterial requires extensive evaluation to assess how it interacts with living tissue, how long it survives in the body, what wear-products it produces and how it degrades. The ability to generate new chemistry currently far outpaces the speed at which their biological advantages and disadvantages can be thoroughly assessed.

There is also considerable interest in the use of natural biopolymers for tissue engineering applications, such as the long chain polysaccharide hyaluronan and its chemically derivatised forms, and various preparations of natural and recombinant proteins, including collagens and even silk. Many of these materials provide the opportunity for chemically linking biological signaling molecules such as peptides or

small proteins, to provide sources of the signaling molecules that will trigger cell responses to help the healing process. For example, these 'smart' materials could be used to provide signals to encourage blood vessel development. When an engineered tissue is placed in the body, it requires the development of a blood supply from the patient for it to become integrated with surrounding tissues; this is essential for the completion of the healing process. The principle exceptions are cartilage, intervertebral disc and cornea, which are largely avascular tissues.

2.3.3 Adult Stem Cells in Tissue Engineering

Mesenchymal stem cells or human bone marrow stromal stem cells are defined as pluripotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. These primitive progenitors exist postnatally and exhibit stem cell characteristics, namely low incidence and extensive renewal potential. These properties in combination with their developmental plasticity have generated tremendous interest in the potential use of mesenchymal stem cells to replace damaged tissues. In essence mesenchymal stem cells could be cultured to expand their numbers then transplanted to the injured site or after seeding in/on shaped biomimetic scaffold to generate appropriate tissue constructs.

Adult human bone marrow contains a minority population of Mesenchymal stem cells that contribute to the regeneration of tissues such as bone, cartilage, muscle, ligaments, tendons, fat, and stroma. Evidence that these Mesenchymal stem cells are pluripotent, rather than being a mixture of committed progenitor cells each with a restricted potential, includes their rapid proliferation in culture, a characteristic

morphology, the presence of typical marker proteins, and their consistent differentiation into various mesenchymal lineages. These attributes are maintained through multiple passages and are identifiable in individual stem cells.

Bone and cartilage generation by autogenous cell/tissue transplantation is one of the most promising techniques in orthopedic surgery and biomedical engineering. Treatment concepts based on those techniques would eliminate problems of donor site scarcity, immune rejection and pathogen transfer. Osteoblasts, chondrocytes and mesenchymal stem cells obtained from the patient's hard and soft tissues can be expanded in culture and seeded onto a scaffold that will slowly degrade and resorb, as the tissue structures grow in vitro and/or in vivo. The scaffold or three-dimensional (3-D) construct provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the ultimate shape of the new bone and cartilage.

The requirement for new bone to replace or restore the function of traumatized, damaged, or lost bone is a major clinical and socioeconomic need. Bone tissue engineering has been heralded as the alternative strategy to regenerate bone. In essence, the discipline aims to combine progenitor or mature cells with biocompatible materials or scaffolds, with or without appropriate growth factors, to initiate repair and regeneration.

2.4 Literature Review

In 1934, Formhals [12] patented a process and an apparatus for the production of the polymer filaments using electrostatic force. An electric field was applied to a free surface of polymer solution, such as cellulose acetate. One electrode was placed into the spinning solution and the other electrode was attached to a moving collector. The liquid jets that were ejected dried and formed charged fibers. Small holes in a metal plate allowed the formation of several jets. The distance between the solution feeding device and the collecting device was variable. When the distance was short, spun fibers tended to stick to the collecting device as well as to each other, due to incomplete solvent evaporation [Figure 2.5]. The electrospinning process never became competitive with melt spinning, partly because the electrospun fibers were generally much smaller in diameter than the fibers that are used to make textiles.

In 1977, Martin and co-workers [21] manufactured a mat of fibers by electro statically spinning organic polymers. The mat could be used for wound dressings. Here the small diameter of the nanofibers fibers was advantageous. The mats of small fibers had small interstices between the fibers, and a high surface area per unit mass. If the dressing was formed from a wettable polymer, blood or serum escaping from the wound tended to penetrate the dressing, but readily clotted, owing to the high surface area and small interstices. Such dressings were usually sufficiently porous to allow interchange of oxygen and water vapor between the atmosphere and the surface of the wound. These mat dressings could be combined with woven textile fiber mats that provided strength and other useful properties, or with materials having antiseptic or wound healing properties.

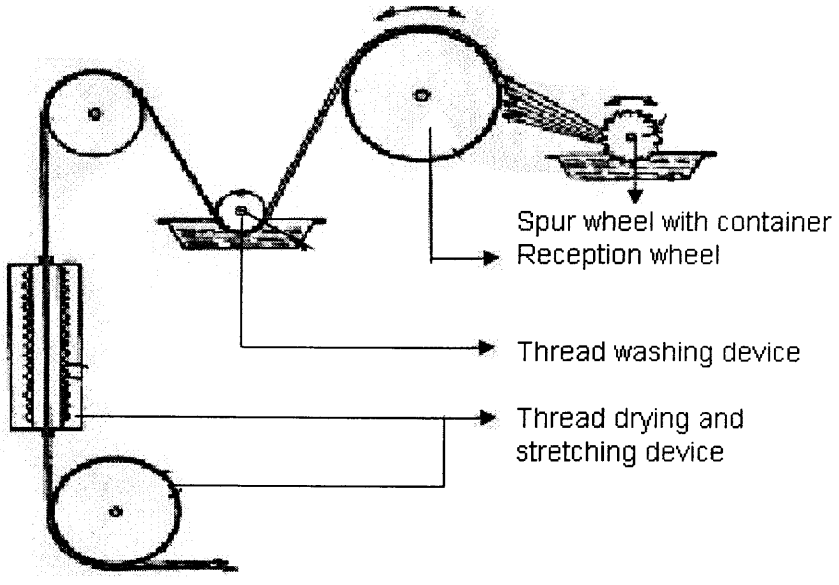

Figure 2.5 Apparatus designed for the production of artificial threads.

Materials that they electrospun include polymeric substances such as fluorinated hydrocarbons, e.g. PTFE and aqueous solutions of polyvinyl alcohol, polyvinyl pyrrolidone and polyethylene oxide, which conveniently may be spun from a dispersion of the material in a suitable dispersing agent, and polyurethanes which may be spun from solution. The spinning material was fed from a syringe reservoir to the tip of an earthed syringe needle, the tip being located at an appropriate distance from an electro statically charged surface. Upon leaving the needle the material formed fiber between the needle tip and the charged surface. The spinning material is fed into an electrostatic field from a syringe reservoir to the tip of an earthed syringe needle [Figure 2.6]. An electrostatically charged surface was placed at an appropriate distance. Fiber is formed between the tip of the syringe needle and the charged surface. Potential maintained was around 20 kV at a distance of 5–35cm between syringe needle and collecting surface. Mats collected on a rotating non-conducting belt had a few microns to a few centimeters thick, highly porous, low diameter, and high surface area.

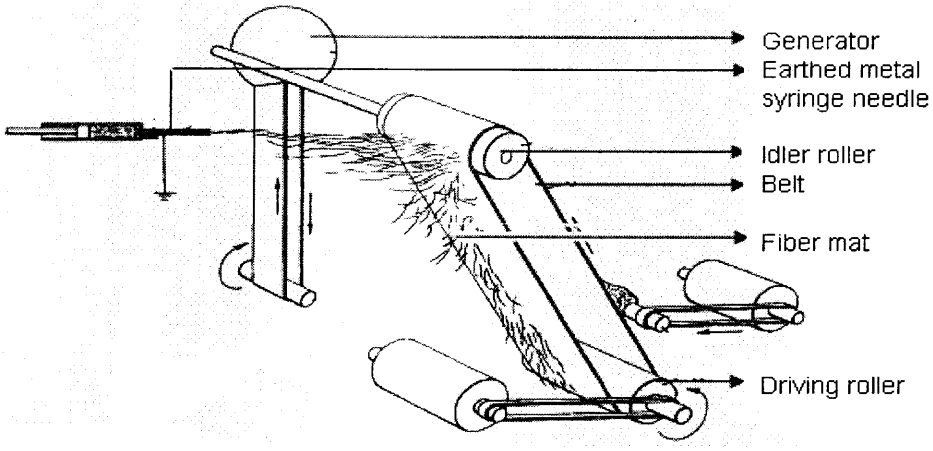

Figure 2.6 Apparatus designed for the continuous production of fibers.

In 1982, Bornat [5,6] described an electrostatic fiber spinning process that produced a tube of nanofibers on a rotating metal mandrel. In this process biologically acceptable polymeric substances were used such as PTFE, polyurethanes, dispersions of polytetrafluoroethylene, thermoplastics such as polyamides, polyacrylonitrile, water-soluble polymers such as polyvinyl alcohol, polyvinyl pyrrolidone and polyethylene oxide.

The apparatus [Figure 2.7] onto which the fibers were collected consisted of a metal mandrel or core 20 mm in diameter and 25 cm long having a sheath of aluminum foil 0.02 mm and 20 cm long wrapped around it. The voltage used was up to 50 KV. The polymer was fed into the electric field from a bank of 3 syringes, the needles of which were 3 cm long and 0.05 cm inner diameter at the rate of 1 gm/hr/needle. Upon introduction of the polymer into the electric field the droplet instantly disintegrated into fibers, which were drawn to the mandrel, and deposited upon it in a tubular layer.

This system also involved several capillaries for higher production rate and spinning solution was filled in these capillaries. The potential was maintained between these capillaries and the collecting surface. The collecting device is a rotating mandrel or

a rod so as to impart a core-sheath structure to the collected fibers so as to obtain a tubular product. The core may be conductive or non-conductive whereas the sheath is a electrically conducting material made of sheet metal or metal foil and is collapsible. The collected composite fibers in tubular form have low diameter, circular cross-section and porous.

How [14] described a process for the production of synthetic vascular grafts from polyurethane. Natural arteries are in general anisotropic, and the degree of anisotropy and the elastic moduli of the arteries increase with the distance from the heart, with the exception of coronary arteries.

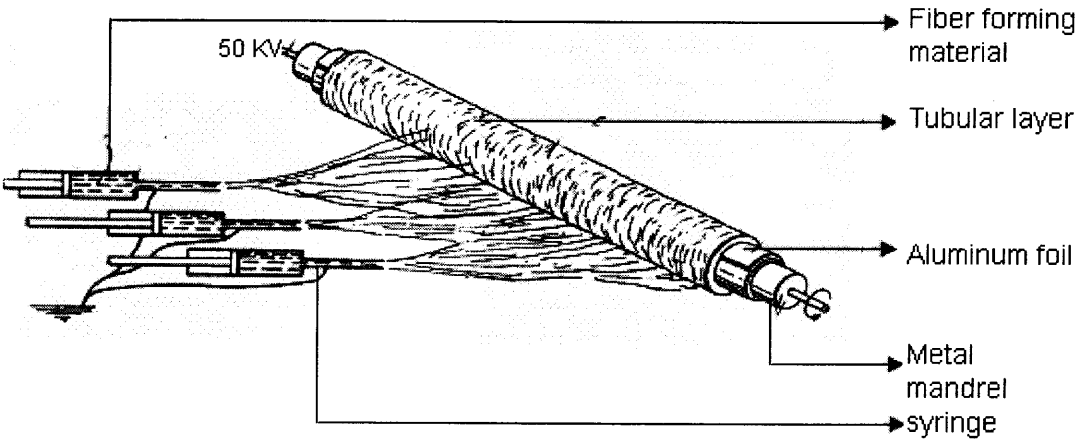

Figure 2.7 Apparatus designed for the production of tubular products.

The polymer solution is ejected from a syringe through a nozzle, the nozzle being earthed. To provide a constant flow of polymer solution through the nozzle, syringe piston is subjected to a constant hydraulic force [Figure 2.7]. Flow of the polymer solution through nozzle is maintained by the syringe piston, which is subjected to a constant hydraulic force. Fluid from the nozzle is fed to an electrostatic field surrounding

a charged mandrel, which is charged to around –12kV. When a droplet of the polyurethane was introduced into the electrostatic field, the droplet elongated to form a cone or jet. From the end of the jet, fine fibers of diameter in the range 1 to 2 micrometers were produced and were attracted onto the mandrel. Layers of fibers were gradually built up forming a porous and micro fibrous tube.

This invention essentially relies on the controlling the speed of rotation of the mandrel (between 2000 to 20000 rpm) such that a desired degree of anisotropy is present in the graft. The speed may be kept uniform during production of a particular graft, or alternatively there may be means for varying the rotational speed of the mandrel in accordance with the traverse position of the fluid directing means. In such a way a vascular graft having varying anisotropic properties along its length could be produced. The electro statically spun products for medical use may be made of any biocompatible, non-absorbable or absorbable fiberizable material.

In 1995, Doshi and Reneker [7,8] described electrospinning conditions, fiber morphology, and some possible uses of electrospun fibers made from several polymers. In electrospinning, the polymer solution is charged to a high electrical potential that produces a high electric field at the surface of the liquid. When the electrical force per unit area equals the pressure of surface tension at the surface of the polymer solution, a charged droplet hanging at the end of the capillary tube is in equilibrium, assuming that no other pressures are present. Increasing the potential makes the charged drop unstable, and usually a charged jet forms to carry the charge away. The jets stretch and dry in flight into solid, electrically charged fibers and are finally collected on an electrically grounded screen.

Electrospun fibers can be formed from nonvolatile solvents by removal of the solvent in a liquid. Srinivasan [27,28] electrospun a liquid crystal polymer, poly(p-phenylene terephthalamide) (PPTA), and an electrical conducting polymer, polyaniline, using sulfuric acid as the solvent for both polymers. Fine fibers of both PPTA and polyaniline, ranging in diameter from 40 nanometers to a few hundreds of nanometers, were obtained. The fibers of PPTA had a circular cross section, were birefringent and stable at temperature above 400°C.

An important characteristic of electrospinning is the ability to make fibers with diameters in the range of nanometers to a few microns. The diameter of these fibers is one to two orders of magnitude smaller than the 10 to 20 micron diameters of conventionally spun fibers. These thin fibers have a high ratio of surface area to volume and a high ratio of length to diameter. It is difficult to make nanometer diameter fibers using typical spinning processes. Equipment required to make electrospun fibers is simple, many jets can be created simultaneously, and nanofibers can be made from a small amount of polymer solution. The electrospun fibers provide a very large surface area per unit mass, due to their small diameter. Non-woven fabrics of nanofibers collected on a screen can be used for example, for filtration of sub -micron particles in separation industries, cleaning glass, and wound dressing in the medical industry.

CHAPTER 3

RESEARCH OBJECTIVE

The objectives of this research were to:

1. Produce scaffolds using the process of electrospinning.
2. Fabricate scaffolds composed of:
    a. Large fibers (micron range) –PLLA-LF
    b. Nanofibers – PLLA-SF
3. Study the effect of the two different fiber sizes on human mesenchymal stem cell proliferation.

The material used for the fabrication of scaffolds was poly-L-lactide. Characterization of the electrospun fibers was carried out using scanning electron microscopy (SEM), Mercury Intrusion Porosimetry and Differential Scanning Calorimetry. The stem cell proliferation on the scaffolds was characterized using the MTT assay.

CHAPTER 4

EXPERIMENTAL METHODS

4.1 Electrospinning Equipment

To achieve the research objectives an electrospinning apparatus was designed and constructed. The apparatus consists of two main devices, the sprayer and the collecting device.

The spraying setup (Figure 4.1) has a syringe fitted with a needle, mounted on a Harvard Syringe Pump Model 901. The polymer solution to be electrospun was loaded into the syringe. A constant flow rate of 0.103ml/min was maintained using the syringe pump. . The positive output lead of a high voltage power supply (Gamma High Voltage Power Supply ES30P; Gamma High Voltage Research, Ormond Beach, FL), set to 15-30 kV, was attached to a blunt, 16-20 gauge (inner diameter 0.047" – 0.023") needle on the syringe. The 16-gauge needle was used for making micro fibers, and the 20-gauge for the nanofibers.

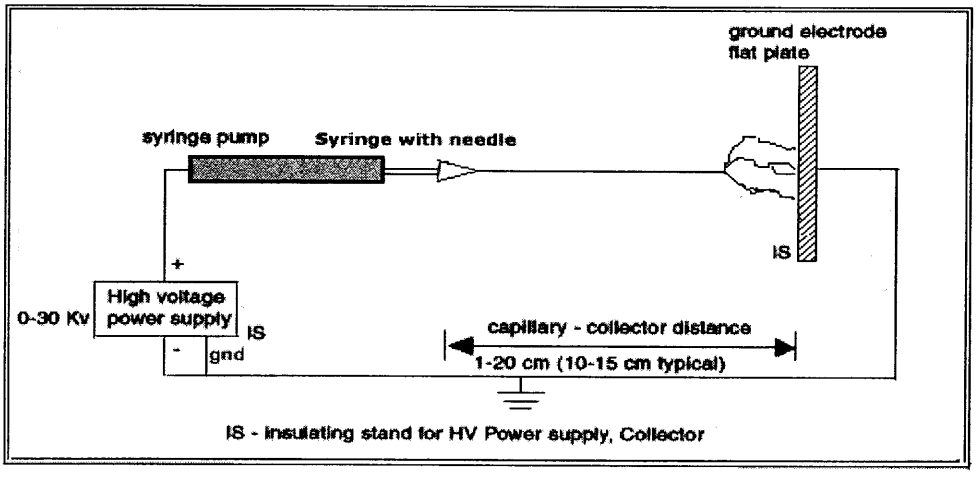

Figure 4.1 Diagrammatic representation of electrospinning equipment.

As has been explained in the principle of electrospinning process (2.2), when the charge of the polymer at increasing voltage exceeds the surface tension at the tip of the spinneret, the polymer gets ejected. The charged jet comes out of the spinneret, first in the form of Taylor cone and then is splayed randomly as fibers. The collector used was a stainless steel plate of dimensions 25x30 cm, which was electrically grounded. Typical operating regimes were flow rates between 0.1 and 1 ml/min, voltages between 10 and 20 kV and a plate distance of 15 to 20 cm.

Electrical sparks were observed if a small metal part was around the system. As the process needs voltage of very high order, proper care must be taken in understanding the electrical connections of the system. A detailed version of the chemical and electrical safety factors is listed in Appendix A.

The electrospinning process is affected by varying the electric potential, flow rate, concentration, distance between the capillary and collection screen, size of the needle, and also by the ambient parameters such as temperature and humidity.

The two variables considered for this work were the concentration of the polymer in the solution, and the diameter of the needle. The needles used were 16-gauge and 20-gauge. The electric field is defined as the ratio of electric voltage and the collecting distance, wherein electric voltage is the applied voltage to the polymer solution and collecting distance is the shortest distance from the tip of the spinneret to the surface of the cylindrical drum. The voltage used for the process was 25 Kilovolts. The collector to needle distance was 20 centimeters.

For the fabrication of PLLA-LF nanofibers mats, a 16-gauge needle was used for spinning a solution of 10% w/w concentration, and PLLA-SF was electrospun using a 20-gauge needle and a solution concentration of 5%w/w.

4.2 Materials

Electrospinning experiments were carried out using Poly-L-lactide, Resomer® L207 [Boehringer Ingelheim Fine Chemicals]. Poly-L-lactide is a biocompatible and biodegradable polymer that makes it an ideal candidate for tissue engineering. Poly-L-lactide has been used in medical applications, such as biodegradable sutures, since the 1960s. PLA exists as L-PLA (mainly crystalline) or DL-PLA (mainly amorphous). Through the manipulation of the co-polymer characteristics (such as the interconnectivity of the internal 3-D geometry, mechanical and structural integrity, and biodegradability) these scaffold structures can be designed and fabricated to suit a particular tissue engineering application.

The solvent used for preparing a solution of Poly-L-lactide was Methylene Chloride (Fisher Scientific). Resorbable polymeric biomaterials are traditionally processed in organic solvents such as methylene chloride. Potential advantages of the use of organic solvents in tissue engineering applications are that they can prevent hydrolysis of substrates/products and their use may allow a better integration with chemical steps/processes.

4.3 Solution Preparation

The solutions of Poly-L-lactide were prepared in glassware using the following technique. Glassware was cleaned using an initial rinsing with tap water. The desired amount of Poly-L-lactide, according to the concentration required was weighed. These polymer chips were poured into a glass bottle containing a measured amount of methylene chloride. The glass bottle was closed with an airtight lid to maintain the concentration throughout. A homogeneous solution was achieved by slow agitation. This was either by keeping the solution for sometime or by using a magnetic stirrer. The agitation was slow to avoid mechanical degradation of the polymer chains. All solutions were prepared at room temperature.

A solution concentration of 10% w/w was achieved by dissolving 20 grams of PLLA in 180 grams of Methylene Chloride. This concentration was used for electrospinning fibers of large diameter (microfibers). The concentration was reduced to 5%w/w for making the nanofibers. To get a concentration of 5% w/w, 10 grams of PLLA was dissolved in 190 grams of Methylene chloride.

4.4 Characterization of Electrospun Scaffold

4.4.1 Fiber Diameter Distribution

Electrospinning process produces very fine fibers down to a few nanometers. The scanning electron microscope (SEM) has many advantages over traditional microscopes. The SEM has a large depth of field, which allows more of a specimen to be in focus at one time. The SEM also has much higher resolution; so closely spaced specimens can be magnified at much higher levels. Because the SEM uses electromagnets rather than

lenses, the researcher has much more control in the degree of magnification. All of these advantages, as well as the actual strikingly clear images, make the scanning electron microscope one of the most useful instruments in characterizing the nanofiber webs produced by electrospinning.

The SEM is an instrument that produces a largely magnified image by using electrons instead of light to form an image. A beam of electrons is produced at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample (Figure 4.2).

Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen. This produces the final image.

Because the SEM utilizes vacuum conditions and uses electrons to form an image, special preparations must be done to the sample. All water must be removed from the samples because the water would vaporize in the vacuum. All metals are conductive and require no preparation before being used. All non-metals need to be made conductive by covering the sample with a thin layer of conductive material.

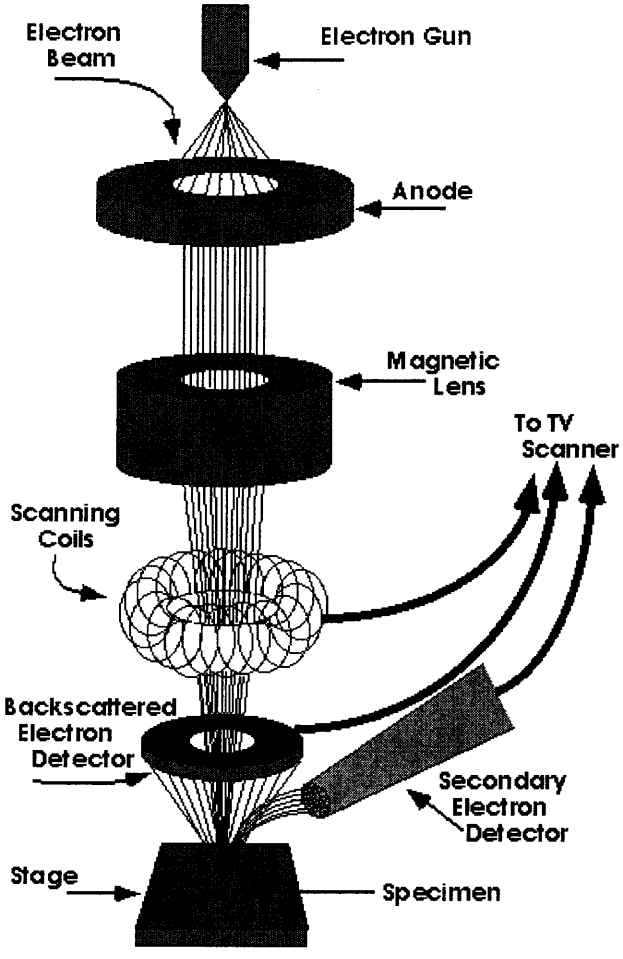

Figure 4.2 Schematic of the Working of a SEM.

The electrospun samples need no preparation, as they are electrically conductive. The samples were prepared by cutting from the electrospun web dimensions of around 4mm x 4mm. The stub has an area of around 120 square millimeters, so the specimen samples are cut in such a way that 4 to 5 could fit into the stub. A layer of carbon tape (double sided) is pasted to the stubs and the sample is stuck to the other side of the tape. Carbon tapes are used to prevent the charging of the sample. Carbon tapes dissipate the excessive charge buildup. The specimen fixed stubs was then mounted on the microscope chamber. To begin with the image capturing, proper spot size and accelerating voltage

should be selected. Higher accelerating voltage will increase resolution, but also increases specimen damage, contamination, and charging, and decreases surface detail. So, a voltage of 1 KV was used for all the samples. After setting the voltage, the samples were magnified to the desired size. The processes of stigmation, apertures align and focus was repeated for proper black level and gain level for clarity and was digitally scanned and stored. The SEM used for the study was Leo 1530VP.

4.4.2 Porosity and Pore Size Measurement

The Mercury Porosimeter is a versatile and accurate instrument used to determine properties such as pore size distribution, total pore volume, surface area, and bulk and absolute densities of solid and powder samples. The Mercury Porosimeter uses mercury intrusion to determine pore volume. The Mercury Porosimeter fills the penetrometer and sample chamber with mercury under vacuum and takes a volume reading. The sample, however, is not initially intruded with mercury because of the high surface tension. Gradually, increasing amounts of pressure are applied. For each incremental increase in pressure, the change in intrusion volume is equal to the volume of the pores whose diameters fall within an interval that corresponds to the particular pressure interval.

The theory of all mercury Porosimeters is based on the physical principle that a non-reactive, non-wetting liquid will not penetrate pores until sufficient pressure is applied to force its entrance. The relationship between the applied pressure and the pore size into which mercury will intrude is given by the Washburn equation:

$$\mathbf{PD = -4\gamma \cos \theta}$$

where P is the applied pressure, D is the diameter, γ is the surface tension of mercury (480 dyne cm^{-1} and θ is the contact angle between mercury and the pore wall, usually near 140°. As pressure increases, the instrument senses the intrusion volume of mercury by the change in capacitance between the mercury column and a metal sheath surrounding the stem of the sample cell. As the mercury column shortens, the pressure and volume data are continuously acquired and displayed by an attached personal computer.

The electrospun samples were cut in to 2.7 cm H x 2 cm D dimensions and were characterized for pore volume, pore size distribution, surface area and porosity.

4.4.3 Thermal Analysis

Thermal analysis for the electrospun web was carried out using TA Q100 DSC (Conventional and modulated). The Q100 can be used over the temperature range -180 to 725°C. DSC provides rapid and precise determinations of transition temperatures using minimum amounts of sample.

Common temperature measurements include the following Melting, Crystallization, Glass Transition, Polymorphic Transition, Thermal Stability, Liquid Crystal, Oxidation Onset, Protein Denaturation, Cure Onset and Solid-Solid Transition.

The system uses aluminum pans for holding the sample/standard (left hot plate) and the reference (right hot plate) and these aluminum pans can either be volatile or non volatile. The equipment gives out the output in terms of the thermogram curves and the following information can be obtained from these thermograms. It essentially provides

the melting point (endothermic peak upward), crystallization (exothermic peak downward) and glass transition temperature.

The operation sequence of the equipment involves running the system base line to zero instrument, running the standard to calibrate temperature range and finally running the sample. In one of the pans, the sample is placed. The other pan is the reference pan, which is kept empty. Both the pans sit on the top of a heater. Now, the system is switched on where the computer turns on the heaters and it heats the two pans at a specific rate, usually $10^{o}C$ per minute. The computer makes absolutely sure that the heating the rate stays exactly the same throughout the experiment. But more importantly, it makes sure that the two separate pans, with their two separate heaters, heat at the same rate as each other.

As the two pans are heated, the computer will plot the difference in heat output of the two heaters against temperature. That is, the heat absorbed by the polymer against temperature is plotted. As the heating of the polymer continues, there is more heat flow. There is an increase in the heat capacity of the polymer sample. This happens because the polymer has just gone through the glass transition. Above the glass transition, the polymers have a lot of mobility. When they reach the right temperature, they will have gained enough energy to move into very ordered arrangements, in other terms called crystals. When polymers fall into these crystalline arrangements, they give off heat. There is a drop in the heat flow versus temperature curve.

The temperature at the lowest point of the dip is usually considered to be the polymer's crystallization temperature, or *T*c. If the heating continues over its *T*c, eventually it'll reach another thermal transition, called melting. When the polymer's

melting temperature, or *T*m, is reached, the polymer crystals begin to fall apart, that is they melt. The extra heat flow during melting shows up as a big peak on the DSC plot.

The thermal analysis was carried out on electrospun web using differential scanning calorimetry. The weight of the samples was 3.8 – 4.8 mg, which was consistent with the weight of the standard sample, which is 3.5 ± 0.5 mg. For calibration, run the baseline to zero. The heating starts at room temperature and increment of 25°C can be given. Temperature should not exceed 600°C, since aluminum pans are being used.

4.5 Scaffold Fabrication for Cell Study

The electrospun fibers of PLLA-LF and PLLA-SF were made in to non-woven mats approximately 1 millimeter thick. The 1mm thick mats were cut in to circles of 6mm diameter.

The scaffolds (6 mm diameter circular mats) were placed in 96 well cell culture plates (Figure 4.3). Each well had a diameter of 6.4mm. The cell culture plates with the scaffolds were sterilized with UV light for 12 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**PLLA-LF**		**PLLA-SF**									
B	**PLLA-LF**		**PLLA -SF**									
C	**PLLA-LF**		**PLLA -SF**									
D	**PLLA-LF**		**PLLA –SF**									
E	**PLLA-LF**		**PLLA –SF**									
F	**PLLA-LF**		**PLLA –SF**									
G	**PLLA-LF**		**PLLA -SF**									
H	**PLLA-LF**		**PLLA -SF**									

Figure 4.3 Schematic diagram of the scaffold setting in the culture plates.

4.6 Cell Seeding on Scaffolds

Human Mesenchymal stem cells (hMSC) were used for this study. hMSCs were isolated from whole, subcultured and cryopreserved according to previously reported methods [2].

Mesenchymal stem cell growth medium (MSCGM) was prepared using Dulbecco's modified eagle medium (DMEM) in 10% Fetal bovine serum (FBS), and 1% of antibiotic-antimycotic (Gibco-BRL).

The cryovial was removed from storage. Holding the cryovial, the bottom ¾ of the cryovial was dipped in a 37°C water bath for 1-2 minutes until contents were thawed. Thawing the cells for longer then 3 minutes results in less than optimal results. The cryovial was removed from the water bath immediately, wiped dry, and transferred to a sterile field. The cryovial was rinsed with 70% alcohol, and then wiped to remove excess. Using a micropipet, gently the thawed cell suspension was added to temperature-equilibrated medium in the ratio 1:9. The cells were transferred from the vial to the centrifuge tube. The tube was centrifuged at 1200-1400 rpm for 5-7 minutes at room temperature. The pellet was resuspended in 3ml of temperature equilibrated MSCGM by gently pipetting up and down. The total number of viable cells was counted with a hemacytometer or cell counter and the total number of cells was calculated. The cell viability was assessed using Trypan Blue (Appendix B).

For cell seeding on to the culture plates, a total of 500,000 cells were needed. The scaffolds (PLLA-LF and PLLA-SF) and the controls (tissue culture plastic) were determined to be a total of 24 wells. Eight samples/group per time point were used. One time point (20^{th} day) was studied. Each well had to be seeded with 10000 cells each. To get 500,000 cells, 0.495ml of suspension was pipetted out and resuspended in a total of

10ml (9.505ml MSCGM + 0.495ml suspension). To each well of the cell culture plates, 100 μl (5000 cells) of the suspension was added.

The remaining suspension was transferred to a T175 (175 square area) flask for use as standards for cell proliferation assay. The suspension was equally divided and pipetted in to the 3 flasks. To each flask, 35ml of MSCGM was added. The flasks were gently rocked to disperse the cell suspension over the growth surface. Then, they were incubated at 37°C, 5% CO2 and 90% humidity.

Human Mesenchymal stem cells cultures should be fed 3-4 days after plating. To feed the cultures, gently and completely the MSCGM was removed from the culture vessel. It was replaced with an equal volume of temperature equilibrated MSCGM and the culture vessels were kept in the incubator.

Human Mesenchymal Stem Cells (hMSC) appeared round when first plated. Within four hours, greater than 90% of the cells were attached and began to flatten and elongate. Within 24-36 hours, the cells were adherent, elongated and spindle shaped with the culture at 20-30% confluence. This observation was made for cells on tissue culture plastic. Scaffolds were opaque; therefore cells could not be viewed using a conventional inverted microscope.

4.7 Cell Proliferation

The cell proliferation was studied using Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes). Vybrant® MTT Cell Proliferation Assay Kit provides a simple method for determination of cell number using standard microplate absorbance readers. Determination of cell growth rates is widely used in the testing of drug action, cytotoxic

agents and screening other biologically active compounds. Several methods can be used for such determinations, but indirect approaches using fluorescent or chromogenic indicators provide the most rapid and large-scale assays. Among such procedures, the MTT assay is still among one of the most versatile and popular assays.

The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan. The formazan is then solubilized, and the concentration determined by optical density at 570 nm. The result is a sensitive assay with excellent linearity up to approximately 10^6 cells per well.

In general, cells seeded at densities between 5000-10,000 cells per well should reach optimal population densities within 48-72 hours. The reagents required for the assay were prepared as given below:

Reagent 1: A 12 mM MTT stock solution was prepared by adding 1 mL of sterile PBS to one 5 mg vial of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). It was mixed by vortexing or sonication until dissolved.

Reagent 2: To one tube containing 1 gm of sodium dodecyl sulfate (SDS), 10 mL of 0.01 M HCl was added. The solution was mixed gently by inversion or sonication until the SDS dissolved.

For the cells, the medium was removed and replaced it with 100 µL of fresh culture medium. 10 µL of the 12 mM MTT stock solution (Reagent 1) was added to each well. A negative control of 10 µL of the MTT stock solution added to 100 µL of medium alone was included. These were incubated at 37°C for 4 hours. After the 4-hour incubation, 100 µL of the SDS-HCl solution (Reagent 2) was added to each well and

mixed thoroughly using the pipette. The microplate was incubated at 37°C for 4-18 hours in a humidified chamber. Longer incubations will decrease the sensitivity of the assay. The samples were mixed using a pipette; absorbance at 570 nm was determined.

Standards were prepared using hMSCs at 5000 up to 50000 cells per well (Figure 4.4). To each well of the standards and controls, 100 µl (suspension + phenol free media) and 100 µl of phenol free media was added respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C			**Ctrl**	**Ctrl**	**Ctrl**	**Ctrl**	**Ctrl**	**Ctrl**	**Ctrl**			
D												
E												
F			**50000 cells**	**30000 cells**	**20000 cells**	**10000 cells**	**5000 cells**	**Blank**				
G			**50000 cells**	**30000 cells**	**20000 cells**	**10000 cells**	**5000 cells**	**Blank**				
H												

Figure 4.4 Schematic diagram of standards and controls in culture plates.

CHAPTER 5

RESULTS

5.1 Morphology of Electrospun Scaffold

The electrospun samples were characterized with the scanning electron microscope for fiber diameter. The PLLA-LF fibers were electrospun at a voltage of 25kv, a collector to needle distance of 20 centimeters and a solution concentration of 10% w/w. The needle used was a 16 gauge and the flow rate was 0.103 ml/min. The PLLA-LF fibers were in the size range of 8 microns to 18.4 microns (Figure 5.1,5.2).

Figure 5.1 SEM image of PLLA-LF (18.4 µm).

Figure 5.2 SEM image of PLLA-LF (8 µm).

The PLLA-SF nanofibers were electrospun at a voltage of 25kv, a flow rate of 0.103 ml/min and a solution concentration of 5%w/w. The distance between the collector and the needle was 20 cm and the needle used was a 20 gauge. The PLLA-SF fibers were 132 nanometers to 3.5 microns in size (Figure 5.3,5.4). The fibers of both samples PLLA-LF and PLLA-SF were cylindrical.

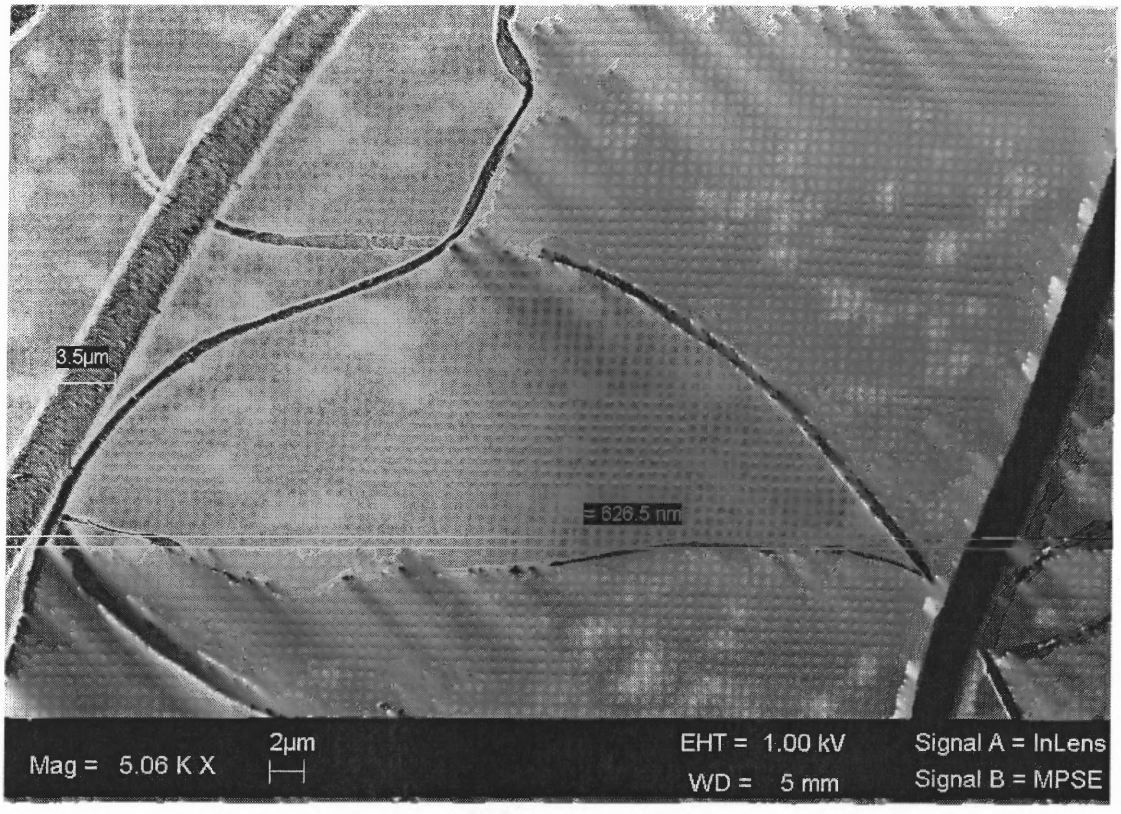

Figure 5.3 SEM image of PLLA-SF (3.5 µm).

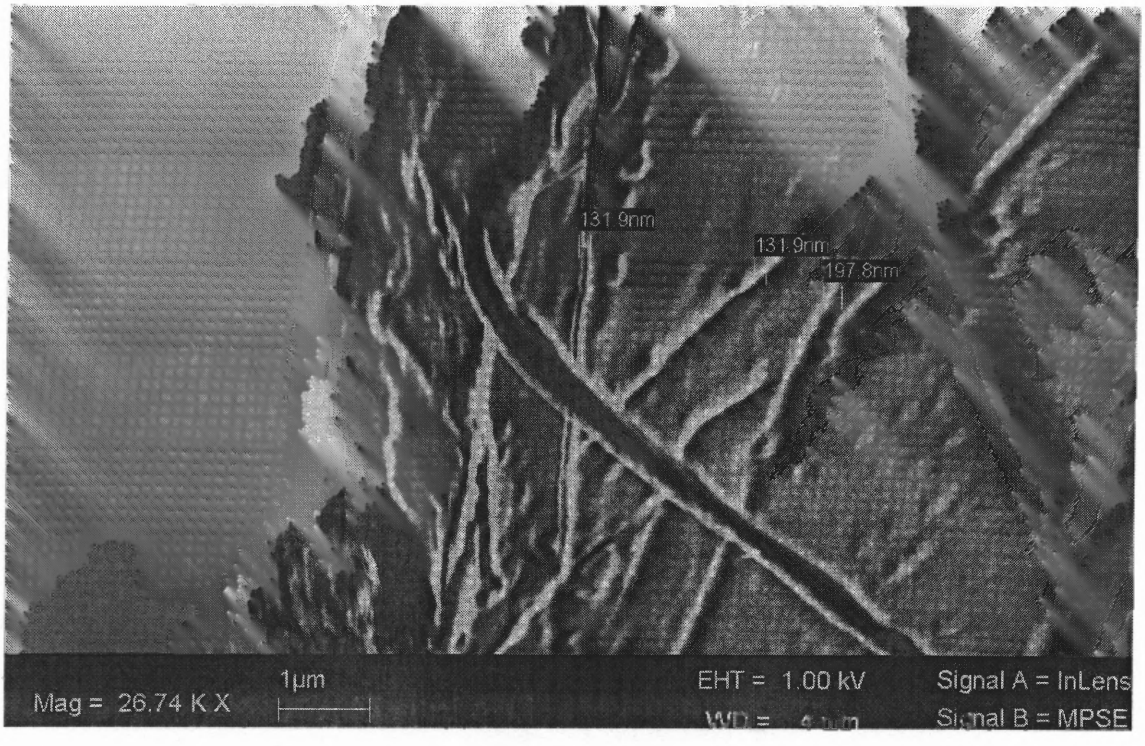

Figure 5.4 SEM image of PLLA-SF (132 nm).

5.2 Porosimetry of Electrospun Scaffold

The mercury porosimetry results of PLLA-SF:

Total intrusion volume =	2.2072 cc/g
Total % Porosity =	39.2653
Total Surface area =	14.5964 m2/g
Median Pore Diameter (Based on Volume) =	31.5093 Microns
Median Pore Diameter (Based on Surface Area) =	0.0095 Microns
Standard Deviation (Based on Volume) =	0.0789 Microns
Standard Deviation (Based on Surface Area) =	0.0035 Microns
Average Pore Diameter (4V/S) =	0.6048 Microns
Bulk Density=	0.1779 g/cc
Absolute Density=	0.2922 g/cc

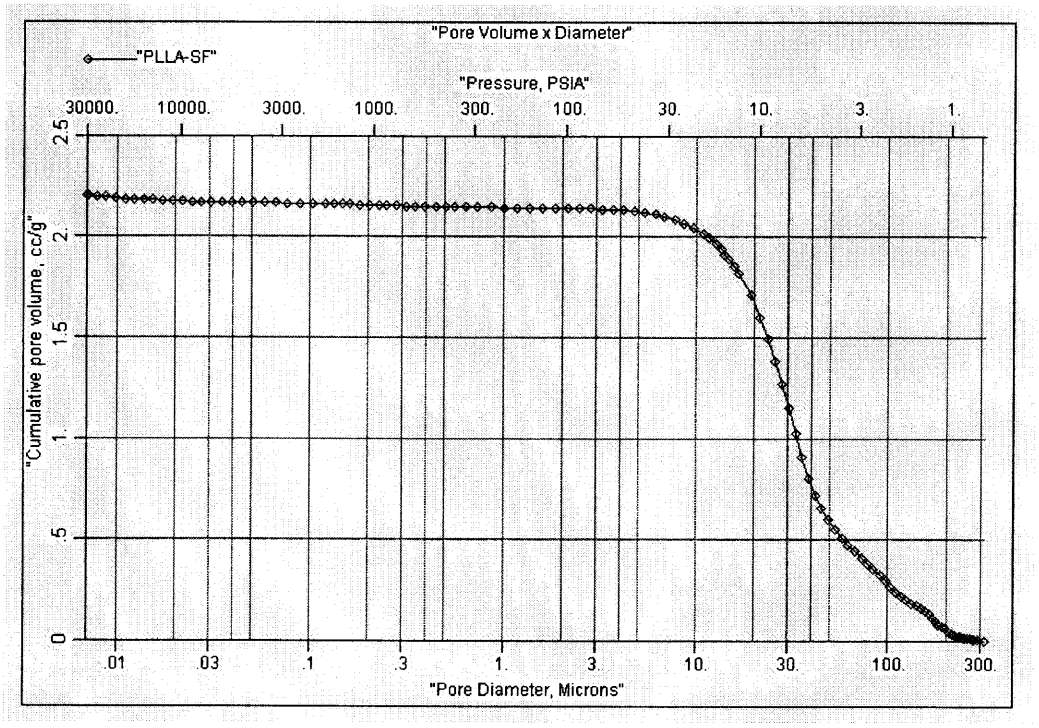

Figure 5.5 Pore volume x Pore Diameter of PLLA-SF.

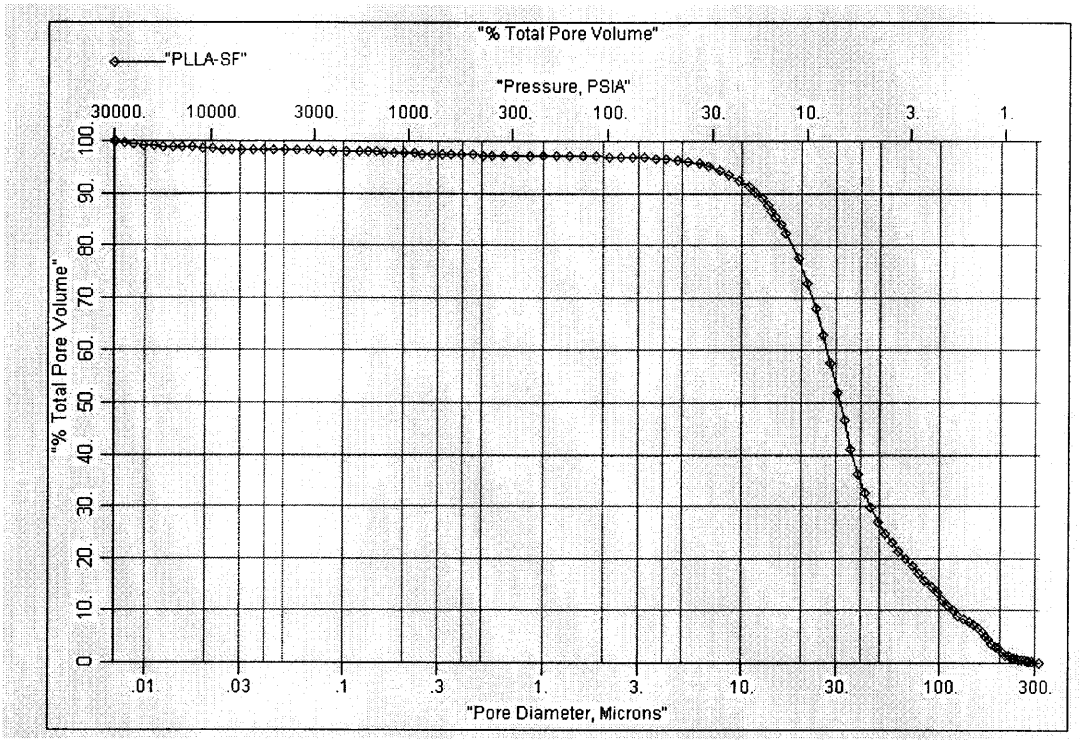

Figure 5.6 Total Pore Volume of PLLA-SF.

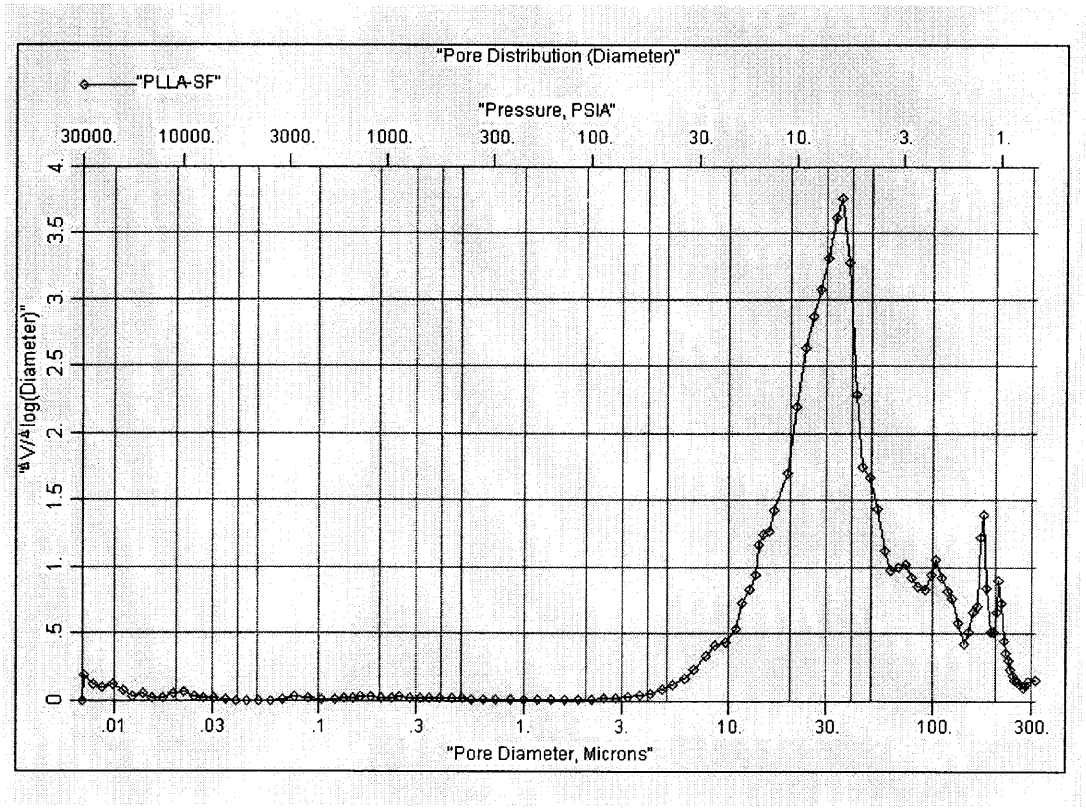

Figure 5.7 Pore Distribution of PLLA-SF.

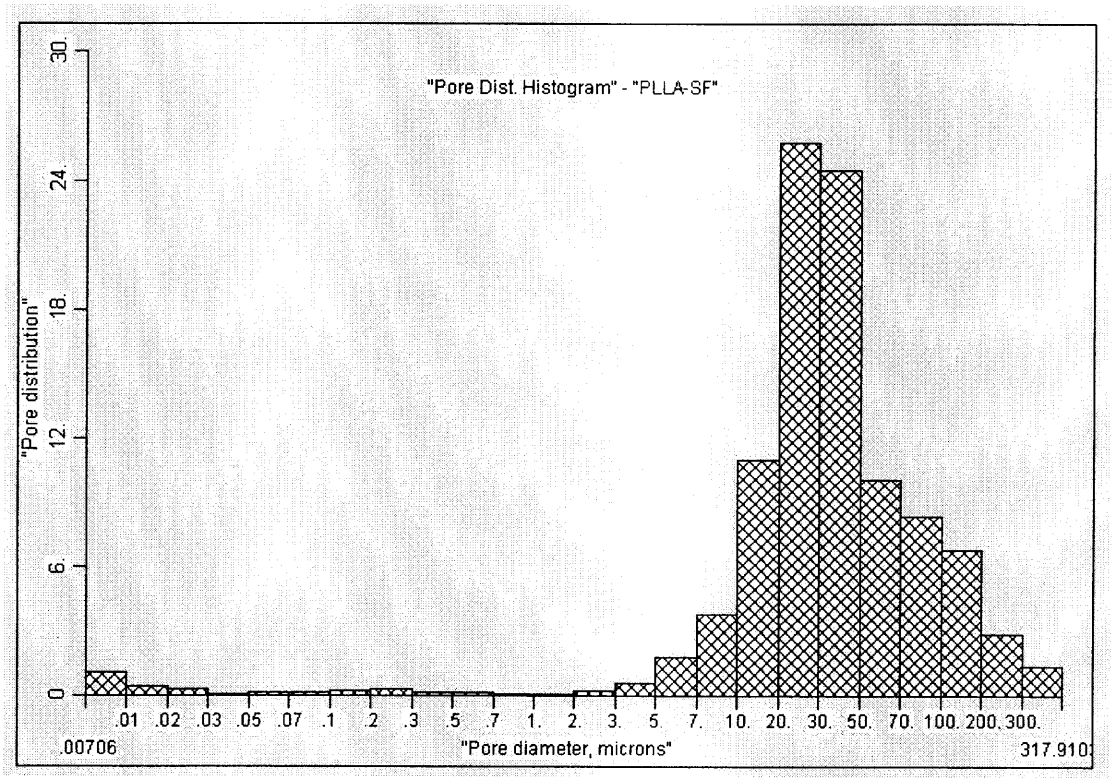

Figure 5.8 Histogram of Pore Distribution of PLLA-SF.

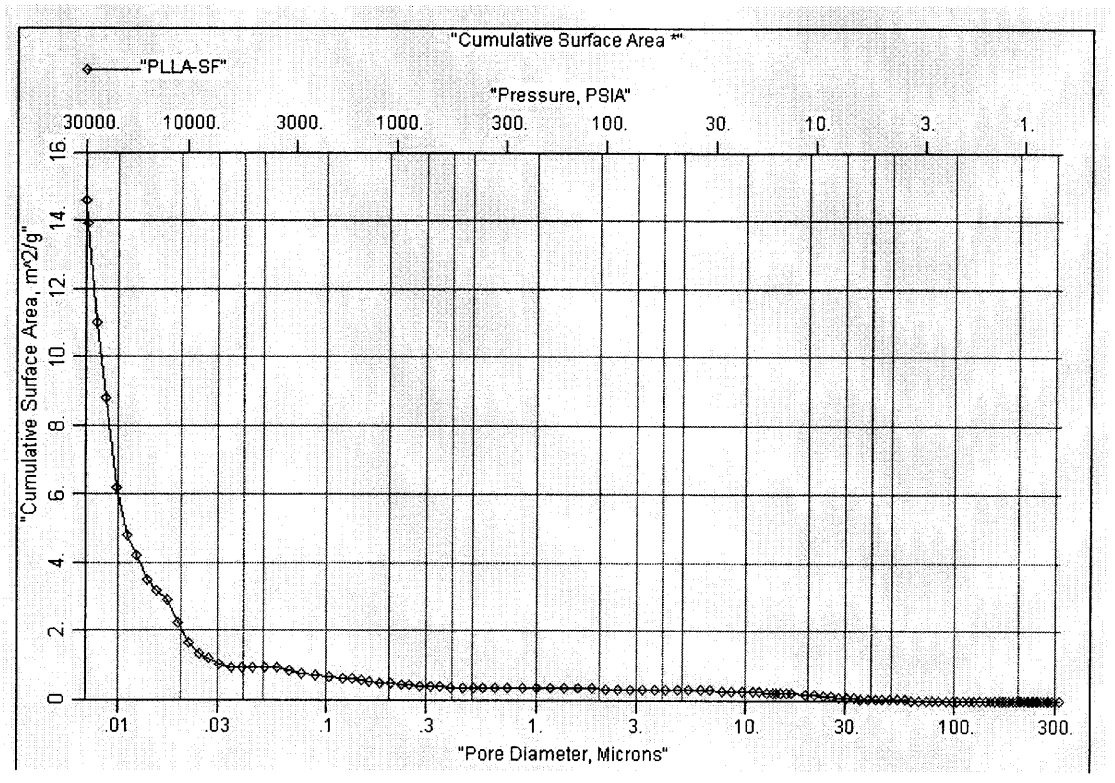

Figure 5.9 Cumulative Surface Area of PLLA-SF.

The mercury porosimetry results of PLLA-LF:

Total intrusion volume =	5.0281 cc/g
Total % Porosity =	47.5659
Total Surface area =	27.3076 m2/g
Median Pore Diameter (Based on Volume) =	108.9927 Microns
Median Pore Diameter (Based on Surface Area) =	0.0095 Microns
Standard Deviation (Based on Volume) =	0.0867 Microns
Standard Deviation (Based on Surface Area) =	0.0036 Microns
Average Pore Diameter (4V/S) =	0.7365 Microns
Bulk Density=	0.0946 g/cc
Absolute Density=	0.1807 g/cc

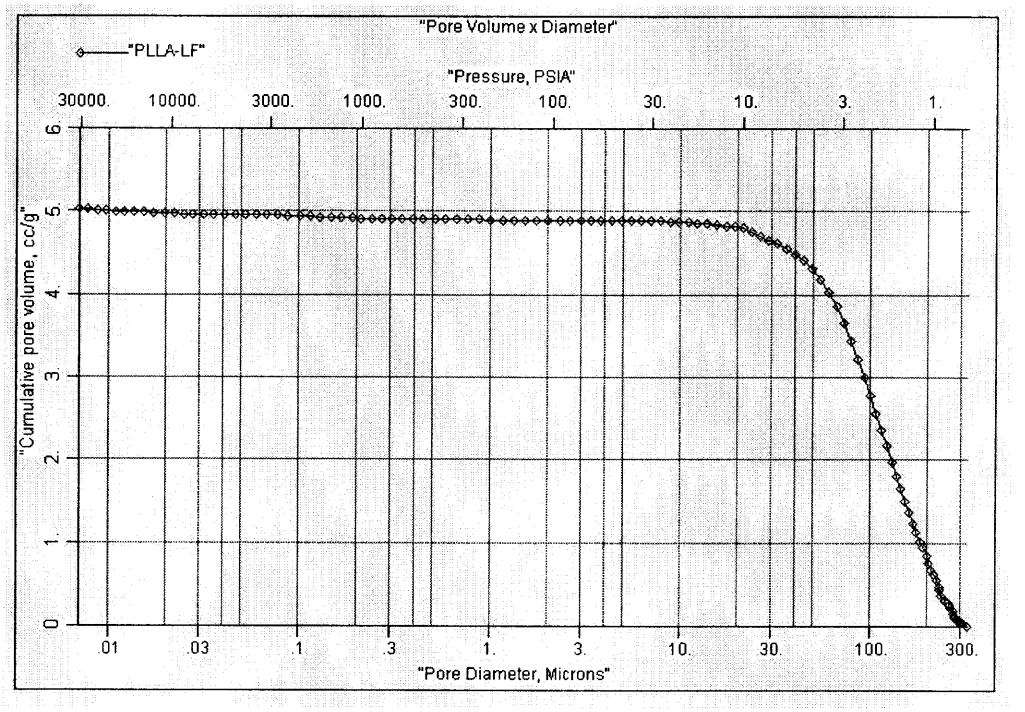

Figure 5.10 Pore volume x Pore Diameter of PLLA-LF.

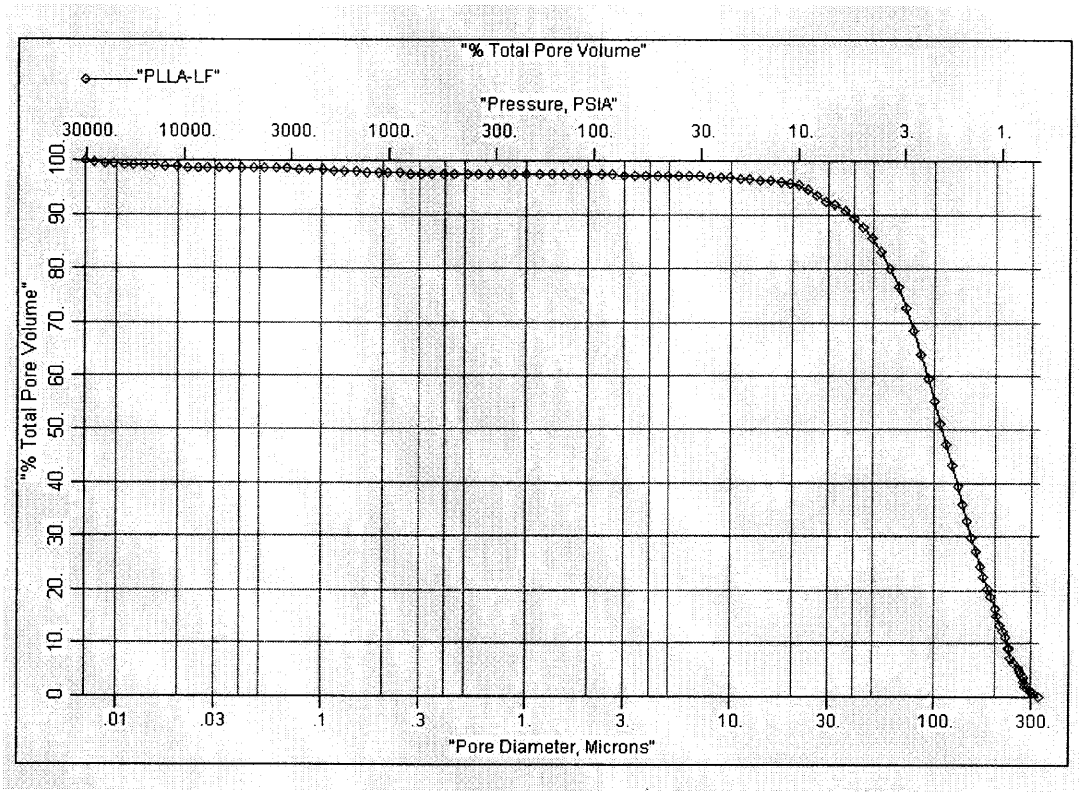

Figure 5.11 Total Pore Volume of PLLA-LF.

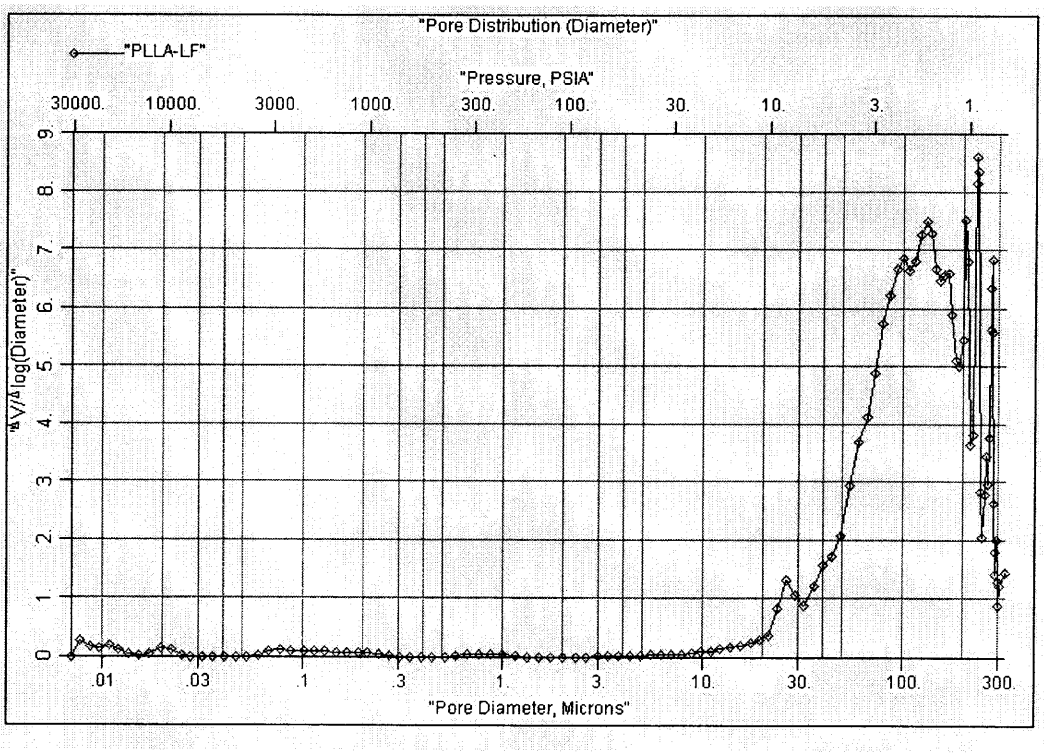

Figure 5.12 Pore Distribution of PLLA-LF.

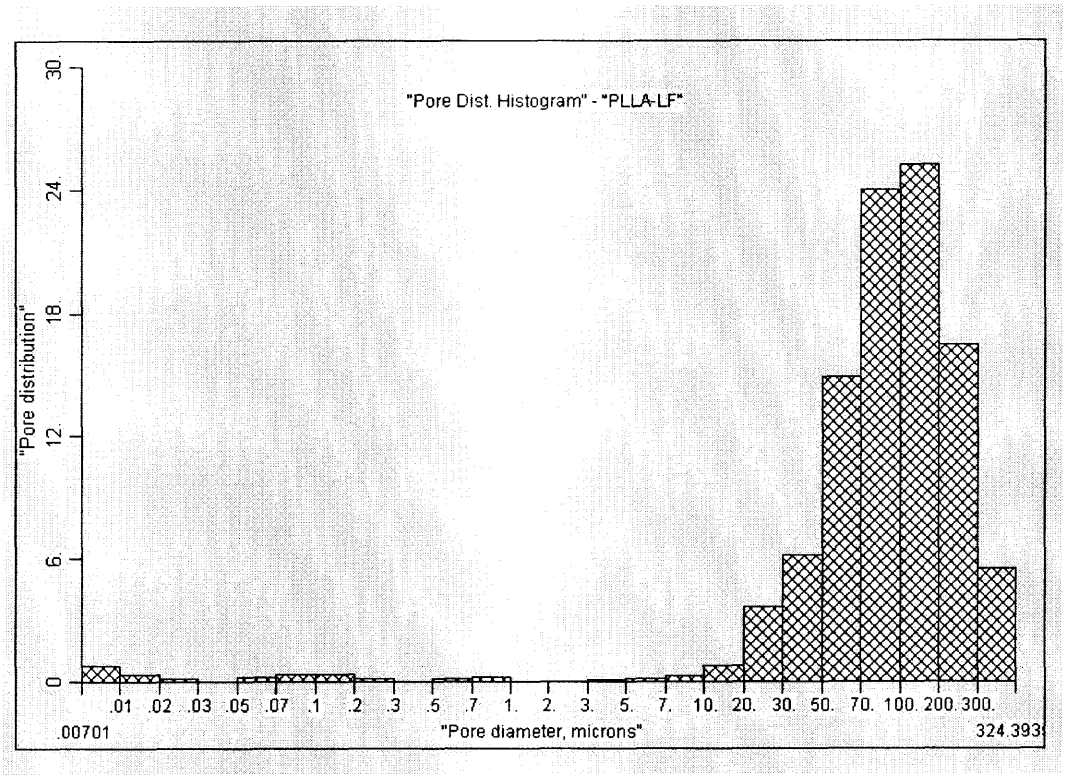

Figure 5.13 Histogram of Pore Distribution of PLLA-LF.

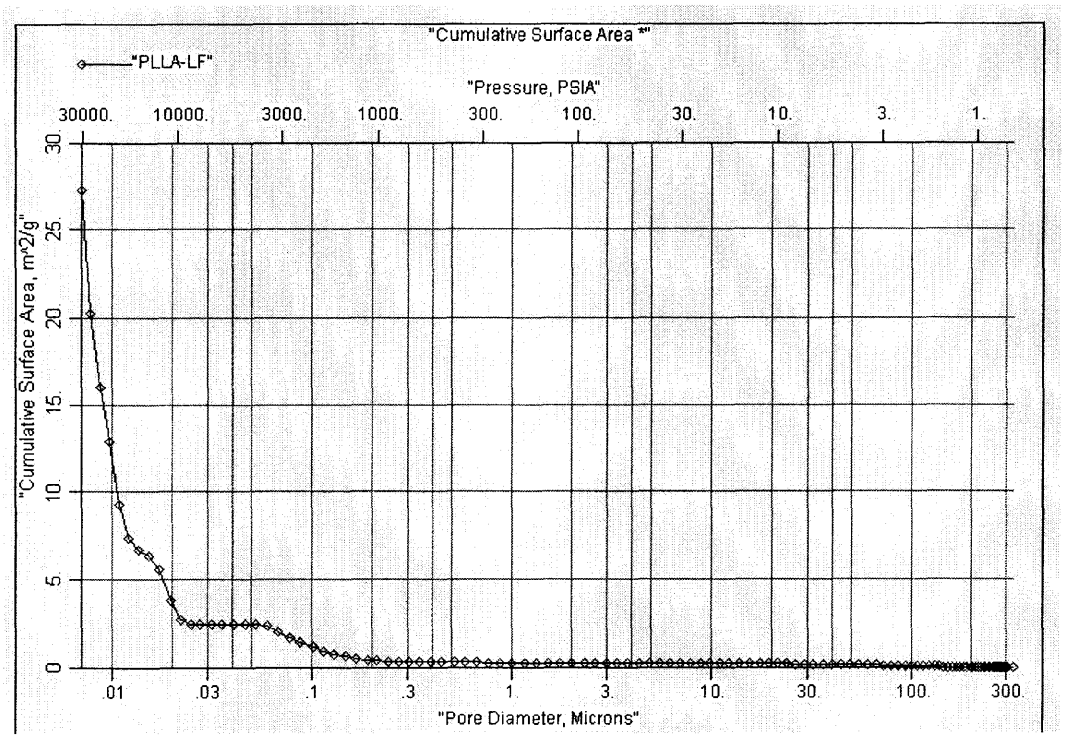

Figure 5.14 Cumulative Surface Area of PLLA-LF.

5.3 Thermal Analysis by DSC

The thermal analysis was carried out on electrospun web using differential scanning calorimetry. The polymer Resomer L 207 and the electrospun fibers of PLLA-LF and PLLA-SF were analysed to see if the melting point (Tm) and the glass transition temperature (Tg) of the electrospun fibers are similar to the PLLA polymer (Resomer L 207) even after dissolving it in methylene chloride.

The weight of the polymer was 3.8 mg. The cycle used was heat/cool/heat. The rate of increment for the heating cycles was 10°C/min. The first heat cycle was from -20°C to 250°C. The cooling cycle was from 250°C to -90°C and the rate of cooling was 5°C/min. The final heating cycle was -90°C to 250°C (Figures 5.15 and 5.16).

The weight of PLLA-LF was 4.8 mg. The rate of increment for the heating cycles was 10°C/min. The first heat cycle was from 0°C to 250°C. The final heating cycle was 0°C to 200°C (Figure 5.17 and 5.18).

The weight of PLLA-SF was 4 mg. The heating cycles were from 0°C to 250°C (Figures 5.19 and 5.20).

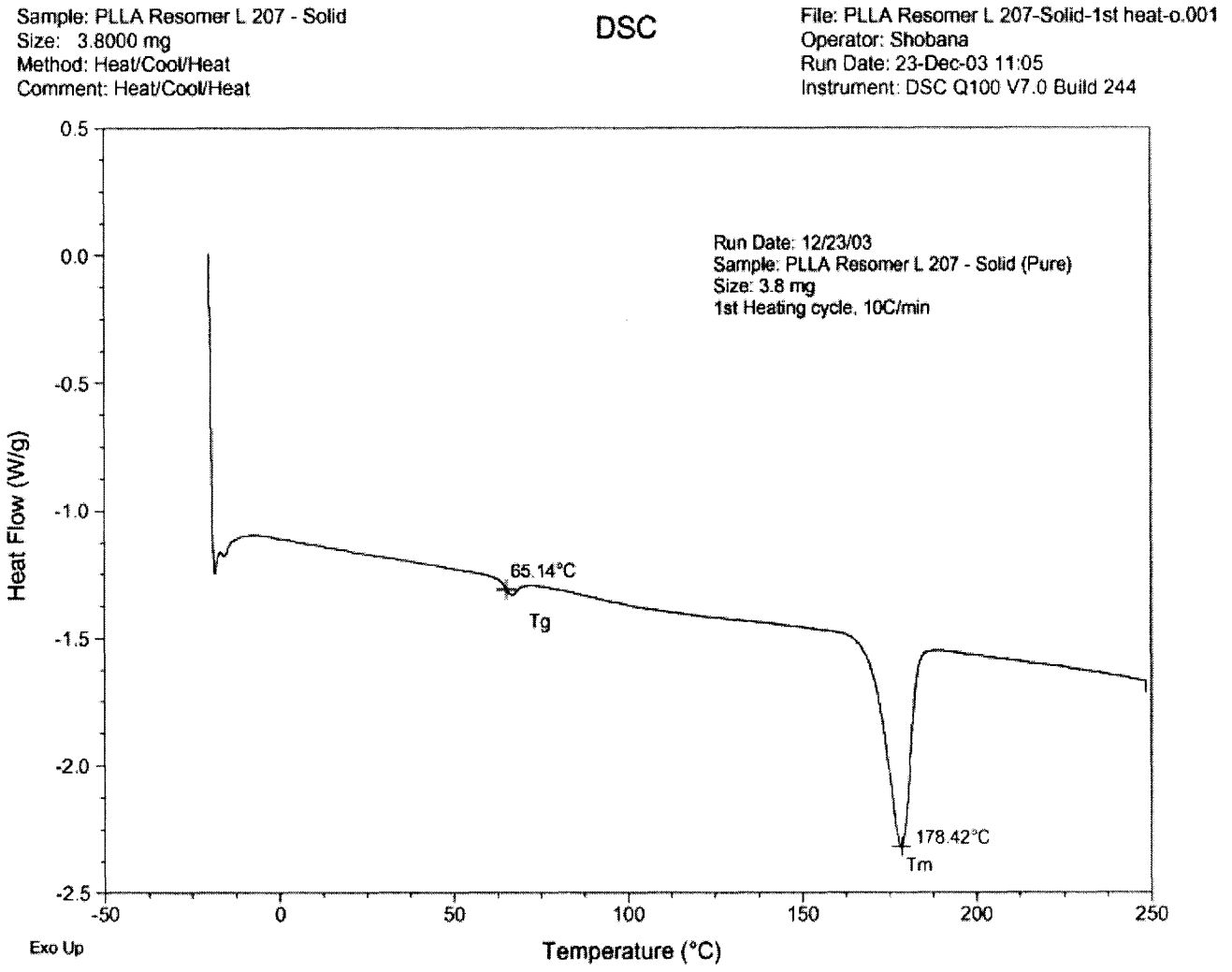

Figure 5.15 Tg and Tm of PLLA polymer, 1st Heat cycle.

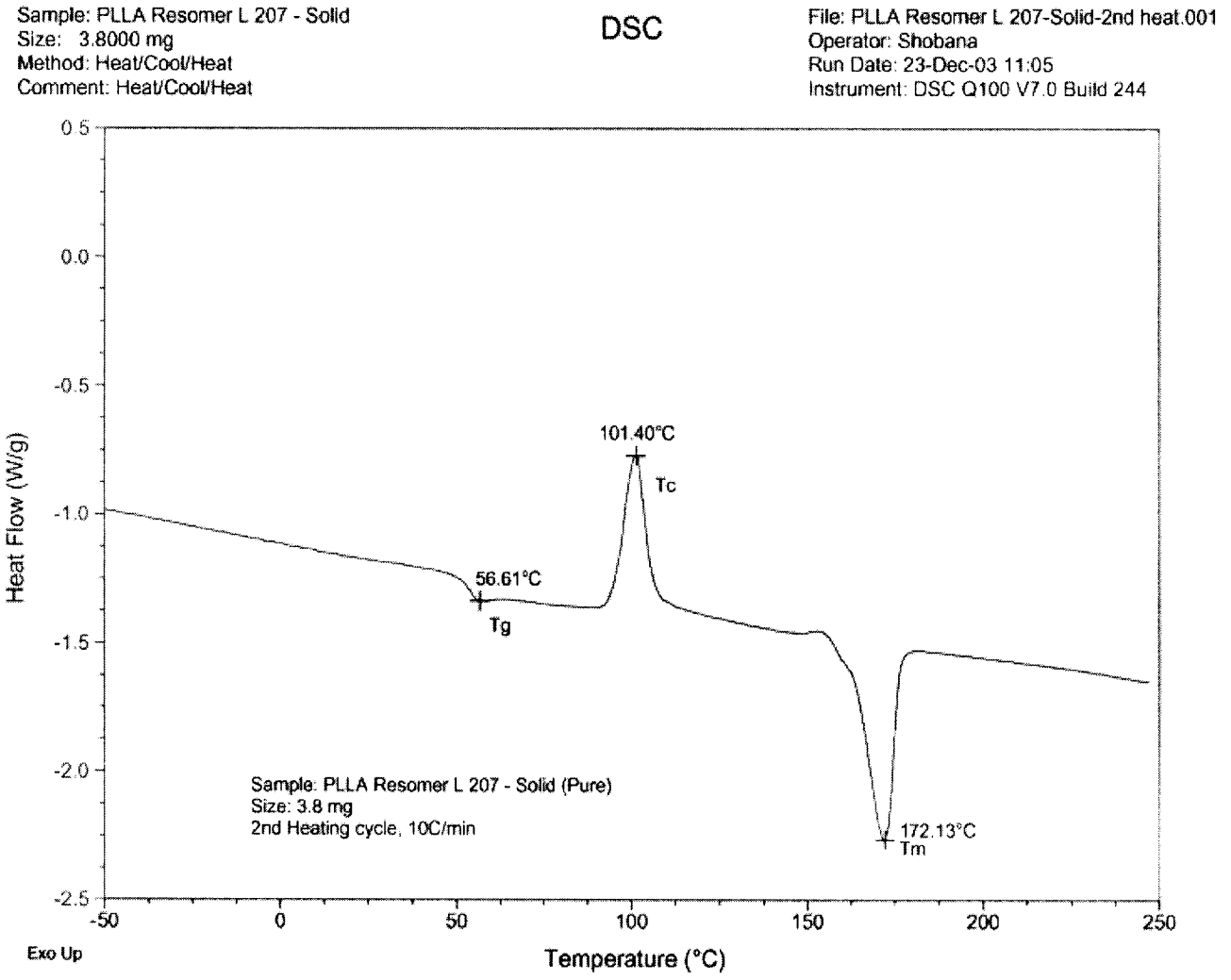

Figure 5.16 Tg and Tm of PLLA polymer, 2nd Heat cycle.

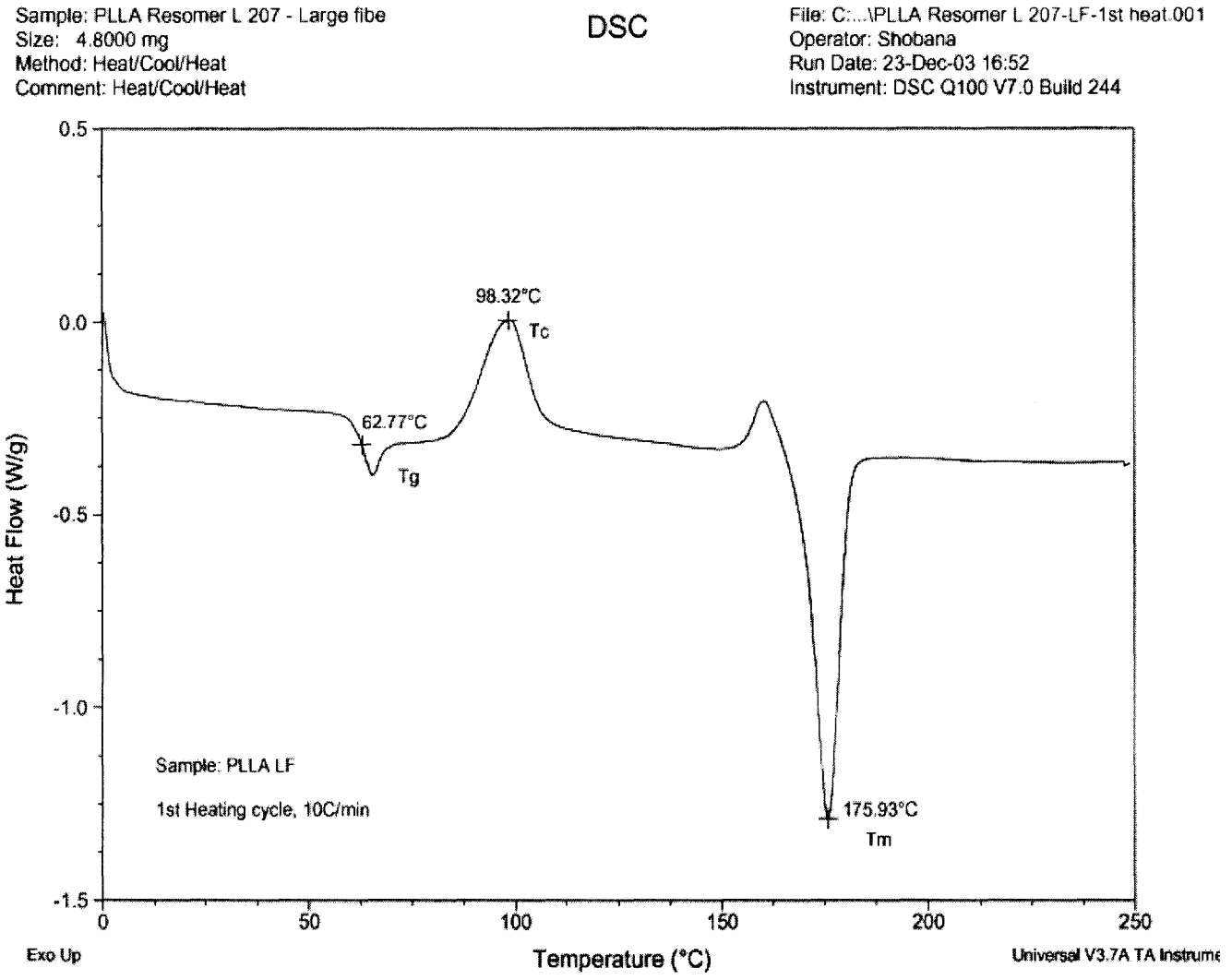

Figure 5.17 Tg and Tm of PLLA LF, 1st Heat cycle.

Above the glass transition, the polymers have a lot of mobility. When they reach the right temperature, they gain enough energy to move into very ordered arrangements i.e., crystals. When polymers fall into these crystalline arrangements, they give off heat. This is the big peak (Tc) in the plot of heat flow versus temperature. The PLLA-LF and PLLA-SF had Tc of 103°C and 92°C that was withing the range of the PLLA polymer (Tc=100°C).

When the melting temperature is reached, the polymer's temperature remains constant until all the crystals have melted. This also means that the furnace will put additional heat into the polymer in order to melt both the crystals and keep the temperature rising at the same rate as that of the reference pan.

This extra heat flow during melting shows up as a large dip in the DSC plot as heat is absorbed by the polymer. The Tm is determined by measuring the area of this dip. The temperature at the apex of the dip is taken to be the point where the polymer is completely melted.

The Tm of PLLA-SF and PLLA-LF were 172°C, which was within the range of the PLLA polymer's melting point of 175°C.The melting point and the glass transition temperatures of the polymer were similar to the electrospun samples of PLLA-SF and PLLA-LF.

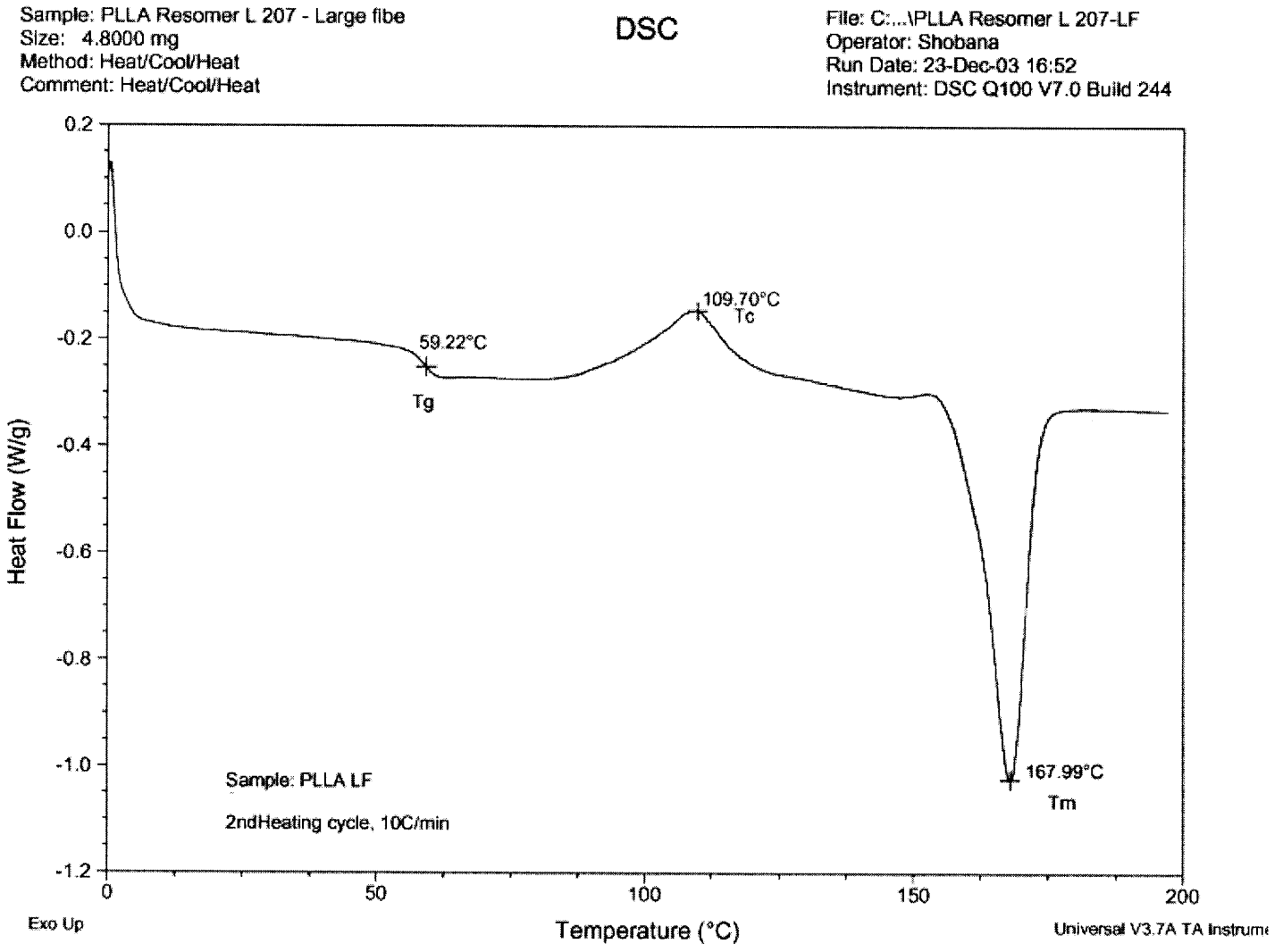

Figure 5.18 Tg and Tm of PLLA LF, 2nd Heat cycle.

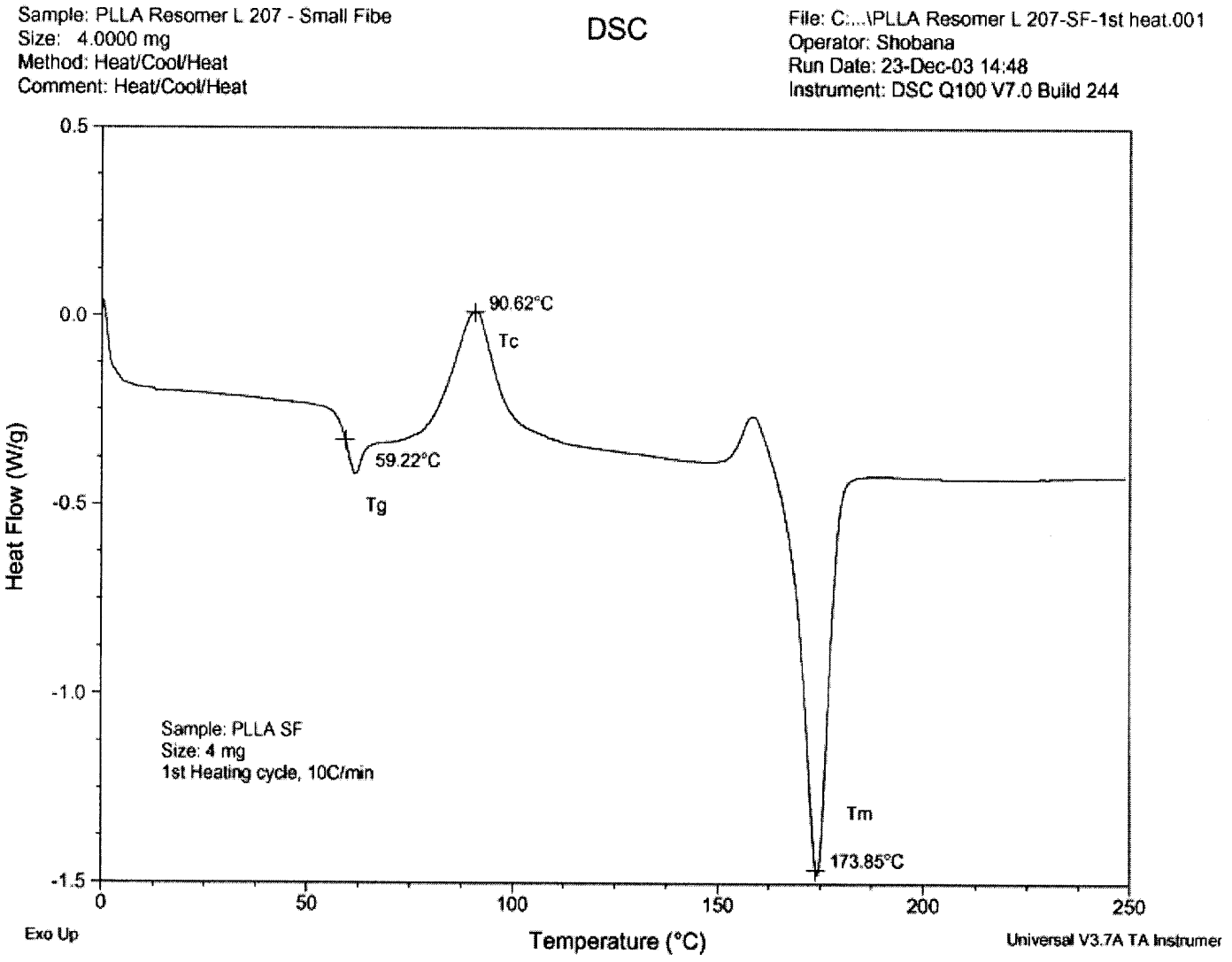

Figure 5.19 Tg and Tm of PLLA SF, 1st Heat cycle.

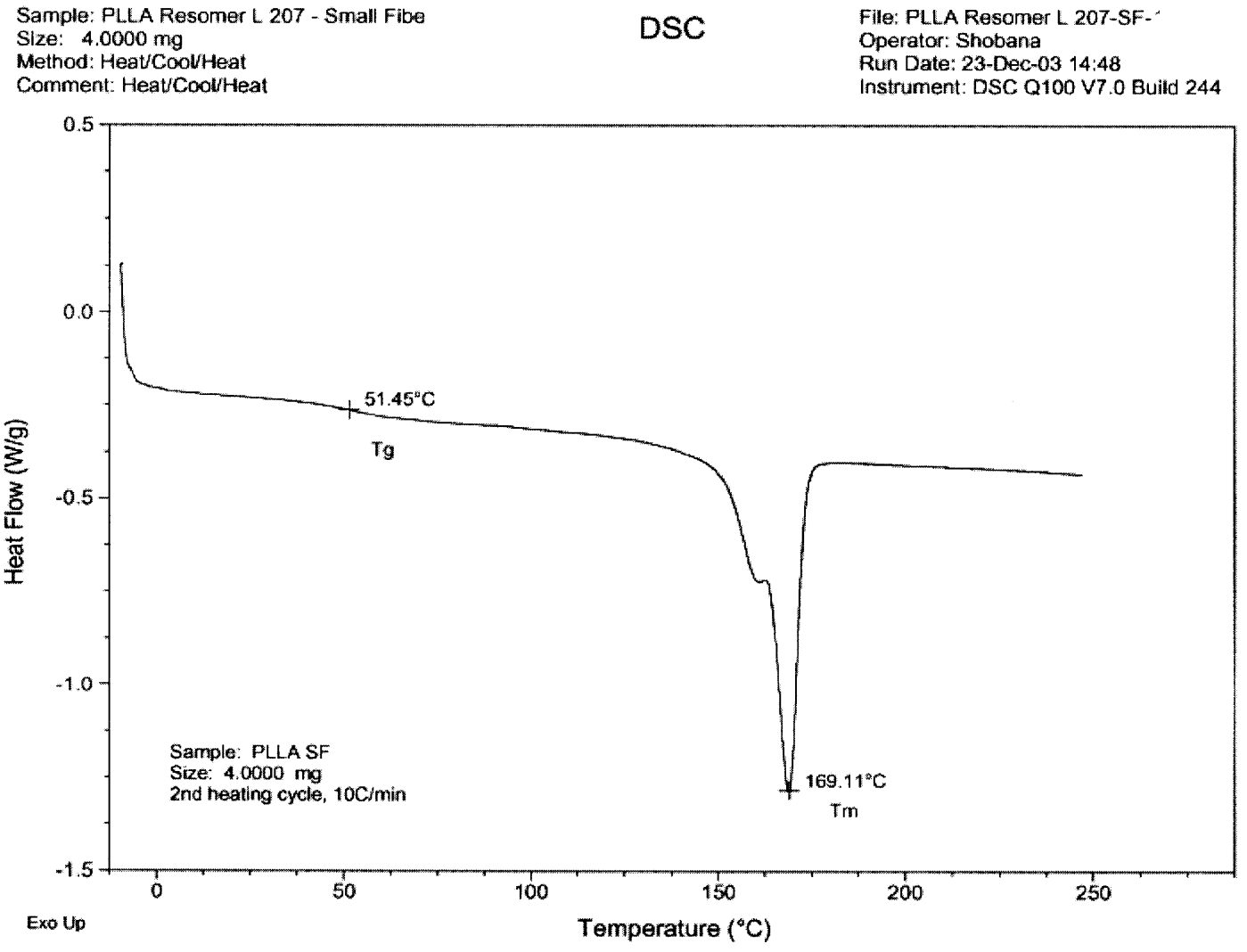

Figure 5.20 Tg and Tm of PLLA SF, 2nd Heat cycle.

The results of DSC showed that the electrospinning process did not affect the polymer. The change in the glass transition temperature takes place over a temperature range. So, the middle of the incline in the curve is taken as the Tg. The PLLA polymer had a Tg of 61°C. The DSC analysis showed that the Tg of PLLA-LF and PLLA –SF were 61°C and 55oC respectively.

5.3 Cell Proliferation

The stem cells were allowed to grow in culture on the two scaffolds and tissue culture plastic (as a positive control) for 20 days. The initial cell seeding (day 0) was 10,000. The following data represents the cell number determined by the MTT assay at day 20.

Statistical analysis was performed on the data using an unpaired T test between cells grown on PLLA-LF and PLLA-SF. The null hypothesis was that the cell number on PLLA-LF is equal to that of PLLA-SF i.e., the mean of the number of cells on both the samples were equal. The P value was 0.15.

The T test showed that the means of the two groups were not equal. The cells proliferated more on the scaffolds of PLLA-SF (Mean $\pm$ Std = 24005 $\pm$ 7227) than in the PLLA-LF (Mean $\pm$ Std = 16529 $\pm$ 6529) scaffolds. The Mean $\pm$ Std of the cell culture plastic (Control) was 53840 $\pm$ 17464.

CHAPTER 6

CONCLUSION

The process of electrospinning was successfully carried out using PLLA as the polymer and methylene chloride as the solvent. By varying the concentration and the needle diameter, the process of electrospinning from yielded very fine fibers with diameters in the nanometer range as well as fibers in the micron range. The nanofibers and the microfibers were fabricated in to scaffolds for cell proliferation study using human mesenchymal stem cells.

The electrospun web of nanofibers and microfibers were composed of smooth, cylindrical fibers of varying diameters. The electrospun fibers had a porosity that was favorable for stem cell proliferation. The results of the thermal analysis showed that the electrospinning process does not alter the characteristic property of the polymer even after dissolving in methylene chloride and even when processed at high voltage.

Human Mesenchymal stem cells proliferated well on both nanofibers and microfibers, but the results of the proliferation assay show that the cells tend to proliferate more on the nanofibers when compared to the microfibers.

Human cells can attach and organize well around fibers with diameters smaller than those of the cells [20]. In this regard, nanoscale fibrous scaffolds may provide an optimal template for cells to proliferate. For the regeneration of biological tissues and organs, fibrous structures with fiber architectures that are beneficial for cell deposition and cell proliferation must be developed. Of particular interest in tissue engineering is the creation of reproducible and biocompatible three-dimensional scaffolds for cell in growth resulting in bio-matrix composites for various tissue repair and replacement procedures.

Recently, people have started to pay attention to making such scaffolds with synthetic biopolymers and/or biodegradable polymer nanofibers [16]. It is believed that converting polymers into fibers and networks that mimic native structures will ultimately enhance the utility of these materials, as large diameter fibers do not mimic the morphological characteristics of the native fibrils. In this study, the stem cells proliferated more on the nanofibers than on the microfibers, which may be due to the fact that these fibers are closer in resemblance to the architecture of the natural extra cellular matrix than the microfibers.

6.1 Recommendations

A critical requirement for the cell studies is that the scaffold should stay at the bottom of the cell culture plate, so that the cells attach to them and proliferate. But, during the course of this study, in a few wells of the culture plates, the scaffolds started to float when media was added to it. Attaching the scaffolds to the cell culture plates with glue that would not be toxic to the stem cells may help the cell study. In this study, only one time point was considered. Studying the cell proliferation over a period of time may be helpful in knowing the cell proliferation on different fiber sizes better.

APPENDIX A

SAFETY PROCEDURES – ELECTROSPINNING

The safety procedures of electrospinning have been summarized in this appendix.

Chemical Safety

- Always wear eye protection, hand protection and protective clothing.
- Handle the chemicals listed above with care and store them at proper/regular places.
- Make sure the solution is prepared inside the hood and the fumes emerging from the chemicals should be properly ventilated.
- Clean beakers and bottles should be used for transferring of chemicals and preparing of solution.
- Close the bottle of methylene chloride immediately after use and also close the glass lid of the bottle containing the prepared solution.
- The polymer chips added to the solution to dissolve can be assisted using a magnetic stirrer and an external source for stirring.
- Dispose of all contaminated materials properly.
- Keep spilled materials out of drains and water supplies.
- Get medical help immediately if someone is overexposed to chemicals.

Electrical Safety

The electrospinning system itself uses electrical power for its operation. Any of these smaller pieces of equipment may produce a potentially damaging or lethal shock or serve to ignite flammable materials. Although such shocks and fires may result from defective equipment, most often they may result from the unsafe practices of the user.

- Proper care should be taken while connecting the wire to the needle.
- The other wire from the high voltage instrument is connected to the collector. Make sure that the end is properly fixed.
- Make sure that the line side and high voltage sides are grounded.
- Make sure that there are no metal parts near the set up or in the hood.

- Check that the wires coming from the power supply and the collecting device do not cross over.

- Close the sash of the hood to at least 18 inches.

- If the above lines are OK, start the experiment with switching on the power switch and slowly increasing the voltage through regulator.

- The high voltage instrument has variable output from, 0 to 30 kilovolts; the range being used for the electrospinning process is 15 to 25 kilovolts.

- To turn down the system, slowly decrease the voltage, turn the power switch off and then take out the power supply plug.

- Turn the system down when the polymer level is near the ending stage and polymer is near the vicinity of the submerged wire or when the polymer is still around 1 ml in the syringe.

- Open the sash, take out the positive end from the needle, transfer remaining solution to a container, and stop the external drive.

- The mesh containing electrospun fibers can now be removed from the plate for analysis.

- Use the power supply controls to cut off the power in an emergency.

APPENDIX B

ASSESSMENT OF CELL VIABILITY (TRYPAN BLUE)

Trypan Blue is a dye that enables easy identification of dead cells. Dead cells take up the dye and appear blue with uneven cell membranes. By contrast, living cells repel the dye and appear refractile and colorless.

Using Trypan Blue

1. Prepare the hemacytometer for use.
    a. Carefully clean all surfaces of the hemacytometer and cover slip.
    b. Take care to ensure that all surfaces are completely dry using non-linting tissue.
    c. Center the cover slip on the hemacytometer.
2. Transfer 50 µl of 0.4% Trypan Blue into a clean tube.
3. Add 50 µl of the prepared cell suspension into the tube containing the stain.
4. Mix the solution thoroughly, but gently. Take care to avoid making excessive bubbles.
5. Allow the mixture to sit for 2-3 minutes after mixing. *(Do not let the cells sit in the dye for more than five minutes because both the living and dead cells will begin to take-up the dye after five minutes.)*
6. Pipet approximately 25 microliters of the Trypan Blue/cell suspension mixture into one of the two counting chambers.
    a. Use a clean pipet tip.
    b. Be sure that the suspension is mixed thoroughly but gently before drawing the samples.
    c. Fill the chambers slowly and steadily.
    d. Avoid injecting bubbles into the chambers.
    e. Do not overfill or under fill the chambers.
7. Determine Cell Viability.

a. Allow the suspension to settle in the chambers for at least 10 seconds.

b. Count all of the stained cells in each of the four corner squares of the hemacytometer.

c. Separately count all of the unstained cells in the same squares.

d. Calculate the cell viability using the equation:

Total # of Viable Cells:

Average (cell count) x dilution factor x hemacytometer factor

REFERENCES

1. Antonios G. Mikos, Johnna S. Temenoff. Formation of highly porous biodegradable scaffolds for tissue engineering. Electronic Journal of Biotechnology, Vol. 3 No. 2, August 15, 2000.

2. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effects of implants loaded with autologus mesenchymal stem cells on the healing of canine segmental bone defects.J Bone Joint Surg Am. 1998;80:985-96.

3. Bognitzki.M, H. Hou, M. Ishaque, Th. Frese, M. Hellwig, Ch. Schwarte, A. Schaper, J. H. Wendorff, A. Greiner. Polymer, Metal and Hybrid Nano- and Mesotubes by Coating of Degradable Polymer Template Fibers (TUFT-process). Adv. Mater. 12, 637 (2000).

4. Bognitzki, W. Czado, Th. Frese, A. Schaper, M. Hellwig, M. Steinhart, A. Greiner, J. H. Wendorff. Nanostructured Fibers via Electrospinning. Adv. Mater. 13, 70 (2001).

5. Bornat A. Electrostatic spinning of tubular products. US Patent. (1982).

6. Bornat A. Production of electrostatically spun products. US Patent (1987).

7. Doshi J, Reneker DH. Electrospinning Process and Applications of Electrospun fibers. Journal of Electrostatics. 35, 151-160, 1995.

8. Doshi J. The electrospinning process and applications of electrospun fibers. Doctoral Dissertation, The University of Akron. 1994.

9. Entov VM, Shmaryan LE. Numerical modeling of the capillary breakup of jets of polymer liquids. Fluid Dynamics 1997; 32 (5): 696-703.

10. Fang X, Reneker DH. DNA fibers by electrospinning. Journal of Macromolecular Science- Physics, B36 (2), 169-173 (1997).

11. Fong H, Chun I, Reneker DH. Beaded nanofibers formed during electrospinning. Polymer 40 (1999) 4585-4592.

12. Formhals A, Process and apparatus for preparing artificial threads. US Patent (1934), 1,975,504.

13. Gibson, P, Schreuder-Gibson, H.L, Rivin, D. Transport properties of porous membranes based on electrospun nanofibers.

14. How TV. Synthetic vascular grafts, and methods of manufacturing such grafts. US Patent (1985).

15. Huang L, McMillan RA, Apkarian RP, Pourdeyhimi B, Conticello VP, Chaikof EL. Generation of synthetic elastin-mimetic small diameter fibers and fiber networks. Macromolecules 2000; 33(8): 2989–97.

16. Jaeger R, Bergshoef MM, Batlle CM, Schonherr H, Vancso GJ. Electrospinning of ultra-thin polymer fibers. Macromolecular Symposium 1998; 127: 141-150.

17. Jaeger R, Schonherr H, Vancso J. Chain packing in electrospun poly (ethylene oxide) visualized by atomic force microscopy. Macromolecules 1996; 29 (23): 7634-7636.

18. Kim J-S, Lee DS. Thermal Properties of Electrospun Polyesters. Polymer Journal, Vol.32, No.7, 616-618 (2000).

19. Kim J-S, Reneker DH. Mechanical Properties of Composites Using Ultra fine Electrospun Fibers. Polymer Composites, Feb 1999, Vol.20, No.1, 124-131.

20. Laurencin CT, Ambrosio AMA, Borden MD, Cooper Jr JA. Tissue engineering: orthopedic applications. Annu Rev Biomed. Eng 1999; 1:19–46.

21. Martin GE, Cockshott ID, and Fildes JT. Fibrillar lining for prosthetic device. US Patent (1977).

22. Martindale D. Scientific American 2000:34–6.

23. Rayleigh FRS. Philosophical Magazine, 14, 184 (1882).

24. Reneker DH, Yarin AL, Fong H, Koombhongse S. Journal of Applied Physics, 87, 4531 (2000).

25. Shin YM, Hohman MM, Brenner MP, Rutledge GC. Electrospinning: A whipping fluid jet generates submicron polymer fibers. Applied Physics Letters. Vol. 78, No.8, 1149-1151, 19 February 2001.

26. Shin YM, Hohman MM, Brenner MP, Rutledge GC. Experimental characteriz- -ation of electrospinning: the electrically forced jet and instabilities. Polymer, 42 (2001) 9955-9967.

27. Srinivasan G, Reneker DH. Structure and Morphology of Small diameter Electrospun Aramid fibers. Polymer international 36 (1995) 195-201.

28. Srinivasan G. Structure and morphology of Electrospun Polymer Fibers. Doctoral Dissertation, The University of Akron. 1994.

29. Stem Cell Research and Applications: Monitoring the Frontiers of Biomedical Research. American Association for the Advancement of Science, November 1999.

30. Talbott JW, Logan JD. Method of forming a composite mat of directionally oriented lignocellulosic fibrous material US Patent (1978), 4,113,812.

31. Taylor GI. Disintegration of Water Drops in an Electric Field. Proceedings of the Royal Society of London Series A Mathematical and Physical Sciences. Vol. 280, 383, 1964.

32. Taylor GI. Electrically Driven Jets. Proceedings of the Royal Society of London Series A Mathematical and Physical Sciences. Vol. 313, 453-475, 1969.

33. Taylor GI. Studies in Electro hydrodynamics. I. The Circulation Produced in a Drop by Electrical Field. Proceedings of the Royal Society of London Series A Mathematical and Physical Sciences. Vol. 291, 159, 1966.

34. Taylor GI. The Force Exerted by an Electric Field on a Long Cylindrical Conductor. Proceedings of the Royal Society of London Series A Mathematical and Physical Sciences. Vol. 291, 145, 1966.

35. Weissman IL, Baume CM, Tsukamoto AS, Buckle A, and Peault B, Isolation of a Candidate Human Hematopoietic Stem-Cell Population. Proc Natl Acad Sci U S A. 1992 April 1; 89 (7): 2804–2808.

36. Y.K.Luu, K.Kim et al., Characterization of an Electrospun Poly (lactide-*co*-glycolide) and Block Copolymer-based, Nanostructured Matrix for DNA Delivery. Biomedical Engineering Society Conf., 2002.

37. Zeleny J. Journal of the Franklin Institute. 219, 659 (1935).

38. Zeleny J. Physical Review, Vol. 10, 1 (1917).

39. Zeleny J. Physical Review, Vol. 3, 69 (1914).

40. Zeleny J. Proceedings of the Cambridge Phil Society. Vol. 18, 71 (1915).